Membrane Anchorage Brings About Fusogenic Properties in a Short Synthetic Peptide[†]

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Received August 30, 1996; Revised Manuscript Received January 16, 1997[⊗]

ABSTRACT: The fusogenic properties of an amphipathic net-negative peptide (wae 11), consisting of 11 amino acid residues, were studied. We demonstrate that, whereas the free peptide displays no significant fusion activity, membrane fusion is strongly promoted when the peptide is anchored to a liposomal membrane. The fusion activity of the peptide appears to be independent of pH, and membrane merging is an essentially nonleaky process. Thus, the extents of lipid mixing and contents mixing were virtually indistinguishable. Vesicle aggregation is a prerequisite for fusion. For this process to take place, the target membranes required a positive charge which was provided by incorporating lysine-coupled phosphatidylethanolamine (PElys). The coupled peptide, present in one population, could thus cause vesicle aggregation via nonspecific electrostatic interaction with PElys. However, the free peptide failed to induce aggregation of PElys vesicles, suggesting that the spatial orientation of the coupled peptide codetermined its ability to bring about vesicle aggregation and fusion. With the monitoring of changes in the intrinsic Trp fluorescence, in conjunction with KI-quenching studies, it would appear that hydrophobic interactions facilitate the fusion event, possibly involving (partial) peptide penetration. Such a penetration may be needed to trigger formation of a transient, nonbilayer structure. Since lysophosphatidylcholine inhibited while monoolein strongly stimulated peptide-induced fusion, our data indicate that wae 11-induced fusion proceeds according to a model consistent with the stalk−pore hypothesis for membrane fusion.

Membrane fusion is a crucial event in numerous intracellular processes, including endo- and exocytosis, during cell−cell interactions, and in the infectious entry of enveloped viruses into mammalian cells (Hoekstra, 1990; Hughson, 1995; Monck & Fernandez, 1994; Rothman, 1994; White, 1990). It is well-established that the fusion process consists of several stages, i.e. aggregation and close apposition of the membranes about to fuse, followed by membrane destabilization. Subsequently, mixing of the outer leaflet lipids ("hemifusion") occurs while a fusion intermediate is formed, which leads to complete lipid mixing and mixing of aqueous contents of the membrane-bounded compartments (Chernomordik et al., 1995a; Siegel, 1993). A common feature in cellular and viral fusion phenomena is the involvement of specific fusion proteins (Hoekstra, 1990; Rothman, 1994; White, 1992). Among such fusion proteins, the viral spike glycoproteins, mediating the penetration of enveloped viruses into their host cells (Gallaher, 1987), have been widely characterized. In their sequences, these proteins possess a "fusion peptide" (Hoekstra, 1990; White, 1990, 1992). Typically, these peptides are short segments (up to some 20 amino acids) composed of relatively hydrophobic amino acids and are always found in a membrane-anchored polypeptide chain (Stegmann et al., 1989; White et al., 1982). Some fusion proteins have to undergo a pH-dependent conformational change to expose their fusogenic peptide. This is the case, for example, for the influenza hemagglutinin (Bullough et al., 1994), for the E protein of the Semliki Forest virus (Schlesinger & Schlesinger, 1986), for the G protein of the vesicular stomatitis virus (Kreis & Lodish, 1986), and for the E glycoprotein of the tick-borne encephalitis virus (Rey et al., 1995).

Many studies on peptide-induced membrane fusion have been conducted on model membranes such as liposomes and have employed synthetic peptides corresponding to the putative fusion domains of fusogenic viral proteins (Düzgünes & Gambale, 1988; Düzgünes & Shavnin, 1992; Epand & Epand, 1994; Martin et al., 1991, 1996; Nieva et al., 1994) or other, nonviral-related *de novo* synthesized peptides (Murata et al., 1992; Parente et al., 1988; Puyal et al., 1994). Usually, these fusogenic peptides exhibit a propensity that causes extensive vesicle leakage, thus demonstrating a strong membrane-destabilizing potential. Accompanying leakage, lipid mixing and contents mixing take place, reflecting the occurrence of membrane fusion. Yet the extensive perturbation of the membranes suggests that these systems suffer from poor control of the fusion event. Moreover, since the fusion-inducing peptides are added to the vesicle suspension as free monomers, which contrasts their normal membrane-anchored environment, the significance of these model systems as a truly membrane fusion-mimicking system is limited (Hoekstra, 1990). To simulate more closely membrane fusion, induced by a membrane-bound protein, we have synthesized a series of novel peptides in an effort to develop a model

[†] We acknowledge the financial support obtained from the Association pour la Recherche contre le Cancer (Grant 4006).

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[⊗] Abstract published in *Advance ACS Abstracts,* March 1, 1997.

S0006-2960(96)02212-X CCC: $14.00

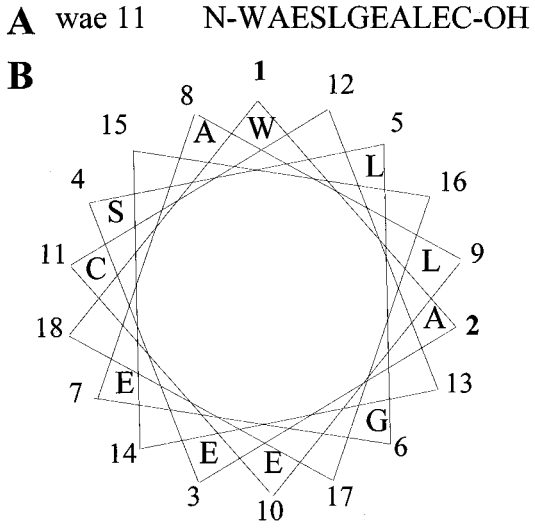

FIGURE 1: (A) Amphiphilic peptide wae 11. (B) Helical representation of wae 11.

system that displayed little if any leakage during fusion, and in which merging could be triggered by the membrane-anchored peptide. In this manner, geometrical constraints, dictated by the peptide's association with a membrane, would be implicitly taken into account.

In the present work, we describe the fusogenic properties of a small peptide consisting of 11 amino acids (Figure 1), called wae 11, which is capable of inducing membrane fusion at neutral pH, without causing any significant leakage of vesicle contents. Most interestingly, anchorage of wae 11 to a lipid membrane appears to be a prerequisite for its ability to exert its fusogenic properties. The role of charge and orientation in promoting the fusogenic ability of the peptide will be discussed.

