Membrane Fusion by Peptide Analogues of Influenza Virus Haemagglutinin

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SUMMARY

We have studied the interactions of synthetic peptides corresponding to the sequence of the amino terminus of the HA₂ subunit of influenza virus haemagglutinin with artificial lipid membranes. The peptides could fuse cholesterol-free liposomes at neutral as well as acid pH; however, liposomes containing cholesterol could only be fused below pH 6. The fusion process caused leakage of aqueous liposomal contents. Peptides with amino acid substitutions had fusion properties similar to whole haemagglutinin molecules with the corresponding sequence changes. Non-fusogenic peptides still interacted with the membrane but did not cause leakage of liposomal contents. A correlation between the α-helical content of peptide and its fusogenicity was noted, but this was not absolute. The results reported here support suggestions for a role of the amino terminus of HA₂ in virus-endosome fusion.

INTRODUCTION

Influenza virus haemagglutinin (HA) mediates the fusion of viral and endosomal membranes which is required to initiate infection of cells (reviewed by White et al., 1983; Wharton, 1987). At the low pH of the endosome (between 5 and 6) a conformational change occurs in HA, which is a prerequisite for fusion activity. A number of studies have shown that this change involves a decrease in subunit contacts within the HA trimer (Skehel et al., 1982; Daniels et al., 1983b, 1985; Ruigrok et al., 1986) and the exposure of the highly conserved hydrophobic amino terminus of HA₂ (Skehel et al., 1982; Daniels et al., 1983a, 1985; Doms et al., 1985). It has been proposed that this hydrophobic region interacts with lipid membranes during the fusion process and support for this proposition comes from the observation that aggregation of bromelain-released HA (BHA), which is specifically induced at the pH of fusion, involves the HA₂ amino-terminal region (Daniels et al., 1983a; R. W. H. Ruigrok et al., unpublished results). Also, variant HAs with amino acid substitutions in the amino-terminal region of HA₂ constructed by site-directed mutagenesis have fusion and membrane association characteristics different to those of wild-type HA (Getting et al., 1986). In a recently published report, Lear & De Grado (1987) have shown that the peptide corresponding to the amino terminus of HA₂ B/Lee/40 is capable of fusing liposomes at neutral pH. In addition, Murata et al. (1987) have demonstrated pH-dependent fusion of liposomes by a peptide corresponding to the amino terminus of HA₂ of A/PR/8/34 virus. In the study reported here we synthesized a peptide with the sequence of the 23 amino-terminal residues of HA₂ of X31 (H₃N₂) which was capable of interacting with and fusing membranes. Using specifically modified analogues of this region, we also showed that interaction with membranes was not sufficient for fusion, that the fusion characteristics of peptides were affected by the lipid composition of the liposomes, and that peptides with sequences equivalent to the amino-terminal regions of variants had fusion properties similar to those of the whole HA molecule.
**METHODS**

**Materials.** N-4-Nitrobenzo-2-oxa-1,3-diaze phosphatidylethanolamine (NBD-PE) was purchased from Avanti Polar Lipids Inc., Birmingham, Ala., U.S.A. and cholesteroyl-anthracene-9-carboxylate (CAC) from Molecular Probes Inc., Junction City, Ore., U.S.A. 1-Aminonaphthalene-3,6,8-trisulphonic acid (disodium salt) (ANTS), xylenebispyridinium bromide (DPX), diphenylhexatriene and terbium chloride were purchased from Molecular Probes Inc., Junction City, Ore., U.S.A. 1-Aminonaphthalene-3,6,8-trisulphonic acid (disodium salt) was also purchased from Sigma or BDH.

**Peptide synthesis.** Peptides were synthesized by the F-moc procedure of Atherton & Sheppard (1985) using pentafluorophenyl esters and pepsyn KB resin. The sequences of the five peptides synthesized are shown in Fig. 1. Wherever possible, the peptides were synthesized on columns linked in series with the amino acid addition occurring simultaneously. HPLC and amino acid analysis showed the peptides to be >90% pure.

