Comparison of lipid vesicle fusion induced by the putative fusion peptide of fertilin (a protein active in sperm-egg fusion) and the NH2-terminal domain of the HIV2 gp41

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Abstract A peptide representing a putative fusion domain of fertilin, a sperm surface protein involved in sperm-egg fusion was synthesized. Its interaction with model membranes was characterized and compared with that of a synthetic peptide representing the fusion peptide of HIV-2rod gp41. The fertilin fusion peptide interaction with lipid vesicles is dependent upon the presence of negatively charged lipids in the membrane. Its fusogenic activity does not require PE and is not inhibited by addition of lysolecithin in the medium. These conditions are quite opposite to those obtained with the HIV2 peptide and suggest that the lipid mixing mediated by the two peptides corresponds to two different molecular mechanisms.

Key words: Fusion peptide; Membrane interaction; HIV-2 gp41; Fertilin

1. Introduction

Membrane fusion is an essential event in cell biology and plays a crucial role in important physiological processes such as endocytosis/exocytosis, fertilization or viral infection. Studies on the molecular mechanism of viral fusion have allowed identification and characterization of a specific fusion protein [1]. One of the main characteristics of most viral fusion proteins is the presence of a relatively hydrophobic amino acid stretch, often referred as 'fusion peptide'. The biophysical properties of such fusion peptides, especially the mechanism of their membrane action, are the subject of intense research [2–6].

Recently, Blobel et al. [7,8] have proposed that fertilin, a protein located on the surface of guinea pig sperm, is implicated in the process of sperm-egg fusion. This protein is composed of two subunits (α and β) and shares many characteristics in common with viral fusion proteins, suggesting that fusion events which occur during fertilization and penetration of enveloped viruses into host cells may share a common mechanism. In this context, particular interest exists in identifying and characterizing a fusion peptide in the sperm surface protein. The sequence recently suggested as a likely candidate for this role comprises residue 89–111 of the α-subunit of fertilin [8]. This region fulfills all three criteria of an internal fusion peptide: (1) located in a membrane anchored subunit; (2) relatively hydrophobic and (3) capable of being modelled as a 'sided' α-helix with most of the bulky hydrophobic residues on one face and negatively charged amino acids on the other [9]. Moreover, this peptide has been shown to interact with the membrane bilayer of liposomes and to induce lipid mixing of POPC/POPG/PE large unilamellar vesicles [10]. The resemblance between a viral fusion peptide and the fertilin fusion peptide may therefore not be entirely coincidental.

In the present work, we studied the ability of a synthetic peptide corresponding to the putative fusion peptide of fertilin: NH_2-His-Pro-Ile-Gln-Ile-Ala-Ala-Phe-Leu-Ala-Arg-Ile-Pro-Pro-Ile-Ser-Ser-Ile-Gly-Thr-Cys-Ile-Leu-Lys-COOH, to destabilize and to induce lipid mixing of large unilamellar vesicles made of lipids mimicking the plasma membrane. These properties of the fertilin fusion peptide were compared with those of a synthetic peptide corresponding to the N-terminus extremity of gp41 of HIV2rod virus: NH_2-Gly-Val-Phe-Val-Leu-Gly-Phe-Leu-Gly-Phe-Leu-Ala-Thr-Ala-Gly-Ser-Ala-Met-Gly-Ala-Ala-Ser-Leu-Thr-Val-COOH. A comparison of the role of negatively charged lipids in the membrane vesicles, of lysoPC in the external medium and of temperature, on the interaction of these peptides with lipids suggests that the two peptide-induced lipid mixing processes have different mechanisms.

2. Materials and methods

2.1. Materials

Egg phosphatidylethanolamine (PE), egg phosphatidylcholine (PC), sphingomyelin (SM), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA) and cholesterol (Chol) were purchased from Sigma Chemical Co. (St Louis, MO, USA). *N*-(Nitrobenzo-2-oxa-1,3-diazol)phosphatidylethanolamine (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (Rh-PE) were from Avanti Polar Lipids Inc. (Birmingham, AL, USA).