MATERIALS AND METHODS

Chemicals. The protected amino acids and the PepSyn KA type resin used for the peptide synthesis were obtained from Neosystem and Millipore, respectively. Egg yolk phosphatidylcholine (PC),[1] L-α-dipalmitoylphosphatidylethanolamine (DPPE), cholesterol (chol), dipicolinic acid (pyridine-2,6-dicarboxylic acid, DPA), palmitoyl-L-α-lysophosphatidylcholine (LPC), and 1-monooleoyl-*rac*-glycerol (monoolein) were obtained from Sigma. $TbCl_3 \cdot 6H_2O$ (99.9% pure) was from Alfa. *N*-Succinimidyl 3-(2-pyridyldithio)-propionate (SPDP)-derivatized DPPE (PE-PDP) was synthesized as described earlier (Martin et al., 1990). DPPE covalently coupled to L-lysine (PElys) was obtained according to Puyal et al. (1995). The fluorescent lipid analogs *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (*N*-Rh-PE) and *N*-(biotinoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (biotinPE) were purchased from Molecular Probes. Saint-2 (1-methyl-4,19-*cis*,*cis*-heptatritiaconta-9,28-dienylpyridinium chloride), a cationic amphiphile, was synthesized as described elsewhere (van der Woude et al., 1997). All other reagents were of analytical grade.

Peptide Synthesis. Wae 11 (Figure 1A) (N-Trp-Ala-Glu-Ser-Leu-Gly-Glu-Ala-Leu-Glu-Cys) was synthesized via its C-terminal extremity and purified as described earlier (Puyal et al., 1994). Cysteine at the C terminus was used in coupling the peptide to liposomes, and the presence of glutamic acid residues promotes the peptide's water solubility. The peptide was dissolved in 20 mM ammonium bicarbonate at pH 8 and stored at −20 °C.

Preparation of Liposomes. All liposomes were prepared by sonication followed by extrusion. Briefly, lipids in chloroform/methanol (9:1, v:v) solutions were mixed, dried under nitrogen, resuspended in the adequate buffer (10 mM Tris and 150 mM NaCl at pH 7.4 unless otherwise stated), and sonicated for 30 min with a 50% active cycle on a VibraCell (Bioblock) at 4 °C. They were then extruded 10 times through a polycarbonate membrane with a pore size of 0.1 μm (Nucleopore Corp.). This procedure yields liposomes with a diameter of about 150 nm, as determined by dynamic light scattering in a Coulter N4S submicron particle analyzer. The size distribution was verified and confirmed by electron microscopy (Philips EM400) (not shown). In some experiments, liposomes were used that had been prepared by reverse phase evaporation (Szoka & Papahadjopoulos, 1978) and extruded as mentioned above. Since the results obtained on fusion were very similar to those obtained with sonicated liposomes, prepared as described above, we chose for reasons of convenience to use the latter ones throughout this study. Cationic liposomes were composed of PC/chol/PElys (11:6:3), or 13:6:1 in some experiments.

Peptide-coupled liposomes composed of PC/chol/PE-PDP (3.5:1.5:0.25) with or without 2 or 6 mol % biotinPE were obtained by an overnight conjugation of the peptide with liposomes via the C-terminal cysteine in a PE-PDP:peptide molar ratio of 1:5. The coupling efficiency was 10−20%, as evaluated by measuring spectrophotometrically (343 nm) released 2-mercaptopyridine (Martin et al., 1981). The peptide-coupled liposomes were purified either by gel filtration through a Sephadex G25 (PD-10 columns, Pharmacia) column or by centrifugation through a two-step dextran gradient (10 to 20%). The phospholipid concentration was measured by the method of Bartlett (1959).

Tryptophan Fluorescence Measurements. Target lipid vesicles were added to the peptide-coupled liposomes in 10 mM Tris and 150 mM NaCl at pH 7.4. Emission spectra were recorded from 300 to 400 nm with a λ_{exc} of 280 nm. The spectra were corrected for the vesicle blank (scatter) and for the dilution caused by the liposome addition.

Assays for Monitoring Vesicle Fusion. Lipid mixing of vesicles was assayed as described by Struck et al. (1981). Peptide-coupled liposomes containing 1 mol % *N*-NBD-PE and 1 mol % *N*-Rh-PE were added to a suspension of PElys-containing vesicles in 10 mM Tris and 150 mM NaCl at pH 7.4 at a lipid molar ratio of 1:6 (total lipid concentration of 70 μM). The increase in NBD fluorescence was monitored as a function of time in an SLM Aminco fluorimeter (λ_{exc} = 460 nm, λ_{em} = 534 nm) under continuous stirring. The excitation and emission band slits were 4 nm. Peak

[1] Abbreviations: PC, phosphatidylcholine; DPPE, L-α-dipalmitoylphosphatidylethanolamine; chol, cholesterol; DPA, pyridine-2,6-dicarboxylic acid; LPC, palmitoyl-L-α-lysophosphatidylcholine; PE-PDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate-derivatized DPPE; PElys, lysine coupled to L-α-dipalmitoylphosphatidylethanolamine; *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; *N*-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; biotinPE, *N*-(biotinoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; Saint-2, 1-methyl-4,19-*cis*,*cis*-heptatritiaconta-9,28-dienylpyridinium chloride; PS, phosphatidylserine.

absorbance of samples was kept at <0.1 to reduce inner filter effects. Zero percent and 100% fluorescence were taken as the intrinsic fluorescence intensity of NBD/Rh-labeled liposomes and the fluorescence obtained, after addition of 0.2% Triton X-100, corrected for detergent-induced quenching of NBD fluorescence, respectively.

Internal content mixing was assayed by using $TbCl_3$/DPA as described by Wilschut et al. (1980). Peptide-coupled liposomes, prepared in 2.5 mM $TbCl_3$, 50 mM sodium citrate, and 10 mM Hepes at pH 7.4, were added to the target liposomes loaded with 50 mM DPA, 20 mM NaCl, and 10 mM Hepes at pH 7.4 (lipid molar ratio of 1:6), suspended in 10 mM Hepes, 100 mM NaCl, and 1 mM EDTA at pH 7.4. The Tb/DPA complex was excited at 276 nm, and the fluorescence emission was detected at 545 nm. Zero percent fluorescence was taken as the intrinsic fluorescence intensity of Tb-loaded peptide-coupled liposomes. To determine the 100% fluorescence, sodium cholate (0.5% w:v) was added to a cuvette containing 20 μM DPA and Tb vesicles in Hepes buffer without EDTA.

Measurements of Peptide-Induced Leakage. Wae 11-induced leakage was determined by adding wae 11-coupled vesicles to a suspension of positively charged target liposomes loaded with a 1:1 mixture of the Tb and DPA solutions as described above. Leakage will be revealed as a decrease of Tb/DPA fluorescence due to immediate dissociation of the complex by EDTA, present in the incubation medium (10 mM Hepes, 100 mM NaCl, and 1 mM EDTA at pH 7.4). The 0% fluorescence was obtained by adding sodium cholate (0.5% w:v) to the cuvette.