**Liposome fusion assay.** Small unilamellar liposomes were prepared by sonication as described by Wharton et al. (1986). They contained palmitoyl-oleoyl-phosphatidylcholine (POPC) or POPC with cholesterol at a molar ratio of 1:1 (POPC/C). The fluorescent liposomes contained 2 mol percent of the fluorescent probes CAC and NBD-PE. Details of the fusion assay are given in Wharton et al. (1986). Fluorescent and non-fluorescent liposomes were mixed in a ratio of 1:9 at a final lipid concentration of 660 μM in phosphate-buffered saline (PBS). Peptide from a 330 μM stock solution in dimethylsulphoxide was added to a final concentration of 16.5 μM (lipid:peptide molar ratio of 40:1); the increase of CAC fluorescence at 460 nm due to the decrease in resonance energy transfer between the fluorescent probes was an indication of the fusion of fluorescent with non-fluorescent liposomes. When required, the pH of the solution was adjusted by the addition of 0.15 M-citrate buffer pH 3.6. All fusions were done at 37 °C. The microviscosity of the membranes was determined by steady-state fluorescence polarization using diphenylhexatriene as described in Wharton et al. (1980).

**Tryptophan fluorescence.** Fluorescence spectra were recorded using a Perkin-Elmer MPF-4 spectrofluorimeter with excitation at 288 nm and slit widths of 5 nm. The peptide concentration was 9 μM and liposomal lipid was 1.35 mM. Spectra were recorded at 37 °C, 10 min after the addition of peptide to liposomes. Brominated Brij 96 was prepared by adding 60 μl bromine to 10 ml of 10% (w/v) Brij 96 (Blewitt et al., 1984). Tryptophan fluorescence at 330 nm was compared in the presence of 0.5% (w/v) brominated and non-brominated Brij 96.

**Assay for the mixing of liposomal contents.** Two assays were used: (i) the mixing of dipicolinic acid and terbium ions and the subsequent increase in terbium fluorescence as described by Wilschut & Papahadjopoulos (1979) and (ii) the mixing of DPX and ANTS and the resulting quenching of ANTS fluorescence (Ellens et al., 1985). Only the latter assay was used for experiments at pH 5 since dipicolinic acid is only weakly bound by terbium at this pH and fluorescence changes are small (Barela & Sherry, 1976). Leakage of liposomal contents was also measured by the release of entrapped calcein and the subsequent relief of its self-quenching upon dilution (Bondeson et al., 1984). Liposomal and peptide concentrations were the same as those used in the fusion experiments. The values for 100% leakage were determined by adding 0.5% (v/v) Brij 36T to the mixture.

**Circular dichroism (CD) experiments.** CD measurements were made on a Jasco J41C spectropolarimeter equipped with a model J-D DPY data processor. Spectra were recorded from 260 to 200 nm at a sensitivity of 1 mdeg/cm with an instrumental time constant of 1 s. Fused silica cells (2 mm) were used for all measurements. The final spectra were the average of at least eight scans and the CD intensity at 220 nm was estimated by averaging the data in the range 221 to 219 nm (eight data points). The concentration of liposomal lipid was 450 μM and the peptide concentration 23 μM. All data were collected at 37 °C 10 min after addition of peptide to the liposomes. Intensity values were corrected by subtraction of the value obtained with a peptide-free solution and the molar CD absorption coefficient (Δε) was then calculated based on a mean residue Mr of 110.

**Haemolysis studies.** Haemolysis was estimated at 37 °C as described by Daniels et al. (1985) using 1% (v/v) human group A erythrocytes.

**RESULTS**

**pH and lipid dependence of the fusion of liposomes by the synthetic peptide corresponding to the amino terminus of HA₂**

The pH dependence of fusion of small POPC unilamellar liposomes at 37 °C by a synthetic 23 amino acid peptide analogue of the amino terminus of HA₂ (Fig. 1) is shown in Fig. 2(a). Fusion occurred readily at pH 7, was more extensive at pH 5 but did not occur at pH values greater than 10. Fusion at pH 7 and pH 5 was rapid and essentially complete within 5 min. At the concentrations of peptides studied, between 8 μM and 40 μM (i.e. between a peptide:lipid molar ratio of 1:100 and 1:20), the extent of fusion was proportional to the concentration of peptide. It was also highly dependent upon salt concentration. Fusion at pH 7 in 300 mM-NaCl was approximately double that in 150 mM-NaCl, whereas fusion in 10 mM-phosphate buffer (pH 7.4) alone was less than one-quarter that in 150 mM-NaCl. In all these experiments the salt
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Wild-type 23 amino acid: GLFGAIAGFIENGWEGMIDGWYG
Wild-type 20 amino acid: GLFGAIAGFIENGWEGMIDG
G4E: GLFEAIAGFIENGWEGMIDG
G1E: ELFGAIAGFIENGWEGMIDG
ΔG1: LFGAIAGFIENGWEGMIDG

Fig. 1. Amino acid composition of the various peptides.

