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Enhanced efficiency of a targeted fusogenic peptide

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Abstract

Membrane targeting was investigated as a potential strategy to increase the fusogenic activity of an isolated fusion peptide. This was achieved by coupling the fusogenic carboxy-terminal part of the β-amyloid peptide (Aβ, amino acids 29–40), involved in Alzheimer's disease, to a positively charged peptide (PIP_2-binding peptide, PBP) interacting specifically with a naturally occurring negatively charged phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2). Peptide-induced vesicle fusion was spectroscopically evidenced by: (i) mixing of membrane lipids, (ii) mixing of aqueous vesicular contents, and (iii) an irreversible increase in vesicle size, at concentrations five to six times lower than the Aβ(29–40) peptide. In contrast, at these concentrations the PBP-Aβ(29–40) peptide did not display any significant activity on neutral vesicles, indicating that negatively charged phospholipids included as targets in the membranes, are required to compensate for the lower hydrophobicity of this peptide. When the α-helical structure of the chimeric peptide was induced by dissolving it in trifluoroethanol, an increase of the fusogenic potential of the peptide was observed, supporting the hypothesis that the α-helical conformation of the peptide is crucial to trigger the lipid–peptide interaction. The specificity of the interaction between PIP_2 and the PBP moiety, was shown by the less efficient targeting of the chimeric peptide to membranes charged with phosphatidylserine. These data thus demonstrate that the specific properties of both the Aβ(29–40) and the PBP peptide are conserved in the chimeric peptide, and that a synergetic effect is reached through chemical linkage of these two fragments.

Keywords: Fusogenic peptide; Targeting; Liposome; Amyloid; Phosphatidylinositol 4,5-bisphosphate

1. Introduction

Membrane fusion is a widespread event, that plays a crucial role in important physiological processes such as endocytosis, exocytosis, secretion, fertilization or viral cell invasion [1–5]. A common feature of the above processes is the active participation of hydrophobic sequences within fusogenic proteins termed fusion peptides [6–9]. Fusion peptides have been identified in several proteins, including viral fusion proteins [10], the β-amyloid peptide [11], the prion protein [8] and lipid-transfer proteins (for review see Ref. [8]). Brasseur et al. [7,8] proposed that these peptides act as oblique-orientated helices at the lipid–water interface, as a result of an asymmetric

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distribution of hydrophobic residues along the helical motif. A tilted insertion of these peptides might perturb the regular packing of the phospholipid acyl chains and might in turn account for the fusogenic activity and/or the membrane toxicity of the above proteins [1,9–14].

Most fusion peptides described in the literature are 12- to 14-residue peptides [9–11,14] with only a limited stability in an α-helical conformation, due to their short length. The high hydrophobicity of these peptides contributes to their strong self-association tendency, and accounts for the high peptide concentrations required to induce membrane destabilization.

In the present study, we attempted to design a more physiological fusogenic peptide, with increased length and increased solubility in aqueous solvents compared to natural fusogenic peptides. However, increased solubility obtained by decreasing the overall hydrophobicity might result in a decreased affinity of the fusogenic peptide for lipids. To circumvent this problem, we used a strategy of chemically linking the fusogenic peptide to another polar peptide ligand which should target the fusogenic peptide to the surface of the lipid bilayer. One possibility is targeting the fusogenic peptide to an integral membrane protein, thus mimicking a receptor-mediated interaction [13]. The strategy proposed here instead is the coupling of the fusogenic peptide moiety to a peptide that specifically recognizes target phospholipids, which can be incorporated into the lipid vesicles used to monitor fusion.

As model fusion peptide, we used the fusion peptide identified in the C-terminal part of the β-amyloid peptide at residues 29–40 (Aβ(29–40)) [10]. The Aβ peptide, a 39- to 42-residue peptide, is found in an aggregated, poorly soluble form in extracellular depositions in the brains and leptomeninges of patients with Alzheimer's disease [15]. The mechanism through which the Aβ peptide causes neuronal cell death has not been elucidated yet. Results from several studies suggest that the Aβ neurotoxicity might be mediated through direct interactions between the Aβ peptide and cellular membranes [16–18]. Perturbation of cellular membranes by the carboxy-terminal domain of Aβ is one of the mechanisms proposed to account for the toxicity of the peptide [10]. Indeed, the Aβ(29–40) peptide displays an asymmetric distribution of hydrophobic residues along the peptide sequence and has significant membrane destabilizing effects on lipid vesicles, causing vesicular fusion and perturbation of membrane permeability [10].