Wae 11-induced leakage was also determined by adding wae 11-coupled vesicles to a suspension of positively charged target liposomes loaded with [^{14}C]sucrose in 10 mM Tris and 150 mM NaCl at pH 7.4 (10^7 cpm/mL). Radioactive marker leakage was assayed by gel chromatography on a PD-10 column (Pharmacia, Sweden). The collected fractions (500 μL) were assayed for phosphate and radioactivity.

RESULTS

Construction of Wae 11. As part of their mechanism of action, fusogenic peptides are thought to penetrate into bilayers, which results in substantial membrane perturbation, as reflected by extensive leakage of aqueous contents, bounded by the fusing membranes. Therefore, a relatively short peptide was constructed to avoid the potential ability of the peptide to traverse the membrane upon penetration. It was reasoned that, in this manner, membrane perturbation would be limited, thereby largely preventing release of vesicle contents. Another prerequisite would be its water solubility, thus allowing coupling to and exposure at the liposomal surface. Hence, Ser and Glu residues were included to provide hydrophilicity to the structure. The residues were positioned according to a helical structure (Figure 1B) (Epand et al., 1995) so that a segregated distribution between hydrophilic and hydrophobic surfaces was obtained. It was reasoned that a hydrophobic surface would facilitate the peptide's interaction with a target membrane. Furthermore, to allow liposomal coupling by disulfide bonding, a Cys residue was positioned at the C terminus, while a Trp residue was located at the N terminus. The latter would allow for monitoring of the interaction of the N terminus with the target membrane. Note that the peptide carries an overall negative charge.

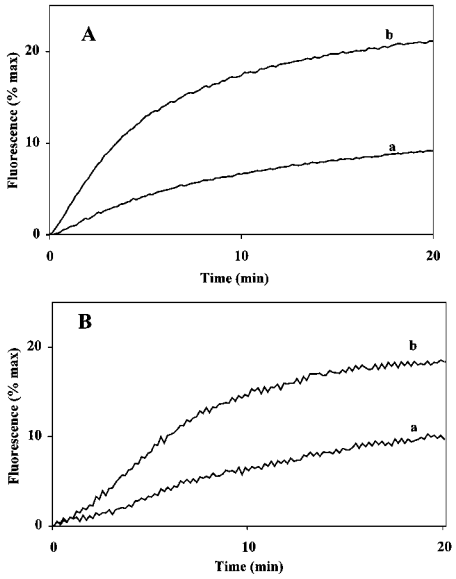

FIGURE 2: Time course of lipid and internal contents mixing upon interaction of positively charged target and peptide-coupled liposomes at neutral pH. (A) Lipid mixing; wae 11-coupled liposomes labeled with 1 mol % *N*-NBD-PE and 1 mol % *N*-Rh-PE were added to PElys vesicles (PElys at 15 mol %) in a lipid molar ratio of 1:6 (total lipid concentration of 70 μM). The buffer was 10 mM Tris and 150 mM NaCl at pH 7.4. The increase in *N*-NBD-PE fluorescence was recorded continuously. (B) Internal contents mixing; wae 11-coupled liposomes containing $TbCl_3$ were added to DPA-loaded PElys vesicles in the same molar ratio as for the lipid-mixing assay, in 10 mM Hepes, 100 mM NaCl, and 1 mM EDTA at pH 7.4. In both assays, the following conditions exist: (a) liposomes without biotinPE and (b) liposomes with 6 mol % biotinPE.

Liposome-Coupled but Not Free Wae 11 Strongly Promotes Membrane Fusion. In preliminary experiments, it was observed that the peptide did not interact with zwitterionic PC/chol liposomes. However, incorporation of Lys, coupled to PE, provided a cationic charge which did serve as a binding site for the negatively charged peptide. This interaction could be revealed by showing that addition of free wae 11 to C_6-NBD-PElys-containing vesicles induced clustering of the fluorescent PElys derivative (not shown), resulting in an increase in fluorescence self-quenching (increased lipid density), analogous to Ca^{2+}-induced phase separation of fluorescent PS analogs (Hoekstra, 1982) (see also below; Table 2). Hence, PElys was therefore included in the target membrane vesicles, serving as a peptide interaction site.

To investigate whether wae 11 displayed membrane fusion properties, the free peptide was added to a vesicle suspension, consisting of PC/chol (donor vesicles) and PC/chol/PElys (target vesicles). Its potency to trigger membrane merging was monitored at neutral pH. Neither by lipid mixing nor by contents mixing did the free peptide, up to molar concentrations 100-fold in excess, relative to that of the coupled peptide (see below), cause a significant change in the fluorescence signal (not shown). Hence, under these conditions, the free peptide failed to induce membrane fusion.

Interestingly, when the same experiments were carried out with PC/chol vesicles to which the peptide had been coupled, lipid mixing was readily observed (Figure 2A). To verify the validity of lipid mixing as a measure of fusion, analogous experiments were carried out using contents mixing. As shown in Figure 2B, the extent of contents mixing was very

Table 1: Effect of BiotinPE in the Membrane of Liposomes on the Initial Rate of Fluorescence Increase of the Lipid-Mixing Process[a]

biotinPE concentration (mol %)	biotinPE in wae 11-coupled liposomes (% min^{-1})	biotinPE in PElys liposomes (% min^{-1})
0.25	0.70 ± 0.060	0.55 ± 0.026
2	3.00 ± 0.100	0.75 ± 0.092
6	5.20 ± 0.130	0.69 ± 0.087

[a] BiotinPE was incorporated at indicated concentrations in the membrane of peptide-coupled or positively charged target liposomes. BiotinPE liposomes were then added to a cuvette containing target or peptide-coupled vesicles. The initial rates of lipid mixing were determined as described in Materials and Methods and expressed as initial rates of fluorescence increase (mean ± SD of at least five different experiments).

similar to that of lipid mixing, and both events leveled off after 10−15 min, reaching values of approximatively 10%. The relative initial rates, as determined from the tangents drawn to the steepest parts of the curves, were 0.89 and 0.84%/min for lipid mixing and contents mixing, respectively. Evidently, however, the initial kinetics differed, contents mixing being slightly delayed compared to the mixing of the lipids (see Discussion). Importantly, *no decrease* in Tb/DPA fluorescence was observed, a phenomenon frequently seen when monitoring fusion by contents mixing. Such a decrease results from EDTA-induced dissociation of the Tb/DPA complex, occurring upon its release into the medium (see Materials and Methods). Hence, these observations imply that the peptide-induced fusion event is essentially a nonleaky process (see below).