Concentration was the same both inside and outside the liposomes. When cholesterol was included in the liposomes at a molar ratio of 1:1 with phospholipid (POPC/C liposomes), fusion occurred only at pH values below 6 (Fig. 2b). Fig. 2(c) shows the pH dependence of fusion with POPC/C liposomes at 37°C. Half-maximal fusion was observed at pH 5.5 at 37°C (Fig. 2c) and pH 5.0 at 20°C (not shown). The decreased extent of fusion of liposomes containing cholesterol at pH 7 appears not to be due to the increased rigidity of POPC/C membranes (Green, 1977) since dipalmitoyl phosphatidylcholine (DPPC) liposomes can be fused by the peptide at neutral pH. Fluorescence polarization studies using the probe diphenylhexatriene have shown that DPPC liposomes are more rigid (anisotropy, r = 0.206) than POPC/C liposomes (r = 0.160) at 37°C (below the phase transition temperature of DPPC). Moreover, if a 1:1 molar ratio of cholesterol is included in DPPC liposomes, fusion does not occur at neutral pH. Cholesterol and cholesta-4-en-3-one gave identical results, thus showing that the β-hydroxyl group of cholesterol, which is important for the cholesterol-induced decrease in the permeability of liposomes (Bruckdorfer et al., 1969), is not involved in the effect on peptide-mediated fusion. Peptide-mediated fusion of POPC liposomes could be stopped by raising the pH to 10 and that of POPC/C liposomes by raising the pH to 7. In both cases, subsequent lowering of the pH reinitiated fusion (Fig. 2d).

Fusion properties of peptides with specific changes in sequence

The fusion activities of peptides corresponding to amino-terminal regions of mutant HAs with different membrane fusion properties were examined. As well as wild-type HA, three peptides were used. The first peptide lacked the amino-terminal glycine (peptide ΔG1) since virus with precursor HA cleaved by the protease thermolysin, which results in an amino terminus of HA2 lacking the glycine, is not infectious (Garten et al., 1981). Table 1 shows the fusion properties of this peptide. Unlike the wild-type peptides, which could fuse POPC liposomes at neutral and acid pH and POPC/C liposomes at acid pH, peptide ΔG1 was incapable of fusing POPC/C liposomes at any pH, and only fused POPC liposomes at acid pH. This decreased fusogenicity of peptide ΔG1 may explain why thermolysin-treated virus is not infectious (Garten et al., 1981). The other two peptides studied had sequences corresponding to those of mutant HAs which have been expressed in CV-1 cells and which have altered fusion characteristics compared to the wild-type (Gething et al., 1986). The fusion properties of these peptides, one with a G to E substitution at position 1 (G1E) and one with the same substitution at position 4 (G4E) are also shown in Table 1. Peptide G1E was incapable of fusing POPC/C liposomes; the corresponding HA molecule was incapable of heterokaryon formation and did not mediate CV-1/erythrocyte fusion (Gething et al., 1986). Peptide G4E had fusion properties similar to the wild-type peptide except that it could not fuse POPC liposomes at neutral pH. The corresponding HA molecule had fusion properties similar to wild-type HA (Gething et al., 1986).