Calcein (Sigma) was purified by chromatography on Sephadex LH-20 (Pharmacia). Calcein was loaded on the column as a sodium salt solution and eluted with water at neutral pH. The concentration of calcein was determined spectrophotometrically by using 7.0×10^4 M^{-1} cm^{-1} as the molar extinction coefficient at 492 nm [11].

HPLC-purified synthetic peptides representing the sequence of the fertilin and HIV2 fusion peptides in their amide form were purchased from Chiron mimotope. The peptides were dissolved in DMSO at a concentration of 1 mg/ml and stored at 4°C.

2.2. Vesicle preparation

Routinely, egg PC and egg PE were dissolved in chloroform at the desired concentrations and the solution was dried under a stream of nitrogen to deposit a thin lipid film on the inside of a glass tube. The film was further subjected to vacuum evaporation for 2–3 h to remove any trace of the solvent. Large unilamellar vesicles (LUV) were prepared by hydrating the dried lipid film with HEPES buffer (10 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, 0.02% NaN_3, pH 7.4), then repeatedly freeze-thawing the suspension five times, and finally extruding it 10 times through two polycarbonate filters of pore size 0.1 μm (Nucleopore Corp., Pleasanton, CA, USA) using an extruder (Lipex Biomembranes Inc., Vancouver, Canada) according to the extrusion

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procedure of Hope et al. [12]. The prepared liposomes were stored at 4°C until use.

2.3. Fusion measurements

Two probes (NBD-PE and Rh-PE) were added to the lipid film and LUV were prepared as described above. Liposomes containing both probes at 0.6% (molar ratio) each, were mixed with probe free liposomes at a 1:9 molar ratio and a final lipid concentration of 3×10^{-4} M. Lipid mixing was determined by measuring the fluorescence intensity change resulting from the fluorescence energy transfer between NBD-PE and Rh-PE inserted into the lipid bilayer, as described by Struck et al. [13]. Briefly, fluorescence energy transfer between the two probes is dependent upon their spatial organization, any fusion between unlabeled and labeled vesicles will result in dilution of the probes in the membrane decreasing the energy transfer and increasing the fluorescence of NBD-PE. Fluorescence was monitored using an SLM 8000 spectrofluorimeter (SLM Instruments Inc., Urbana, IL, USA) with excitation and emission slits of 4 nm. The suspensions were excited at 470 nm and the NBD fluorescence was recorded at 530 nm. Experiments were conducted in a 1-ml stirred cuvette, with right angle illumination. The initial fluorescence of the 1:9 (labeled/unlabeled) suspension was taken as 0% fluorescence and the 100% fluorescence was determined using an equivalent concentration of vesicles prepared with 0.06% of each fluorescent phospholipid.

2.4. Leakage measurements

Liposomes were prepared as described above, except that the dried lipid film was rehydrated in buffer containing 62 mM calcein purified as described above. After extrusion, liposome-entrapped calcein was separated from free calcein on a Sephadex G-50 gel filtration column, and eluted with 10 mM HEPES, 150 mM NaCl, 1 mM EDTA buffer, pH 7.2. Liposome concentration was estimated by measuring the lipid phosphorus content [14]. Leakage from calcein-containing liposomes was detected as an increase in fluorescence, resulting from the relief of self-quenching upon dilution of the dye into external medium [11,15]. Calcein was detected at an excitation wavelength of 490 nm and an emission wavelength of 520 nm, employing a slit width of 4nm. The fluorescence scale was calibrated by setting the initial fluorescence of liposomes to 0% and the fluorescence at infinite probe dilution, after adding Triton X-100 (0.1% (v/v)), to 100%.

3. Results

The fusogenic activity of fertilin peptide was assessed by measuring its ability to induce lipid mixing of vesicles containing the fluorescent probes NBD-PE and Rh-PE and the label free vesicles [13] and was compared to the fusogenic activity of HIV2rod peptide. Addition of the fertilin peptide to LUV of PC/PE/SM/PS/PI/PA/Chol (17, 19, 20, 11, 2, 0.5, 30 wt%) results in an effective intervesicular lipid mixing comparable to fusion activity of HIV2rod peptide. The effect is concentration dependent and increases with increasing amounts of peptide added (Fig. 1). This lipid composition has been chosen as the most representative of the plasma membrane.