The foregoing experiments suggest that the peptide presumably plays a dual role. First, it serves to attach the donor vesicles to the target membrane vesicles by means of its interaction with PElys. The second function involves the actual fusion step. To investigate whether binding *per se* was relevant for subsequent fusion or whether the fusogenic capacity could be enhanced if interaction between the vesicles would be facilitated by an alternative means, the following experiments were developed. The initial approach relied on triggering vesicle aggregation via biotin−avidin interaction. Quite surprisingly, incorporation of biotinPE *per se* in the donor (i.e. peptide-coupled vesicles) was sufficient to cause a drastic enhancement in both the rate and extent of wae 11-induced vesicle fusion (Figure 2), i.e. without a need for avidin to be present in the acceptor vesicle. Thus, upon incorporation of 6 mol % biotinPE, the extent of fusion increased by a factor of 2, leveling off at about 20%, while the rates of contents and lipid mixing increased 3−4-fold, reaching values of 2.7 and 3.0%/min, respectively.

To exclude a direct role of the biotinPE in fusion itself, potential fusion of peptide-devoid vesicles with PElys target vesicles was also examined. No lipid mixing was seen under these conditions. Furthermore, the enhancement of fusion only occurred when biotinPE was located in the peptide-containing bilayer. Thus, identical rates of fusion were obtained irrespective of the presence of biotinPE in the target membrane (Table 1).

These data suggested that biotinPE might therefore facilitate fusion by interacting with PElys, thus providing an alternative means of aggregating donor and target membranes. To obtain further support for this suggestion, we examined whether a correlation existed between the rate of fusion and the biotinPE and PElys concentrations in the various membranes. As shown in Figure 3, at a given biotinPE concentration, the rate of fusion increases almost linearly with the concentration of PElys in the target membrane. These data are consistent with the suggestion that the stimulating effect of biotinPE is accomplished via its interaction with PElys in the target membrane, likely by facilitating vesicle aggregation.

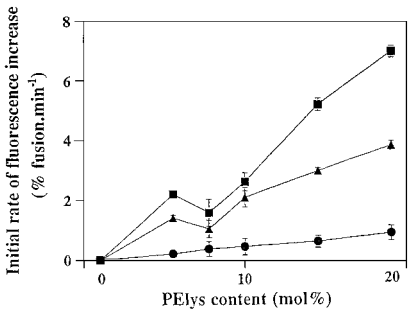

FIGURE 3: Influence of the PElys content on the initial rate of lipid mixing. Experimental conditions are identical to those in Figure 2. Liposomes without (●) and with 2 mol % (▲) or 6 mol % (■) biotinPE. Data are mean ± SD of three different liposome preparations.

We subsequently examined whether the presence of biotinPE in one vesicle population and PElys in the other could affect the ability of the *free* peptide to induce their fusion. When the different populations had been preincubated for 2 min, prior to addition of a 10- or 100-fold molar excess of wae 11, relative to the molar concentration of vesicle-bound wae 11, no significant lipid dilution was observed. When at the same conditions free wae 11 was added to biotinPE vesicles, followed by PElys vesicles, no significant fluorescence development was seen in the case of a 10−20-fold molar excess of the peptide. Only at an excessively high concentration of free peptide, i.e. at a 100-fold excess compared to the bound peptide, was some dilution apparent. However, the extent of dilution reached after 5 min amounted to less than 15% of the fluorescence level reached, when using peptide-coupled liposomes (cf. Figure 2). Hence, also at these extreme conditions, the bound peptide displayed fusogenic properties that were far superior compared to those of the free form.

To further define the role of PElys and biotinPE in the overall fusion process, we next examined their effects on vesicle aggregation.

Vesicle Aggregation Is the Rate-Limiting Step in Wae 11-Induced Fusion. To elucidate the role of biotinPE and PElys in the peptide-induced fusion process, we examined vesicle aggregation as the initial step in the overall event, over the same time interval as the fusion event. As shown in Figure 4, the inability of the peptide-coupled vesicles to fuse with neutral PC/chol vesicles is related to the fact that vesicle aggregation does not occur (curve d *vs* curve a in Figure 4A). However, incorporation of PElys in the target vesicles allowed aggregation to occur. This event is further facilitated when biotinPE is included in the peptide-coupled vesicles (curves a−c, representing 0, 2, and 6 mol % biotinPE, respectively, in Figure 4A), but *not* when incorporated in the target vesicles (Figure 4B). Thus, under these latter conditions, no effect of biotinPE on aggregation is observed. Note that these effects on aggregation entirely match the effects seen on fusion (Figures 2 and 3, Table 1). It is of interest to emphasize that, at the higher concentration range

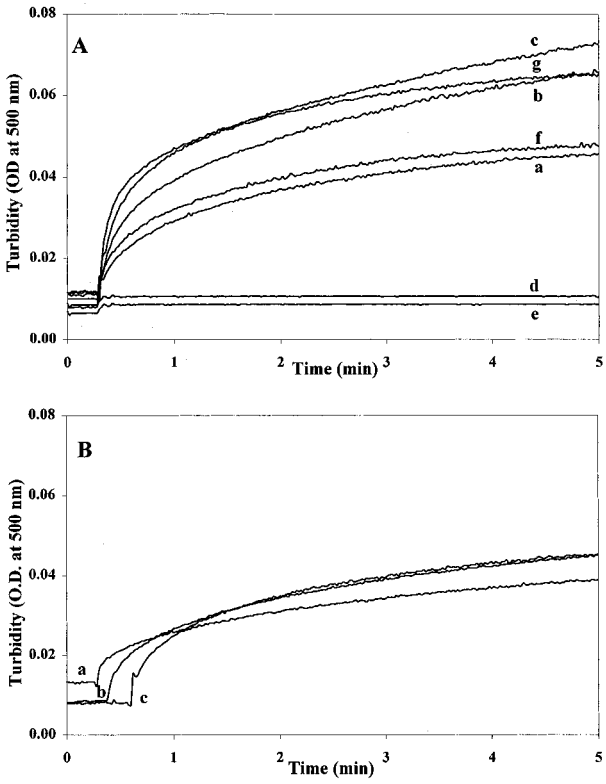

FIGURE 4: Time course of vesicle aggregation between positively charged and peptide-coupled liposomes at neutral pH. Aggregation was followed by measuring the turbidity of the suspensions at 500 nm. (A) Influence of the incorporation of biotinPE in the membrane of wae 11 liposomes on aggregation: cationic vesicles *vs* wae 11 liposomes without (a) and with 2 mol % (b) or 6 mol % (c) biotinPE, (d) PC/chol vesicles *vs* liposomes without biotinPE, and cationic vesicles *vs* peptide-free liposomes without (e) and with 2 mol % (f) or 6 mol % (g) biotinPE. (B) Influence of the incorporation of biotinPE in the membrane of PElys liposomes on aggregation: (a) 0.25, (b) 2, and (c) 6 mol % biotinPE. The total lipid concentration was 70 μM, and experiments were done in 10 mM Tris and 150 mM NaCl at pH 7.4.

of biotinPE (6 mol %, curve g in Figure 4A), the kinetics of vesicle aggregation are independent of the presence of the peptide (compare curves c and g). This finding would thus be consistent with the suggestion that at those conditions vesicle aggregation is entirely mediated by an interaction between biotinPE and PElys, whereas at a lower biotinPE concentration (e.g. 2 mol %, compare curves f and b), the peptide itself also participates in causing vesicle aggregation.