Interaction of the peptides with liposomes

Tryptophan fluorescence

Peptide analogues of the 20 amino-terminal residues were used in these experiments because they contain a single tryptophan residue at position 14 which facilitates fluorescence analysis. Tryptophan residues in aqueous environments fluoresce with an emission maximum at around 350 nm. In contrast, those in a hydrophobic environment, such as in a lipid bilayer or the interior of a protein, emit fluorescence at shorter wavelengths (generally 320 to 335 nm) (the
Fig. 2. (a) Fusion of POPC liposomes by the 23 amino acid wild-type peptide at various pH values. Six-hundred nmol of unlabelled POPC liposomes were incubated with 60 nmol of fluorescently labelled POPC liposomes in 950 μl PBS adjusted to various pH values by 0.15 M-citrate buffer (pH 3.5). At time 0 16.5 nmol of peptide in 50 μl dimethyl sulphoxide was added and the increase in CAC fluorescence, due to a decrease in resonance energy transfer following liposome-liposome fusion, was monitored at 460 nm. Excitation was at 368 nm. The pH of each incubation medium is indicated to the right of each curve. (b) Fusion of POPC/C liposomes by the 23 amino acid wild-type peptide at various pH values. Experimental conditions were the same as in (a) except that POPC/C liposomes were used. The pH of each incubation medium is indicated to the right of each curve. (c) pH dependence of the 23 amino acid wild-type peptide-mediated fusion of POPC/C liposomes. The experimental conditions were as in (a) and (b). The ratio of CAC to NBD-PE fluorescence was measured after 5 min incubation at the various pH values at 37 °C and this was taken as a measure of the extent of fusion. (d) The effect of altering pH after peptide addition upon liposome fusion. The experimental conditions were as in (a) and (b) but the pH was adjusted and readjusted after addition of the peptide (arrow) by adding the appropriate amounts of 0.1 M-citric acid or 0.1 M-sodium hydroxide. The pH of the incubation medium is indicated next to each portion of the curve. Upper curve, POPC liposomes; lower curve, POPC/C liposomes.
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Table 1. Fusion capacities of the various peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>POPC</th>
<th>POPC/C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 7</td>
<td>pH 5</td>
</tr>
<tr>
<td>23 Residue wild-type</td>
<td>41</td>
<td>81</td>
</tr>
<tr>
<td>20 Residue wild-type</td>
<td>29</td>
<td>60</td>
</tr>
<tr>
<td>G4E</td>
<td>2</td>
<td>75</td>
</tr>
<tr>
<td>G1E</td>
<td>4</td>
<td>47</td>
</tr>
<tr>
<td>ΔG1</td>
<td>0</td>
<td>50</td>
</tr>
</tbody>
</table>

* Sixty nmol of fluorescently labelled liposomes was incubated with 600 nmol of unlabelled liposomes at 37 °C at pH 7 or pH 5 in 950 μl PBS-citrate buffer and the fluorescence spectrum was recorded. Peptide was added (16.5 nmol in 50 μl dimethyl sulfoxide) and after a 5 min incubation at 37 °C the spectrum was re-recorded. The fusion capacity is expressed as the percentage change in the ratio of CAC to NBD-PE fluorescence before and after the addition of peptide, a value which, under the experimental conditions used, is proportional to the extent of fusion.

Table 2. Ratio of fluorescence intensities (330 to 350 nm) for the various peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>No liposomes</th>
<th>POPC</th>
<th>POPC/C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 7</td>
<td>pH 5</td>
<td>pH 7</td>
</tr>
<tr>
<td>20 Residue wild-type</td>
<td>0.64</td>
<td>0.68</td>
<td>0.85</td>
</tr>
<tr>
<td>G4E</td>
<td>0.70</td>
<td>0.75</td>
<td>0.92</td>
</tr>
<tr>
<td>G1E</td>
<td>0.69</td>
<td>0.72</td>
<td>0.97</td>
</tr>
<tr>
<td>ΔG1</td>
<td>0.64</td>
<td>0.65</td>
<td>0.77</td>
</tr>
</tbody>
</table>

* The ratio of 330 nm to 350 nm fluorescence intensity was calculated using 9 nmol of peptide (20 μl of a stock solution in dimethyl sulfoxide) incubated at 37 °C for 10 min with 980 μl PBS-citrate buffer pH 7 (or pH 5) or 950 μl of buffer containing 1.35 μmol of POPC or POPC/C liposomes. Excitation was at 288 nm.