In order to explore the influence of vesicle lipid composition on lipid mixing, experiments were carried out with different lipid mixtures. The percentage of fusion was measured after addition of HIV2rod or fertilin peptide to a suspension of vesicles of different compositions (Table 1). The total lipid/peptide molar ratio was 40 and the percentage of fusion measured 20 min after addition of peptide. As previously demonstrated [2], HIV peptide induces lipid mixing only of liposomes containing PE in their bilayer and the efficiency of this fusion process increases with the concentration of PE in the lipid bilayer (Table 1). Analysis of data in Table 1 reveals that negatively charged lipids like PI or PS greatly increase the observed lipid mixing induced by fertilin peptide. Unlike HIV peptide, addition of PE to liposomes containing a negatively charged lipid (PI or PS) reduces the fusion activity.

Table 1 confirms a qualitative correlation between the destabilizing efficiency and the fusogenic activity of the two peptides. Addition of the two peptides caused the rapid release of the calcein from LUV of PC/PE/SM/PS/PI/PA/Chol (17, 19, 20, 11, 2, 0.5, 30 wt%), while HIV2rod fusion peptide is less efficient in inducing calcein release than fertilin fusion peptide (Fig. 3).

3.1. Effect of temperature

Lipid mixing induced by HIV2rod fusion peptide depends on temperature as shown in Fig. 2A. The fluorescence increase between 30 and 40°C is associated with the transition of PE from a bilayer to a non-lamellar lipid structure [15].

In the presence of fertilin peptide, fusion occurs over a wide temperature range (5–45°C) and no increase at the PE transition temperature could be detected, suggesting that these two peptides induced lipid mixing through two different mechanisms.

3.2. Effect of lysoPC

It has been previously shown that addition of exogenous lysoPC to the medium resulted in concentration-dependent inhibition of liposome fusion induced by HIV or SIV fusion peptide [16,17]. We assessed the effect of lysoPC in the fusion buffer, on lipid mixing induced by HIV2rod and fertilin peptide. Liposomes of PC/PE/SM/PS/PI/PA/Chol (17, 19, 20, 11, 2, 0.5, 30 wt%) were incubated with various amounts of lysoPC at 37°C. After 15 min of incubation, the peptides were added to the liposomes, and the NBD-PE fluorescence was measured. LysoPC inhibits lipid mixing induced by HIV2rod but has no effect on the vesicle fusion induced by fertilin peptide even at high concentration (>50 μg) (Fig. 2B).

Table 1
Influence of bilayer lipid composition on fusion peptide-induced lipid mixing and release of vesicle contents

Lipid composition	Fertilin-induced lipid mixing (%)[a]	Fertilin-induced release (%)[a]	HIV2rod-induced lipid mixing (%)[a]	HIV2rod-induced release (%)[a]
Egg PC	0	<10	0	0
PC/PE (50/50)	0	0	50	30
PC/PS (80/20)	50	75	0	0
PC/PI (80/20)	50	80	0	0
PC/PE/PI (50/30/20)	30	35	30	10
PC/PE/PS (50/30/20)	n.d.	40	n.d.	10
Plasma membrane lipids[b]	30	60	30	40

[a]% release or % lipid mixing measured 20 min after addition of fusion peptide (10 μg). Lipid concentration was 13 μM. The values in this table represent means of five different experiments with a standard deviation of ±3%.
[b]An artificial mixture of lipids mimicking the composition of the plasma membrane: PC/PE/SM/PS/PI/PA/Chol (17, 19, 20, 11, 2, 0.5, 30 wt%).
n.d., not determined