As a target peptide for the lipid membrane, we selected a positively charged peptide interacting specifically with phosphatidylinositol 4,5-bisphosphate (PIP_2). This phospholipid carries multiple negative charges and is present at less than 3 mol% in natural membranes. This phospholipid plays a major role in cellular signaling events linked to the organization of the cytoskeleton [19,20]. The PIP_2-binding peptide was further selected in order to mimic the positively charged N-terminal domain of the β-amyloid peptide. It has recently been shown that membrane binding of the Aβ(1–40) and Aβ(1–42) peptides was maximal when these membranes contained acidic phospholipids [21]. Moreover, phosphatidylinositol has been reported as the most efficient acid phospholipid, able to enhance binding of the Aβ peptide to membranes [22].

The PIP_2-binding peptide (PBP) used in this study was identified in fragmin, a member of the actin-binding proteins, involved in actin polymerization [23,24]. This sequence is homologous to the PIP_2-binding segment of villin and gelsolin [25,26]. PIP_2 efficiently competes with actin for binding to these proteins, consistent with the role of PIP_2 as a regulator of the actin network. Binding of the PBP peptide to PIP_2 is not only due to electrostatic interactions, since binding to other negatively charged phospholipids such as phosphatidylserine (PS) is less efficient [23,27]. Specific interactions between phospholipids and other peptides such as that between Penetratin, identified in homeoproteins and phosphatidylserine (PS) headgroups, were also reported [28].

We therefore investigated the fusogenic properties of the synthetic composite peptide (PBP-Aβ(29–40)) on phospholipid vesicles, to which PIP_2 was added at physiological concentrations. We report in this paper that membrane targeting efficiently increases the fusogenic properties of the Aβ(29–40) peptide, since five to six times less PBP-Aβ(29–40) than Aβ(29–40) was required in order to observe the same extent of fusion. Our data also suggest that due to the increased solubility of the chimeric peptide in water, the presence of target ligands on the membrane surface is required to induce fusion. The specificity of the interaction between the PBP-Aβ(29–40) peptide

and PIP_2, was further characterized with respect to the specificity of the anionic lipid target. The specificity of the PBP–PIP_2 interaction was preserved after chemical linkage of the PBP and Aβ(29–40) fragments. Taken together, these observations demonstrate that the separate properties of each peptide component are retained in the chimeric peptide. These data further support the hypothesis that the cytotoxicity of the β-amyloid peptide might be driven by electrostatic interactions between positive charges of the N-terminal domain of the amyloid peptide and the net negative charges of cellular membranes. This would be then followed by the insertion of the Aβ(29–40) moiety into the lipid phase. The chimeric peptide described here stresses the role of fusogenic peptides in the membrane destabilization properties of different proteins.

2. Materials and methods

2.1. Materials

L-α-Phosphatidylethanolamine (PE) from egg yolk, L-α-phosphatidyl-L-serine (PS) from bovine brain, L-α-phosphatidylcholine (PC) from egg yolk and phosphatidylinositol 4,5-bisphosphate (PIP_2) were purchased from Sigma. 1-Palmitoyl-2-pyrene(14)-phosphatidylcholine (Pyr-PC) was from Molecular Probes (Junction City, OR, USA). Reagents for peptide synthesis and sequencing were purchased from Applied Biosystems (Foster City, USA). Trifluoroethanol (TFE) and hexafluoro-2-propanol (HFIP) used for sample preparation were of the highest grade from Sigma. 2′,7′-{bis(carboxymethyl)aminomethyl} fluorescein (calcein) was from Molecular Probes (Junction City, OR, USA).

2.2. Synthesis and purification of peptides

The amyloid peptide (Aβ(29–40)), the PIP_2-binding peptide (PBP), and the chimeric peptide (PBP-Aβ(29–40)) were synthesized by the standard F-moc solid-phase method, on an Applied Biosystems Model 431A peptide synthesizer. The peptide–resin conjugates were cleaved with trifluoroacetic acid, and the peptides were precipitated twice with tributylmethyl ether, and recovered by centrifugation at $2000 \times g$ [10]. The residue was dried for 2 h in a Speedvac concentrator (Savant Instr., Farmingdale, NY). The peptides (20–40 mg) were resuspended in acetonitrile/water (80/10, v/v) to remove all scavengers molecules, and the insoluble peptide was recovered by centrifugation. The washing step was repeated 10 times with water. The PBP and PBP-Aβ(29–40) peptides were purified by reverse-phase HPLC on a C18 column using a linear gradient of 0–80% acetonitrile, with 0.007% trifluoroacetic acid. The purity and correct sequences of all peptides were verified by electrospray ionisation mass spectrometry using a Fisons/VG Platform (Manchester, UK) mass spectrometer.