phenomenon of blue shift). The ratio of fluorescence intensities at 330 and 350 nm may therefore be used as an index for the environment of tryptophan residues. Table 2 shows these ratios for the various peptides at pH 7 and pH 5 at 37 °C in solvent or in the presence of POPC or POPC/C liposomes (150:1 molar ratio of lipid to peptide). In the absence of liposomes, the peptide tryptophans were exposed to solvent (the 330:350 ratio being between 0.64 and 0.70). N-Acetyltryptophanamide, a water-soluble analogue of tryptophan, has a value of 0.5 for this ratio. Lowering the pH to 5 had no significant effect upon the fluorescence of the tryptophan residues in the absence of liposomes. However, when peptides were added to either POPC or POPC/C liposomes there was a significant increase in the ratio for all peptides at pH 7, indicating that the tryptophan residue of the peptide had moved to a more hydrophobic environment and on lowering the pH to 5 this effect was increased. In all cases, the liposome–peptide interaction was accompanied by an increase in the total fluorescence. The tryptophan of the peptide lacking the amino-terminal glycine appeared to be less buried than those of the other peptides; at pH 11, at which no fusion occurred with any peptide, the tryptophan residue of wild-type peptide was not in a hydrophobic environment (a ratio of 0.68). The effect of pH upon the environment of the tryptophan residue was reversible. In a separate experiment, wild-type peptide incubated at pH 7 with POPC liposomes had a ratio of 0.89. When the pH was lowered to 5 the ratio increased to 1.21. If the pH was then raised to 7 the ratio fell to 0.91, and raising the pH to 11 decreased the ratio to 0.74.

Detergent binding properties of the peptides

The degree of hydrophobicity of the peptides was also studied by using brominated Brij 96, a non-ionic detergent which quenches the tryptophan fluorescence of peptides or proteins to which it binds (Blewitt et al., 1984). The fluorescence of wild-type, G4E, G1E and ΔG1 peptides was quenched by 54, 61, 54 and 36%, respectively, by brominated Brij 96. Upon lowering the pH to 5, only ΔG1 showed any significant increase in binding to detergent (quenched 52%). The
Table 3. Δε at 220 nm for various peptides*

<table>
<thead>
<tr>
<th>Peptide alone</th>
<th>POPC</th>
<th>POPC/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7</td>
<td>pH 5</td>
<td>pH 7</td>
</tr>
<tr>
<td>pH 7</td>
<td>pH 5</td>
<td>pH 5</td>
</tr>
<tr>
<td>23 Residue wild-type</td>
<td>-1.15</td>
<td>-1.07</td>
</tr>
<tr>
<td>20 Residue wild-type</td>
<td>-0.92</td>
<td>-1.16</td>
</tr>
<tr>
<td>G4E</td>
<td>-1.05</td>
<td>-1.29</td>
</tr>
<tr>
<td>G1E</td>
<td>-0.51</td>
<td>-0.63</td>
</tr>
<tr>
<td>AG1</td>
<td>-0.41</td>
<td>-0.49</td>
</tr>
</tbody>
</table>

The Δε at 220 nm for the various peptides (23 nM) was measured in the presence or absence of 450 nM-POPC or POPC/C liposomes in PBS-citrate buffer at pH 7 or pH 5 10 min after the addition of peptide to liposomes. The peptide concentration was determined from the absorbance at 280 nm from the tryptophan and tyrosine content as described in Ruigrok et al. (1986) and Δε was calculated assuming a mean residue Mr of 110. Δε is proportional to helical content and a value of -11/M·cm represents 100% helix.

The fluorescence of N-acetyltryptophanamide was unaffected by brominated Brij 96 at pH 7 or pH 5. The results of the detergent binding studies therefore closely parallel those of the peptide–liposome binding experiments.