Finally, curve d was also obtained when the free peptide (40-fold molar excess, relative to the bound peptide) was added to either PElys- or biotinPE-containing vesicles.

Peptide-Induced Fusion Is Not Specific for PElys. As indicated above, electrostatic interactions in all likelihood provide a means for peptide/PElys-mediated aggregation. This led to the question of whether the interaction between the peptide and PElys displayed specificity ("ligand-receptor"-like interaction) with regard to the overall fusion process. However, as can be inferred from the previous paragraph, the biotinPE/PElys-mediated aggregation (curve c *vs* g in Figure 4A) would argue against such a specificity. Nevertheless, to obtain further support for nonspecificity, two different positively charged lipid-like compounds, i.e. stearylamine and a cationic amphiphile, Saint 2 (see Materials and Methods), were examined for their ability to mediate peptide-induced fusion.

Stearylamine, when substituted for PElys, did not show any tendency to promote peptide-induced fusion. This is likely due to the fact that the (small) charged head group is buried at the bilayer−water interface. When the same experiments were carried out with Saint 2 instead, which contains a surface-protruding, charged pyridinium head group, membrane fusion was fully restored and rates that were even slightly higher than those obtained with PElys were observed (not shown). These data thus indicate that charge, providing a means for close apposition of membranes, appears to be the primary parameter in mediating fusion, as induced by the membrane-coupled wae 11 peptide.

Overall Membrane Destabilization Is Negligible in Wae 11-Induced Fusion. The overall similarity between the kinetics of fusion, monitored by lipid and contents mixing, already indicated that the fusion event, as induced by the coupled peptide, is not accompanied by a significant perturbation of the membranes. Indeed, as noted above, no decrease in Tb/DPA fluorescence was seen, implying that the complex was effectively shielded from EDTA present in the incubation medium. To corroborate this suggestion, the fluorescent Tb/DPA complex was encapsulated in the target membrane vesicles, and wae 11-coupled PC/chol vesicles, with or without biotinPE, were added (buffer containing 1 mM EDTA). Immediately after addition of the peptide vesicles, a very small "burst" of leakage was seen, which amounted to less than 2% when biotinPE-devoid vesicles were used, and some 4−5% in the case where vesicles contained 6 mol % biotinPE. These extents of leakage were obtained after 2−3 min, the half-times for the burst being approximately 20−30 s (not shown). Also over an extended incubation time interval, release of contents was essentially negligible. When the target liposomes were loaded with [^{14}C]sucrose, followed by addition of the peptide-coupled liposomes, no leakage was observed over a time span of 2 h, whereas some 4% leakage was seen when the peptide vesicles also contained 6 mol % biotinPE.

In spite of a minimal overall perturbation of the merging membranes in the present system, it is obvious that some departure from the bilayer structure, albeit transiently, must take place to allow fusion to occur. The purpose of the following experiments was to obtain further insight into the nature of such transitions and thereby insight into the mechanism by which wae 11 triggered fusion.

Mechanism of Wae 11-Induced Fusion. Wae 11 is a net-negatively charged peptide, as it contains three Glu residues. Apparently, neutralization of the charges, required for expressing the fusogenic properties of certain viral peptides, is not a prerequisite for wae 11-induced fusion. The experiments described above were carried out at neutral pH. Figure 5A shows wae 11-induced fusion as a function of pH. At a relatively high amount of biotinPE (6 mol %), fusion proceeds largely in a pH-independent manner, with a slight tendency to decrease at pH values below 5.5. Particularly at 2 mol % biotinPE, the decrease in fusion activity at mild acidic pH is quite pronounced. Note that, at the latter conditions, the peptide contributes substantially to the process of vesicle aggregation, as described in the previous paragraph (Figure 4A,B), while at 6 mol %, the kinetics of vesicle aggregation are independent of the presence of the peptide. In Figure 5B, it is shown that this distinction in biotinPE concentration-dependent, peptide-mediated aggregation also holds as a function of pH. Thus, the rate of aggregation of

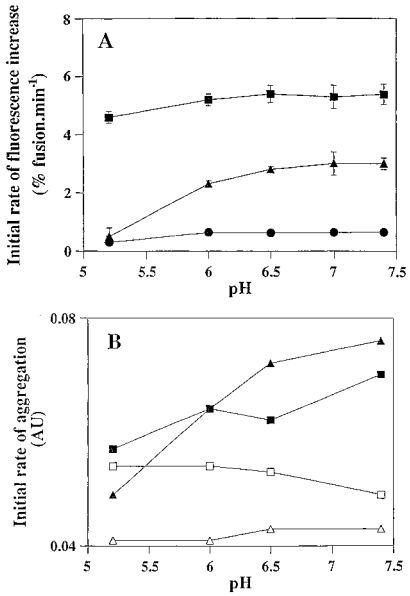

FIGURE 5: Influence of the pH on the initial rate of lipid mixing (A) and aggregation (B). Experimental conditions are identical to those in Figure 2. (A) Liposomes without (●) and with 2 mol % (▲) or 6 mol % (■) biotinPE. Data are mean ± SD of three different experiments. (B) Liposomes without wae 11 (open symbols) and wae 11-coupled liposomes (closed symbols).