2.3. Circular dichroism measurements

The peptides were first dissolved in HFIP at 5 mg/ml, then dried under a stream of nitrogen, and further diluted at a final concentration of 0.1 mg/ml either in a 10 mM phosphate pH 7.5 buffer, in TFE, or in buffer containing either 20 or 50% TFE (v/v). Circular dichroism spectra were obtained at 25°C in a Jasco 710 spectropolarimeter (B α L Systems, Zoetermeer, Holland) calibrated with 1 g/l D-10-camphorsulfonic acid solution [29]. Nine spectra were recorded 15 min after the peptides had been dissolved. The percentages of secondary structure were estimated by curve-fitting on the entire ellipticity curve between 184 and 260 nm according to the variable selection procedure developed by Johnson [30].

2.4. Preparation of vesicles

Phospholipids (PC, PE, PS) were dissolved in chloroform at 10 mg/ml. PIP_2 was dissolved in pure water at 5 mg/ml, by sonication in a water bath sonicator for 10 min at room temperature, as described elsewhere [31]. For the preparation of mixed PC/PE and PC/PE/PS vesicles, the corresponding phospholipids were mixed in chloroform at the appropriate molar ratios, PC/PE 2:1, PC/PE/PS 20:10:5.4 (18 mol% PS), and dried under a stream of nitrogen. Dried lipid mixtures were resuspended in a 10 mM Tris–HCl pH 7.5 buffer, containing 150 mM NaCl, 0.1 g/l Na-EDTA and 1 mM NaN_3. Small unilamellar vesicles (SUV) were obtained by sonica-

tion of the lipid suspensions with a Branson sonifier (Danbury, CT) at 10°C under a stream of nitrogen for four times during 15 min. For the preparation of vesicles consisting of PC, PE and PIP_2 (1.5 and 3 mol% PIP_2), PIP_2 was added to the PC/PE (2:1 M/M) lipid suspension in buffer prior to sonication. The SUV were labeled, when required, with 2.5 mol% Pyr-PC dissolved in chloroform together with the other phospholipids. After sonication, the labeled or unlabeled vesicles were applied to a Sepharose CL 4B column. SUV were separated from larger particles eluting in the void volume of the column. In order to obtain a homogenous preparation, only the top fractions of the SUV elution peak were collected and pooled. Phospholipid concentration was determined by an enzymatic assay (Biomérieux, France). Large unilamellar vesicles (LUV) were prepared from phospholipids as follows. Dry lipids were hydrated in buffer and dispersed by vortexing to produce large multilamellar vesicles. The lipid suspension was freeze-thawed five times, and then extruded 10 times through two stacked 100 nm pore size polycarbonate filters in a pressure extruder (Lipex Biomembranes, Vancouver, Canada). The SUV and LUV preparations could be stored at 4°C for at least 1 week, without significant size change of the vesicles during storage.

2.5. Lipid-mixing experiments

Fusion of pyrene-labeled vesicles together with unlabeled vesicles, at a 1/4 w/w ratio, was followed using a fluorescence probe dilution assay. The pyrene excimer/monomer intensity ratio was measured as a function of time after addition of increasing quantities of the peptides, dissolved in either a 10 mM Tris–HCl pH 7.5 buffer, containing 150 mM NaCl, 0.1 g/l Na-EDTA and 1 mM NaN_3 or in the same buffer containing 20, 50 or in 100% (v/v) TFE. Fusion resulted in a decrease of the excimer intensity, and a slight increase of the monomer fluorescence. Emission spectra were obtained on an Aminco SPF 500 spectrofluorimeter (MD, USA) at 25°C. The pyrene excimer/monomer (E/M) ratio was calculated from the excimer and monomer fluorescence intensity at 475 and 398 nm, respectively, with an excitation wavelength of 346 nm. The excitation and emission band width were set at 2 and 5 nm, respectively. Under these conditions, unlabeled vesicles in the presence of the peptides gave no significant signal due to light scattering. All experiments were performed at a phospholipid concentration of 42 μM, in a 10 mM Tris–HCl pH 7.5 buffer, containing 150 mM NaCl, and 0.1 g/l Na-EDTA. The final TFE concentration in the reaction mixture was less than 5% (v:v), and a correction was made for the effect of the solvent on the excimer/monomer ratio. The final peptide concentrations were in the range of 1 to 40 μM.