CD studies of the peptides

The results of tryptophan fluorescence spectroscopy clearly showed that under certain conditions peptides interact with membranes but do not fuse them, e.g. G1E with POPC/C liposomes at pH 7. To characterize further the interaction between lipid and peptide, the secondary structure of the peptides under different conditions was studied by CD. Table 3 shows the Δε (220 nm) for the peptides in the presence or absence of liposomes. Under the conditions of the experiment, this provides a reasonable estimate for α-helical content, with a value of approximately -11 representing 100% helix. Conventional methods for the estimation of secondary structure could not be used because the highly scattering liposomes limited data collection to a short wavelength range. In the absence of liposomes, the peptides did not assume a helical conformation at either pH 7 or pH 5. Addition of trifluoroethanol, an α-helix-promoting agent, increased the α-helical content; this effect was greater in the wild-type peptides than the G1E, AG1 or G4E peptides. In the presence of POPC liposomes, there appeared to be some correlation between α-helix content and lipid fusion activity, in that peptides which fused liposomes at pH 7 had a greater helical content. In no case was the amount of α-helix greater than 55%. However, it is not known whether these figures reflect the proportion of each peptide in an α-helical conformation or the proportion of the peptides with 100% α-helix. There appears to be a limit to the number of peptides that can insert into the membrane and a lipid:peptide ratio of approximately 75:1 appears to be optimal for insertion of all the peptides into the liposome (Lear & De Grado, 1987). In order to balance the effects of weak peptide signal and excessive light scattering from the liposomes, the CD experiments were performed at the lower ratios of 20:1 in this study and 25:1 in the study of Lear & De Grado (1987). A significant proportion of the peptide would therefore not be inserted into the liposomes and as a consequence the actual α-helical content of the lipid-associated peptide might have been considerably higher than the recorded values indicate. The correlation between α-helicity and fusogenicity was not absolute. For example, the 20 residue wild-type peptide fused POPC liposomes at pH 7, but had a lower helical content than G4E which was not fusogenic under the same conditions. A similar phenomenon was seen with POPC/C liposomes. In support of the correlation, the non-fusogenic peptides AG1 and G1E had a lower α-helical content at pH 5 than the fusogenic peptides, and the 23 residue wild-type peptide at pH 7, which was not significantly fusogenic, had a higher helical content that the fusogenic 20 residue wild-type peptide at pH 5. In addition, the increase in helicity that occurred when peptides were incubated at low pH with POPC liposomes, compared to incubation at neutral pH, was not as
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Fig. 3. The haemolysis activities of the various peptides. Peptide (1-6 nmol in 5 μl dimethyl sulphoxide) was incubated with 0.5 ml of erythrocytes at various pH values in PBS-citrate buffer for 15 min at 37 °C. After removal of the cells by centrifugation, the optical density of the supernatant was measured at 520 nm. (▲) 20 Residue wild-type peptide, (▲) G4E, (■) G1E, (○) AG1, (●) dimethyl sulphoxide control.

marked with POPC/C liposomes. As noted above, at pH 10 and above, peptides no longer associated with membranes or fused POPC liposomes. The α-helical content also decreased at high pH, from approximately 40% at pH 7 to 20% at pH 11. Peptides in buffer alone (10 mM-phosphate pH 7.4) in which their fusion activity was decreased also had a lower helical content, being about 50% of those of peptides in 150 mM-NaCl.

Peptide-induced leakage of liposomal contents

When the ratio of peptide to liposomes was increased 20-fold, to give a lipid to peptide ratio of 2:1 the peptides solubilized the liposomal membranes, as determined by a decrease in resonance energy transfer between the anthroyl cholesterol ester and NBD-PE in the absence of non-fluorescently labelled liposomes. This did not occur above a ratio of 5:1. Ideally, liposome fusion should be monitored by the mixing of aqueous contents as well as of liposomal lipids. However, virus–liposome fusion is a process that allows leakage of approximately 80% of entrapped calcein (unpublished observations) and this is likely to be the case following peptide-mediated fusion. Experiments using the calcein, the terbium/dipicolinic acid and the ANTS/DPX systems for monitoring aqueous content mixing showed that peptide-induced fusion was indeed a process involving leakage of approximately 90% of the liposomal contents. However, in situations where there was peptide insertion but not fusion, e.g. POPC/C liposomes with G1E peptide at pH 7 or 5, there was no leakage of liposomal contents. Thus it was the fusion process and not simply peptide insertion that was responsible for leakage.

We have also used haemolytic activity as an assay of virus fusion activity. This measures leakage following fusion of virus and erythrocytes and not fusion per se. The haemolytic activities of the different peptides are shown in Fig. 3. The dependence of haemolysis on pH was similar to that of POPC/C liposome fusion by the peptides (erythrocyte membranes have a molar ratio of cholesterol to phospholipid of approximately 0.9). Furthermore, the peptide lacking the amino-terminal glycine (AG1) did not haemolyse, the haemolysis profiles of the 20 amino acid wild-type peptide and that of G4E were highly pH-dependent and G1E haemolysed only at pH values below 5. The wild-type peptide was capable of fusing POPC/C liposomes with erythrocyte ghosts, but only at pH 5 (unpublished observations). Therefore, the pH dependence of peptide-mediated fusion of erythrocyte ghosts was similar to that of POPC/C liposomes, as expected from the cholesterol content of the ghosts.
DISCUSSION