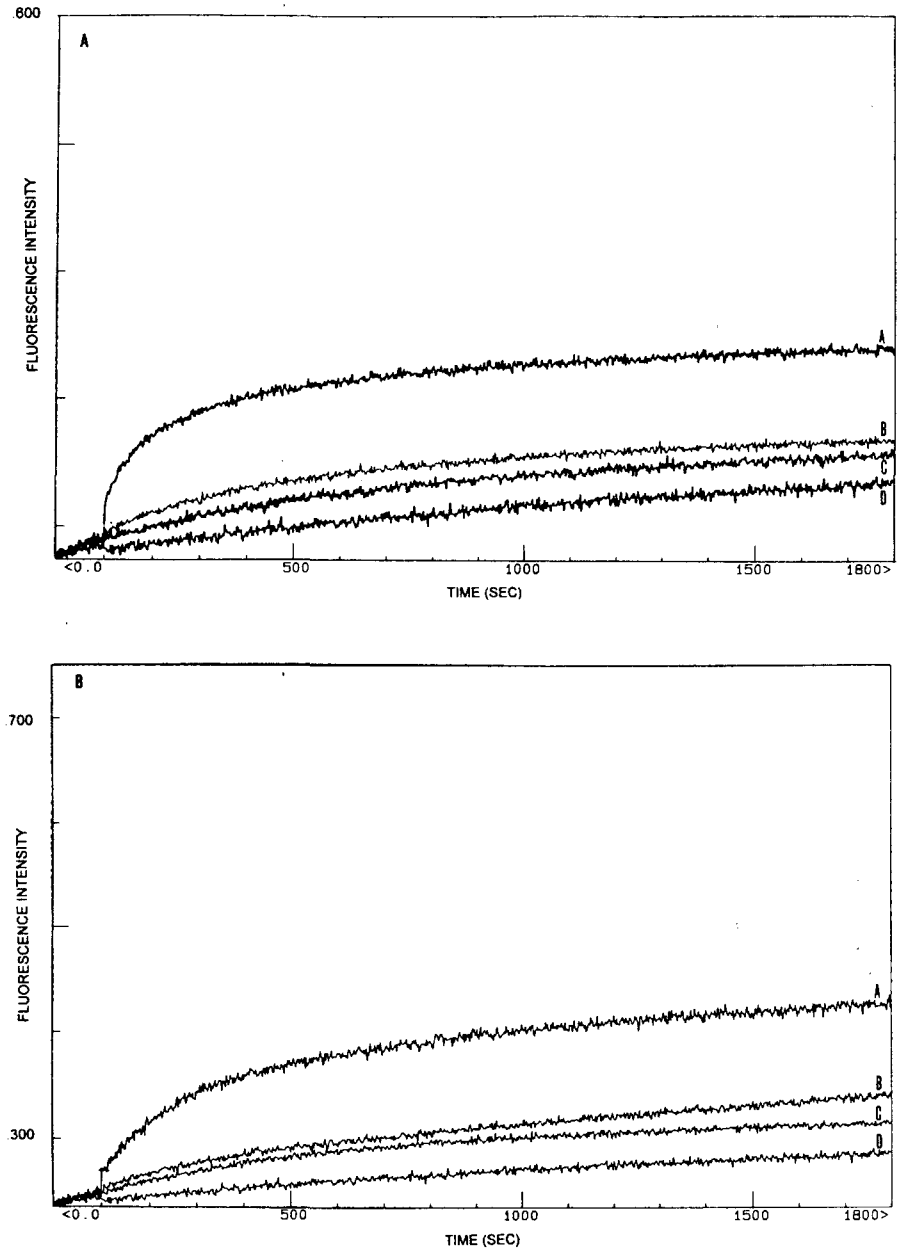

Fig. 1. Time course of fertilin fusion peptide (A) and HIV2rod peptide (B) induced lipid mixing of large unilamellar vesicles (LUV PC/PE/SM/PS/PI/PA/Chol). Small aliquots of fusion peptide/DMSO solution were added at time = 100 s to vesicles at 37°C. Experimental conditions: total lipid concentration was 3×10^{-4} M and the peptide concentration (a) = 30 μg/ml, (b) = 20 μg/ml, (c) = 15 μg/ml and (d) = 10 μg/ml. Dimethyl sulfoxide (DMSO) up to 2% (v/v), which is the maximal concentration used, did not modify the fluorescence.