peptide-coupled vesicles with target membrane vesicles containing 2 mol % biotinPE strongly decreases when the pH decreases. By contrast, only a minor decrease in aggregation was seen at mild acidic pH when the vesicles contained 6 mol % biotinPE. Note that aggregation of PElys- and peptide-devoid, biotinPE-containing vesicles was not affected by pH. It is generally assumed that hydrophobic interactions play a key role in the mechanism of peptide-induced fusion, involving penetration of at least part of the peptide into the target membrane. *A priori*, the diminished tendency of the negatively charged peptide to trigger fusion at mild acidic pH is, therefore, quite remarkable. To obtain further insight into the nature of the interaction of the peptide with the target bilayer, potential changes in Trp fluorescence were investigated. It has been well-established that the intrinsic fluorescence of Trp increases when the amino acid senses a more hydrophobic environment. Concomitantly, a blue shift in the emission maximum is seen. These parameters were subsequently monitored for the wae 11 peptide, taking advantage of the localization of Trp at the N terminus (Table 2). In the presence of PC/chol vesicles, no change in intrinsic fluorescence of Trp was apparent when the free peptide was added, consistent with its inability to induce either aggregation or fusion. Inclusion of PElys in the PC/chol bilayer suggests that in this case some interaction occurs, as reflected by a marginal increase in the intrinsic fluorescence and a small blue shift of the emission maximum. This observation is consistent with results described above, showing that the free peptide causes clustering of fluorescently labeled PElys, which implies that the peptide does interact with the bilayer in this case. However, anchorage of wae 11 to the liposomes had a pronounced effect on the Trp intrinsic fluorescence, provided the target membranes contained PElys. Under those conditions, prominent blue shifts were seen, accompanied by a substantial increase in intrinsic fluorescence (Table 2). Hence, these data indicate a migration of Trp to a hydrophobic environment and would support the view that coupling of the peptide to the vesicle surface promotes the ability of the peptide to engage in hydrophobic interactions with the target membranes.

Table 2: Fluorescence of Free and Liposome-Coupled Wae 11, Interacting with Lipid Vesicles

condition	$\Delta\lambda_{max}$[a] (nm)	F/F_0 (340 nm)[b]
free wae 11 *vs* PC vesicles	0	1
free wae 11 *vs* PElys vesicles	5	1.03
PC/chol−wae 11 *vs* PC vesicles	0	1
PC/chol−wae 11 *vs* PElys vesicles	11	1.25
PC/chol/biotinPE−wae 11 *vs* PElys vesicles		
2 mol % biotinPE	12	1.41
6 mol % biotinPE	12	1.25

[a] Emission spectra (λ_{exc} = 280 nm) of wae 11 peptide, free or coupled to liposomes, were recorded from 300 to 400 nm before and after addition of indicated vesicles. $\Delta\lambda_{max}$ indicates the shift of the maximum spectral position toward lower wavelengths (blue shift). [b] The increase in the fluorescence quantum yield is expressed as F/F_0, where F and F_0 denote the fluorescence intensity at 340 nm after and before the addition of vesicles, respectively. The lipid:peptide molar ratio is 200 in all cases. Experiments were done in 10 mM Tris and 150 mM NaCl at pH 7.4. Data are mean values obtained in two independent experiments.

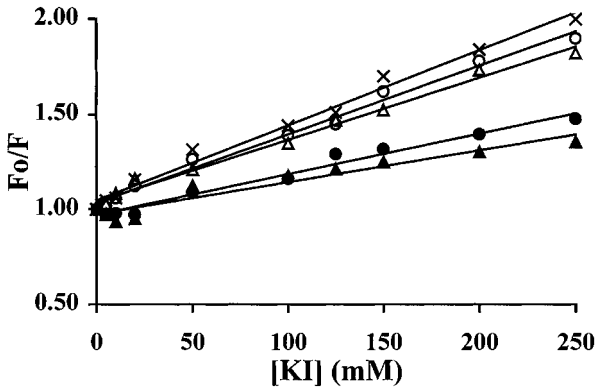

FIGURE 6: Stern−Volmer plots of Trp fluorescence quenching by I^- of wae 11 coupled to PC/chol liposomes in the absence (open symbols) or presence (closed symbols) of PElys vesicles at a lipid:peptide molar ratio of 200. Liposomes without (○, ●) or with 2 mol % biotinPE (△, ▲) were preincubated for 1 min with PElys vesicles, and increasing concentrations of KI were added. Results are expressed as F_0/F, where F_0 denotes the fluorescence intensity at 340 nm in the absence and F the fluorescence intensity in the presence of aqueous quencher. (×) Wae 11-coupled liposomes incubated in the presence of PC/chol vesicles.

Part of these hydrophobic interactions may involve penetration of the peptide. Since the model system used in the present study is essentially nonleaky, penetration must be very subtle, without any significant (overall) membrane perturbation. To obtain some evidence for penetration rather than folding of the peptide along the vesicle surface, Trp fluorescence quenching studies were carried out, using the aqueous quencher KI. As shown in Figure 6, quenching by KI was decreased when the peptide-coupled vesicles were incubated in the presence of PElys-containing target membrane vesicles (compare open *vs* closed symbols). Hence, the inaccessibility of the Trp residues to the aqueous quencher would be consistent with (at least partial) peptide penetration.

Along the line of current concepts concerning mechanisms of membrane fusion, (partial) penetration of a peptide into a bilayer will give rise to increasing negative monolayer curvature. The latter is thought to facilitate fusion, involving the formation of a "stalk" which represents a transient, highly