Membrane fusion mediated by influenza virus HA is dependent on a conformational change in the molecule which is required, at least in part, to allow extrusion of the conserved hydrophobic amino terminus of HA2 from its location buried in the centre of the trimer. Once extruded, it is generally proposed that the amino terminus of HA2 interacts with either the endosomal membrane to form a bridge between it and the virus membrane, or with both virus and endosomal membranes to destabilize their bilayer structure. In this report we have shown that synthetic peptide analogues of the amino terminus can fuse liposomes at rates similar to those observed for virus–liposome fusion (Wharton et al., 1986) and we have analysed similarities between peptide-mediated and virus- or HA-mediated fusion. Our results extend those of Lear & De Grado (1987) which indicated that peptides analogous to the amino terminus of B/Lee/40 HA2 were capable of fusing POPC liposomes at neutral pH. In the results presented here, however, the extent of fusion with cholesterol-containing liposomes at neutral pH was small; fusion occurred only at acid pH and at 37 °C was 50% of the maximum at pH 5.35. In a recent report, Murata et al. (1987) reported that the 20 residue peptide with the sequence corresponding to the amino terminus of HA2 of A/PR/8/34 (H1N1) virus fused egg phosphatidylycholine liposomes lacking cholesterol only at acid pH. It is not clear whether this difference is because of the lower temperature (20 °C) of the experiments of Murata et al. (1987) as opposed to 37 °C in our study or the slightly different sequence of the H1 peptide; for example, one of the glutamic acids is a glycine at position 15. It is not due to the difference in liposomal composition, as the 20 residue peptide used in our study fused egg phosphatidylycholine liposomes at neutral pH (not shown).

There have been many studies on the interactions of natural and synthetic peptides with membranes, some of which are pH-dependent and others of which result in the fusion of vesicles. For example, melittin interacts with and fuses vesicles over a wide pH range (Morgan et al., 1983) and polyhistidine fuses liposomes only at low pH (Uster & Deamer, 1985). In addition, tryptophan fluorescence spectroscopy and CD have been used to study the interactions of polypeptides such as diphtheria toxin (Blewitt et al., 1985) and tetanus toxin (Cabiaux et al., 1985) with bilayers and many polypeptides such as diphtheria toxin (Cabiaux et al., 1984) and colicin (Pattus et al., 1985) fuse liposomes at low pH. In this study we have employed tryptophan fluorescence, detergent binding and CD to assess peptide–liposome interactions. The results show that insertion into the bilayer is not sufficient for fusion since there are conditions, e.g. POPC liposomes at pH 7 with G1E or AG1 peptides or the same peptides with POPC/C liposomes at pH 5, where the peptide tryptophan is buried in the bilayer but no fusion occurs. In addition, leakage of liposomal contents occurs only when there is fusion of the liposomes.

Although peptides which had a larger α-helical content when embedded in membranes tended to be fusogenic, the correlation between fusogenicity and helical content was not absolute, particularly when comparisons were made between different peptides. A correlation between fusogenicity and helicity was also seen by Lear & De Grado (1987) for the 20 residue analogue of B/Lee/40 HA2.

Although the peptides reported here clearly interact with bilayers, it is not clear how they mediate fusion which requires that two membranes are brought together presumably by a bridging action. HA rosettes, for example, are capable of fusing liposomes (Wharton et al., 1986) probably because their polyvalency allows aggregation of liposomes prior to their fusion. On the other hand, BHA, which exists as single molecules, does not fuse membranes (Wharton et al., 1986) and a similar situation would be expected to apply to short peptides. However, electron microscopy has shown that the peptides aggregate in solution (Fig. 4), and this aggregation may enable the peptides to interact simultaneously with more than one liposome. Such aggregation was observed with the wild-type peptide at pH 7, 5 and 11. Peptides ΔG1 and G1E also aggregated at low pH. At neutral pH, although some aggregation of ΔG1 was observed, there was none with peptide G1E. Electron microscopy enabled only larger aggregates to be visualized and smaller aggregates might still have been present. It is clear that aggregation is not the only process required for fusion activity, because there are conditions under which peptide aggregation occurs without fusion, such as with wild-type peptide at pH 11 and G1E or ΔG1.
peptides with POPC/C liposomes at pH 5. CD studies indicated that this aggregation was not accompanied by changes in secondary structure of the peptide, and fluorescence spectroscopy suggested that the tryptophan residues of the peptides in solution remained exposed to solvent. The fusion activities of a number of selected and of site-directed mutant HA molecules have been reported, and confirm the importance of the amino acid sequence of the amino terminus of HA₂ in membrane fusion.