4. Discussion

The hydrophobic region located at the amino-terminal extremity of transmembrane envelope glycoproteins of several enveloped viruses (paramyxo-, orthomyxo-, and retroviruses) has been proposed to play a role in the fusion process and is referred to as the fusion peptide (for review, see [9]). In HIV-1 and SIV, this hydrophobic domain is located at the N-termini of gp41 and gp32. The presence of a putative fusion peptide in the α-subunit of fertilin, a protein involved in sperm-egg fusion has also been suggested [8]. This peptide is not an N-terminal peptide and corresponds to a sequence located internally in the protein (residues from 89 to 111).

The present data demonstrate that synthetic peptides representing the putative fusion peptide of fertilin and of HIV2 gp41 interact with phospholipid bilayers and that binding of the peptides to the vesicles is followed by intervesicular lipid mixing as demonstrated by the resonance energy transfer assay.

Interaction of fertilin fusion peptide with lipid membrane requires the presence of negatively charged lipids in the membrane, since lipid mixing and calcein release were not observed from egg PC vesicles or PC/PE vesicles even at a high lipid/peptide molar ratio. The increased effect of fertilin peptide in the presence of acidic lipids could be due to increased binding of peptide to a negatively charged surface. The absence of fertilin peptide-induced fusion and calcein release from neutral vesicles (Table 1) suggests that negative charges are essen-

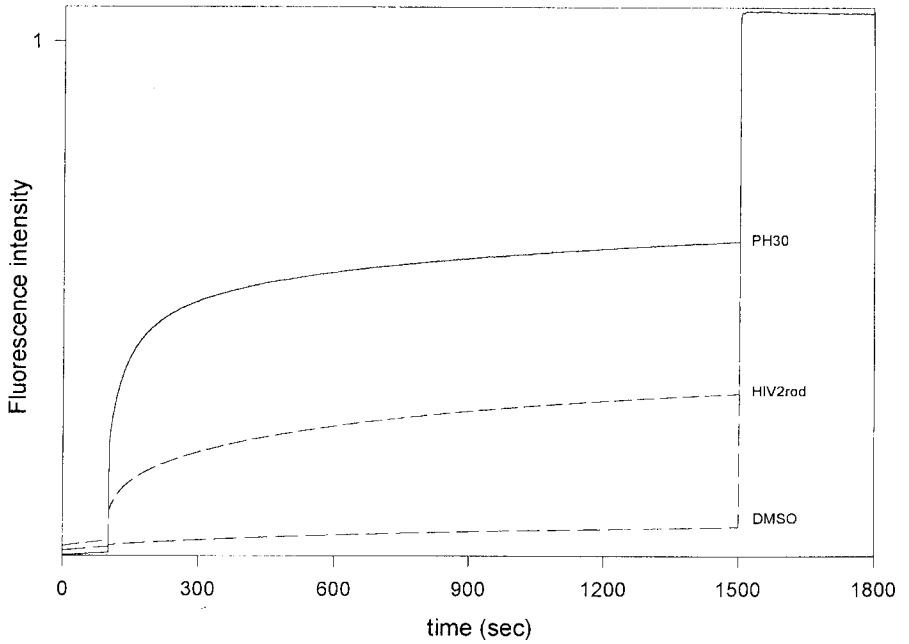

Fig. 2. Time course of fertilin fusion peptide and HIV2rod induced calcein release from large unilamellar vesicles (LUV PC/PE/SM/PS/PI/PA/Chol). Small aliquots of fusion peptide/DMSO solution were added at time = 100 s to calcein-loaded vesicles at 37°C. Experimental conditions: total lipid concentration was 3×10^{-4} M and the peptide concentration 1.3×10^{-5} M. Dimethyl sulfoxide (DMSO) up to 2% (v/v), which is the maximal concentration used, did not modify the fluorescence.

tial for the peptide either to bind at the vesicle surface or to induce a conformational change which would allow its insertion in the lipid membrane. The characterisation of secondary structures of this peptide as a function of the presence of negatively charged vesicles is under investigation using Fourier transform infrared spectroscopy. A first experimental study on this peptide by CD and FTIR measurements indicates that the preferred conformation of the peptide in the lipid environment is a β-sheet structure [10]. However, in these experiments, the unbound peptide was not separated from the inserted peptide, and the observed β-sheet structure could be due to the aggregated population. In the case of HIV fusion peptide, the determining factor for an interaction of the peptide with lipid membrane is the presence of PE in the bilayer as has been previously demonstrated [3]. HIV2 peptide induces release of PC/PE but not of egg PC or PC/PS vesicles and the presence of negatively charged lipid is not required. These results suggest that the first step of fusion process which is the binding of peptide to the lipid membrane is different for the viral infection and the sperm-egg fusion.