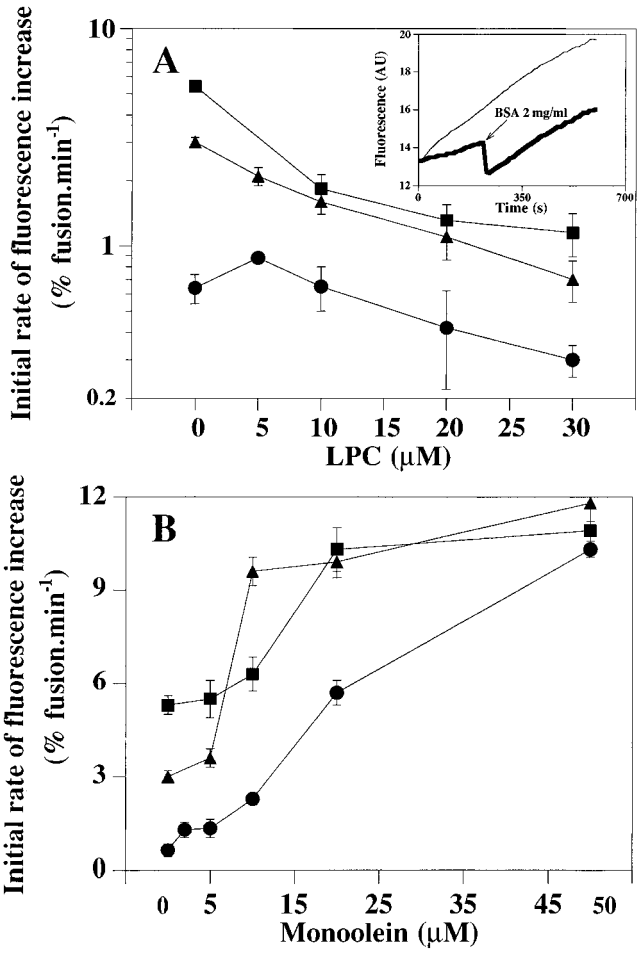

FIGURE 7: Effects of LPC and monoolein on wae 11-induced lipid mixing. PElys liposomes were incubated with increasing concentrations of LPC (A) or monoolein (B) for 3 min at room temperature, and wae 11-coupled fluorescent liposomes without (●) and with 2 mol % (▲) or 6 mol % (■) biotinPE were added. The total lipid concentration was 70 μM, and LPC and monoolein were present at concentrations well below the lytic threshold. Results are expressed as mean ± SD of three different experiments. (Inset) Reversibility of the inhibiting effect of LPC on liposome fusion by bovine serum albumin (uncorrected fluorescence tracing). BiotinPE-containing liposomes were added to a suspension of PElys vesicles preincubated with (thick line) or without (thin line) 30 μM LPC. After 200 s, BSA (2 mg/mL final concentration) was injected into the cuvette containing the LPC-treated liposomes. The sudden drop in the fluorescence intensity is due to the dilution caused by BSA addition.

bent lipid intermediate. This mechanism is known as the so-called "stalk hypothesis" (Chernomordik et al., 1995b; Epand et al., 1994). Hence, the hydrophobic interactions, noted above, could be a reflection of this process.

To examine this possibility further, compounds were therefore included in the wae 11 fusion system, which are known to interfere with the bilayer curvature, lysoPC inhibiting and monoolein facilitating negative spontaneous curvature and, in parallel, stalk formation and fusion, respectively. As shown in Figure 7, inclusion of LPC showed an effective, dose-dependent *inhibition* of fusion (panel A), irrespective of the presence of biotinPE in the wae 11 liposomes. The inhibition of the fusion process could be fully reversed when the inserted pool of lysoPC was removed with BSA (inset of Figure 7A), thus excluding lysoPC-induced artifacts. Moreover, when similar experiments were carried out in the presence of the (nonionic) monoolein, a drastic *increase* in the fusion activity was observed (Figure 7B). Depending on the composition, the rates increased by 3−10-fold, in the presence and absence of biotinPE, respectively. As a control, we examined the occurrence of vesicle leakage at the same conditions, employing the Tb/DPA complex as the aqueous contents marker. However, the rates of leakage in all cases were essentially similar to those obtained at control conditions, i.e. in the absence of monoolein. Thus lysis, followed by membrane reassembly, could be excluded as the cause of the stimulatory effects of the lipid. Finally, since neither the kinetics nor the extent of vesicle aggregation was affected by the presence of lysoPC or monoolein, we exclude the possibility that the effect of either compound was mediated via a direct interaction with the peptide, biotinPE or PElys.

DISCUSSION

In this work, we have shown that a short, negatively charged peptide, 11 amino acids in length, is able to trigger the fusion of zwitterionic PC/chol vesicles, provided that the peptide is coupled to the membrane surface of one population of the interacting bilayers and that the vesicles can be brought into close contact. In contrast, little if any fusion was observed upon addition of the free peptide, even at a molar excess of 2 orders of magnitude, compared to the concentration of the bound peptide.

Part of the distinction between the free and bound peptide is likely related to spatial constraints. Obviously, the multivalent character of the peptide would allow multivalent interactions, which seem to occur when the free peptide is added to PElys-containing liposomes. As revealed with NBD-labeled PElys, the free peptide causes clustering as reflected by fluorescence self-quenching, analogous to Ca^{2+}-induced phase separations triggered in PS-containing systems (Hoekstra, 1982). Presumably, the free peptide is thus capable of forming a so-called "cis" complex, i.e. between PElys molecules within the same plane of the bilayer, but *not* a "trans" complex, which would be needed to bring adjacent bilayers into close apposition. Indeed, as we have shown (Figure 4), neither PElys- nor biotinPE-containing vesicles are aggregated by the free peptide. Hence, these observations would suggest that spatial constraints are important in governing the peptide-induced fusion event. As also supported by the studies concerning changes in intrinsic Trp fluorescence (Table 2), it would appear that the coupled peptide, possibly by virtue of its restricted motional freedom, is engaged in a more "prominent" type of hydrophobic interaction than the free peptide, which appears to be largely involved in multivalent electrostatic interactions. These considerations would explain the different modes of interaction, the free peptide folding along the surface, causing clustering of PElys, while the coupled peptide is restricted from such interactions, thereby facilitating hydrophobic interactions, possibly involving some penetration into the bilayer. The KI quenching experiments revealed that at least some of the Trp residues were hidden from direct accessibility to the aqueous phase in the case of the bound peptide, but not the free peptide (Figure 6, Table 2). These distinctions are therefore consistent with the relationship between hydrophobicity and fusion susceptibility. Yet further experiments will be needed to provide direct proof for penetration. The following notions are of relevance in this context. Preliminary results obtained by Fourier transform infrared spectroscopy (FTIR) show that the peptide

adopts a β-sheet conformation when free in bulk solution but is converted into an α-helix when coupled to the liposomal surface (E. I. Pécheur, and I. Martin, unpublished observations). The latter would provide a structural motive as presented in Figure 1, in which hydrophobic and hydrophilic residues are organized on opposing faces.

The occurrence of penetration is dictated by the current concepts of protein/peptide-induced fusion, suggesting that such events are relevant for facilitating departure of the bilayer structure, a crucial intermediate step for fusion. Direct proof of penetration of some viral fusion peptides has been shown (Harter et al., 1989; Novick & Hoekstra, 1988). Note, however, that the primary sequence of the present peptide does not contain an extended sequence of hydrophobic amino acids, a typical feature of viral fusion peptides. Usually, penetration is inferred from the occurrence of extensive leakage of vesicle contents, which accompanies the fusion event (Düzgünes & Gambale, 1988; Lee et al., 1992; Murata et al., 1987; Parente et al., 1988; Puyal et al., 1994; Szoka et al., 1988). Interestingly, the system we describe is essentially nonleaky, which, to the best of our knowledge, has not been reported before. The nonleakiness can be related to several causes. First, intentionally, we synthesized a short peptide, to avoid its ability to traverse the membrane. Second, the system contains a relative high mole ratio of cholesterol, which will favor membrane sealing (Anholt et al., 1982; Vidal et al., 1984). The relative contribution of each of these parameters to the nonleakiness cannot be assessed from the present data. But whatever the exact contribution of these parameters, it seems fair to conclude that fusion proceeds in the present model system without a major perturbation of overall bilayer structure, in a manner analogous to what one expects to occur in a biological fusion event, which is thought to be a strictly controlled process.