In an attempt to compare the significance of specific residues for peptide-mediated membrane fusion, we have determined the fusion activities of appropriately modified peptide analogues. Deletion of the amino-terminal glycine (ΔG₁ peptide) abolished peptide-mediated fusion of POPC/C liposomes at any pH, as did the substitution in G₁E. These observations are in agreement with the results of Garten et al. (1981) and of Gething et al. (1986) which showed that HA molecules thus modified were not fusogenic. The peptide that contained the substitution G₄E fused liposomes at acid pH but, unlike the wild-type peptide, could not fuse POPC liposomes at neutral pH. This difference may correlate with the reduced efficiency of heterokaryon formation by the equivalent HA mutant (Gething et al., 1986). The correlation between peptide and protein membrane association was also demonstrated by the results obtained at high pH. At a pH greater than 10 the peptide no longer associated with bilayers and BHA, which had previously been acid-treated, became dissociated from liposomes (Doms et al., 1985).

These correlations between peptide and whole-molecule fusion contrast with findings for the vesicular stomatitis virus membrane glycoprotein and a peptide analogue of the region proposed to be involved in membrane fusion. In this case, although changes in the composition of the peptide analogue of the putative fusogenic region of the protein altered haemolytic properties (Schlegel & Wade, 1985), the equivalent changes in the G protein by site-specific mutations did not alter the fusogenic properties of the glycoprotein (Woodgett & Rose, 1986), suggesting that other regions of the glycoprotein are important for fusion.

Recently, Duzgunes & Gambale (1988) have reported that heptapeptide analogues corresponding to wild-type, G₄E and G₁E have different effects upon the conductance
properties of planar membranes lacking cholesterol. Notably, the peptides corresponding to wild-type and, to a lesser extent, G4E increased membrane conductance at pH 7, whereas the peptide corresponding to G1E did not. In addition, the wild-type peptide induced leakage of contents from liposomes, whereas the G4E and G1E peptides did not. Both observations are in agreement with the results presented above. The amino terminus of HA2 is rich in glycine residues (Fig. 1) which allows its unusual helical structure in the native molecule (Wilson et al., 1981). The presence of these glycine residues and the fact that glutamic acid at positions 11 and 15 and aspartic acid at position 19 would align on a predicted α-helix and would exhibit charge repulsion (Gratzer & Doty, 1963) may be the reasons why the peptide does not adopt a significantly helical structure in solution at pH 7 or pH 5. When embedded in a membrane, the above constraints need not apply. Subbaro et al. (1987) designed a peptide based upon the amino terminus of HA2 which interacted with bilayers in a pH-dependent manner. Every fourth residue was a glutamic acid and the remaining residues were chosen for their hydrophobicity and α-helix preference. This peptide had a significant helical content in solution at pH 5 and interacted with membranes, even those without cholesterol, only at low pH. These differences from the results obtained using peptides with the actual sequence of the amino terminus of HA2 are probably attributable to the number of glutamic acid residues (six) and the absence of any glycine residues in the peptide studied by Subbaro et al. (1987).

Finally, the fact that peptides corresponding to the amino terminus of HA2 were able to fuse POPC liposomes at neutral pH supports the conclusion that the low pH trigger required for infection is the conformational change of the HA which exposes this region, rather than an obligatory requirement for protonation of charged residues in the fusion process. This is in accordance with observations that mutants of HA selected by growth of virus in the presence of a weak base, amantadine (Daniels et al., 1985), are capable of fusing liposomes at a pH as high as 6.4 at 37 °C, and that raising the temperature of virus–liposome mixtures can result in virus–liposome fusion at pH 7 (Ruigrok et al., 1986). Although peptide-mediated fusion of liposomes without cholesterol clearly occurs at neutral pH, the extent of fusion of liposomes containing cholesterol (with a cholesterol:phospholipid molar ratio of 1) is small above pH 6. Since the endosomal membrane is known to contain cholesterol at a molar ratio of 0.7 the relevance of these observations for HA-mediated fusion is not clear. It is possible that cholesterol is excluded from regions of membranes participating in fusion so that fusion in vivo is between membranes more equivalent to POPC liposomes in vitro. Alternatively, additional as yet unidentified regions of the HA molecules might be directly involved in the fusion process.

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REFERENCES


Membrane fusion of influenza virus haemagglutinin


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