Another argument to say that the mechanisms used by these two peptides to induce vesicle fusion are different is that a lipid such PE, which tends to destabilize the bilayer under certain conditions, allows the development of high fusogenic activity of HIV fusion peptide but inhibits activity of fertilin peptide. Moreover, lysoPC which inhibits HIV fusion peptide-induced lipid mixing and calcein release has no effect on membrane fusion induced by fertilin fusion peptide. Our data demonstrate that fertilin fusion peptide-induced membrane fusion does not proceed via non-lipid bilayer intermediates. In the case of HIV peptide, it has been demonstrated that it is the oblique orientation of the fusogenic peptide into the lipid bilayer that induces a negative curvature of the lipid membrane, favoring inverted micelle formation, catalyzing the fusion event [15,18]. Lysolecithin was shown to modify the peptide orientation in such a way that in its new orientation, parallel to the lipid-water interface, it prevents inverted mi-

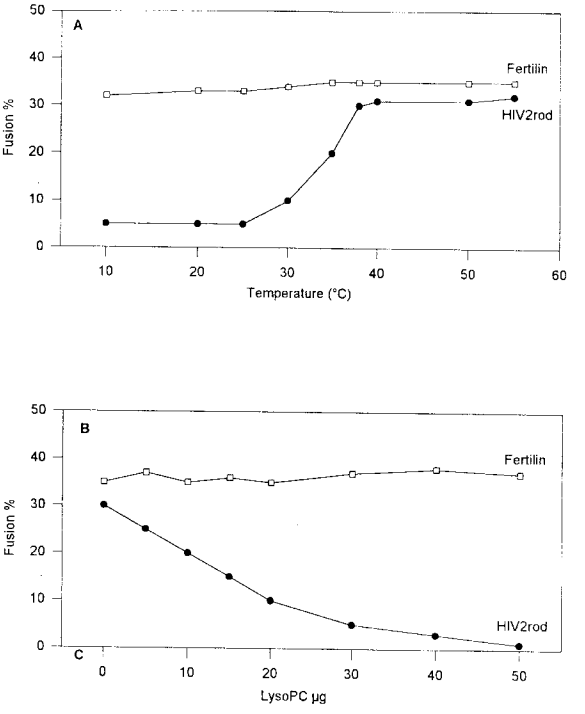

Fig. 3. Effect of temperature (A) and lysoPC (B) on the lipid mixing of LUV of PC/PE/SM/PS/PI/PA/Chol (17, 19, 20, 11, 2, 0.5, 30 wt%) in presence of HIV2rod and fertilin fusion peptides. The percent fusion values after 15 min were plotted as a function of temperature or lysolecithin concentration. The final peptide and lipid concentration were 13 and 300 μM, respectively. In the presence of lysoPC, LUV (300 μM) and lysoPC (at different concentration) were preincubated at 37°C, pH 7.2 in HEPES buffer, for 15 min prior the addition of peptide (13 μM)

celle formation, curvature modification and therefore the fusion process.

In conclusion, this paper provides evidence that the fusion peptide from fertilin and that from HIV2 gp41 act by two different mechanisms. The role of PE and lysoPC in lipid mixing induced by HIV peptide is directly correlated with its capacity to induce the hexagonal phase during fusion process. Since these two molecules have no effect on the fusion induced by fertilin peptide, we suggest that this peptide induces lipid mixing via another mechanism which does not need hexagonal phases as intermediary structures. A possible mechanism could require the formation of a transmembrane pore but this hypothesis has to be demonstrated. However, these differences have been detected only in model systems which have obvious limitations while many features of the biological system are not present. We can suggest that membrane fusion involved in viral fusion and sperm-egg fusion has a different molecular mechanism. Further studies are need to dissect these mechanisms.

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