Although the final extents of lipid mixing and contents mixing are very similar, the initial kinetics of both processes are different, contents mixing being slightly delayed. Possibly, this distinction reflects the sequential occurrence of hemifusion events (outer leaflet and inner leaflet mixing, respectively) and pore formation, only the latter process giving rise to contents mixing. The nature of the transient bilayer-to-nonbilayer transition, which necessarily must occur upon wae 11-induced fusion, fits with the currently advocated model, involving formation of a highly curved nonbilayer structure, a so-called stalk. Originating from molecular factors that influence bilayer curvature, lysoPC and monoolein modulate the wae 11-mediated fusion process as anticipated, showing an inhibition and promotion of fusion, respectively (Figure 7). Note that in our model system negative bilayer curvature, i.e. formation of the fusion intermediate, can be facilitated by cholesterol, a lipid of negative spontaneous curvature, which should facilitate stalk formation (Chernomordik et al., 1995b). Although some amphiphilic peptides are known to counteract negative bilayer curvature, and thereby inhibit fusion (Cheetham et al., 1994; Chernomordik et al., 1995b; Colotto et al., 1996; Damodaran & Merz, 1995; Epand et al., 1994; Siegel, 1993), the wae 11 peptide apparently favors negative bilayer curvature.

Taking into account the fact that the peptide contains three Glu residues which are negatively charged at pH 7, it would have been anticipated that charge neutralization could exert a pronounced effect on the peptide's ability to trigger fusion, for example by facilitating hydrophobic interactions. However, as could be revealed by including biotinPE in the peptide-coupled vesicles, an entirely different picture emerged, when studying the aggregation and fusion activity as a function of pH (Figure 5A,B). As can be concluded from these data, rather than the fusion activity, the binding function of the peptide is affected by pH, showing a diminished reactivity when the pH decreases. By contrast, the fusion activity appears to be *independent* of pH, being sustained at mild acidic pH. These conclusions can be drawn from the observation that the primary function of biotinPE and PElys is causing the vesicles to aggregate (Figure 5B). In the absence of biotinPE, the peptide itself fulfills this function via its ability to engage in electrostatic interactions with the Lys residue coupled to PE. Thus, biotinPE is capable of inducing vesicle aggregation, while wae 11 is needed to induce membrane fusion. As revealed by the aggregation experiments, at a low (2 mol %) biotinPE concentration, aggregation is stimulated in the presence of the peptide. At higher concentrations (e.g. 6 mol %), essentially the same rates and extents of aggregation are obtained, irrespective of the presence of the peptide. Thus, the decrease in fusion activity that is observed as a function of pH when using vesicles with 2 mol % biotinPE must be a reflection of a diminished degree of aggregation, rather than a decrease of fusion *per se*. Indeed, when aggregation was monitored as a function of pH, aggregation of vesicles that contained 2 mol % biotinPE appeared to be severely impaired at low pH, whereas vesicles containing 6 mol % biotinPE aggregate almost as effectively over the entire pH range. Consistent with these notions is the observation that fusion also remains essentially constant between pH 5.2 and 7.4. Evidently, upon the peptide nearing charge neutralization, a diminished tendency of the peptide to engage in electrostatic interaction with PElys at mild acidic pH is readily explained. As a consequence, a decrease in vesicle aggregation may therefore be expected. Finally, it is remarkable that the protonation of the acidic residues does not increase the fusion activity. It would thus appear that hydrophobicity *per se* is not the sole driving force for peptide-induced fusion. Rather, the overall structure, presumably in conjunction with the spatial orientation, is an important parameter in triggering and regulating peptide-induced fusion, as has been recently shown by Gray et al. (1996) for the influenza hemagglutinin fusion peptide. Indeed, preliminary evidence showed that fusion is eliminated when substituting Glu for His, irrespective of the pH of the incubation. Work is in progress to further define these parameters, using wae 11 peptide analogs.

It is important to emphasize that the presence of biotinPE did not seem to affect the properties of the peptide, neither by a direct interaction nor by altering the peptide's vicinity (Table 2). When biotinPE was incorporated into the target membrane, no effect on aggregation or fusion was observed. In fact, the aggregation experiments in the presence and absence of the peptide, as well as the correlation between the concentrations of biotinPE and PElys *vs* fusion activity, clearly indicated that the lipid analogs provide the chance for the vesicles to aggregate, most likely via electrostatic interactions. Indeed, the use of biotinylated PE allowed us to define the pH dependence of wae 11-induced fusion and the requirement for peptide−bilayer anchorage (as opposed to the free peptide) to cause membrane fusion. Besides, it has been reported that biotin has an affinity for Lys residues, as binding to (strept)avidin involves the imidazolidone ring

of the biotin and the ϵ-amino group of Lys residues (Gitlin et al., 1987; Grubmüller et al., 1996; Wilchek & Bayer, 1989). However, substitution of PElys for another cationic lipid (Saint-2) sustained the ability of wae 11 to trigger fusion; therefore, specificity for a target membrane receptor is not required for conveying fusogenic properties to the peptide. Rather, the mode of interaction with the membrane *per se*, when comparing the results of the free *vs* bound peptide, seems relevant for bringing about fusion. The nature of that interaction is then at least in part governed by the covalent binding to a membrane, resulting in motional restrictions or "controlled" conformational changes, such as adopting α-helical secondary structure, as opposed to β-sheet which is exhibited by the free peptide (see above, unpublished observations).

The present observations may also be of relevance in the context of some recent studies in which the fusogenic properties of GPI-anchored mutants of influenza HA (Kemble et al., 1994; Melikyan et al., 1995) and HIV gp120/41 (Weiss & White, 1993) were examined. In these studies, the GPI-anchored fusion proteins did not induce syncytium formation (HIV) or did not carry the fusion step beyond that of hemifusion (merging of outer leaflets only). It could thus be suggested that the transmembrane parts of these proteins are relevant in leading fusion to completion, i.e. triggering the merging of the inner leaflets which would allow formation of the fusion pore, necessary for contents mixing. In conjunction with the present work, it would appear that the nature of the membrane anchor codetermines the ability of peptide or protein-anchored systems to propagate the overall fusion event, leading to contents mixing. Hence, we can conclude that, for a membrane-associated peptide (protein), the presence of a transmembrane moiety is not a prerequisite for inducing fusion.

ACKNOWLEDGMENT

We thank Dr. Michel Vidal for helpful discussions on fluorescence experiments.

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BI9622128