2.6. Monitoring of vesicle size and lipid-bound peptides by gel filtration

A total of 75 μM of labeled SUV were incubated with 5 to 40 μM of PBP-Aβ(29–40) or PBP peptides dissolved in 100% TFE, for 15 min at 25°C in a 10 mM Tris–HCl pH 7.5 buffer, containing 150 mM NaCl, 0.1 g/l Na-EDTA and 1 mM NaN_3. The mixture was then applied to a Sepharose CL 4B column in 0.05 M Tris–HCl buffer, pH 7.5, containing 150 mM NaCl and 1 mM NaN_3, in a FPLC system (Pharmacia, Upsalla, Sweden). The fluorescence emission intensity of Pyr-PC was continuously monitored, and the elution profile was compared to that of vesicles in the absence of peptides. In a parallel experiment, the PBP-Aβ(29–40) and PBP peptides were detected by continuous monitoring of the tyrosine fluorescence emission at 305 nm, as both peptides contain one Tyr residue.

2.7. Core-mixing experiments

Vesicle fusion was also investigated by the core-mixing assay developed by Kendall and MacDonald [32]. Lipid vesicles were prepared as described above, except for the phospholipid mixture which was prepared by hydrating 2 mg phospholipids in 1.5 ml of a 10 mM Tris–HCl pH 7.5 buffer containing 150 mM NaCl and 1 mM NaN_3 and containing 0.8 mM calcein together with 1 mM $CoCl_2$ or 20 mM EDTA. Untrapped solutes were removed by two successive elutions on a Sephadex G-25 column with a 10 mM Tris–HCl pH 7.5 buffer, containing 150 mM NaCl, and 1 mM NaN_3. Phospholipid concentration of the liposome suspensions was determined by an enzymatic assay (BioMérieux, France). In a standard ex-

periment, calcein–Co^{2+}, and EDTA-containing vesicles were mixed at a 1:1 molar ratio (42 μM total phospholipids) in a 10 mM Tris–HCl pH 7.5 buffer, containing 150 mM NaCl and 1 mM NaN_3. Peptides from the concentrated stock solutions in 100% TFE were added at different concentrations and the calcein fluorescence was followed using an Aminco SPF 500 spectrofluorimeter (MD, USA), with excitation and emission wavelengths of 490 and 520 nm, respectively, and with excitation and emission slits of 5 nm. The maximum fluorescence yield was determined in the presence of 0.5% Triton X-100 and 10 mM EDTA. In some experiments, 0.4 mM $CoCl_2$ chelated with 0.4 mM citrate was included in the reaction mixture in order to monitor leakage of encapsulated components. All experiments were performed under constant stirring at 25°C in the fluorimeter cuvette.

2.8. Leakage of liposomal content

Kinetics of the leakage of liposomal content into the external buffer were monitored by measuring release of the calcein trapped inside the vesicles [33]. The vesicles were prepared as previously described, except that dried lipids were rehydrated in a 10 mM Tris–HCl pH 7.5 buffer, containing 150 mM NaCl, 0.1 g/l Na-EDTA, 1 mM NaN_3 and 40 mM calcein. At this concentration, fluorescence self-quenching occurs. Uncapsulated calcein was removed by gel chromatography on Sephadex G-25 after elution with the above buffer. The fluorescence of calcein encapsulated at a self-quenching concentration in the liposomes, increases under leakage from the vesicles. The calcein fluorescence increase was followed upon addition of the peptides dissolved in TFE to the vesicle suspension. Measurements were performed on an Aminco SPF 500 spectrofluorimeter, with excitation and emission wavelengths at 490 and 520 nm, respectively, and with excitation and emission slits of 5 nm. One hundred percent leakage was established by lysis of the vesicles with 0.5% (w/v) Triton X-100. The percentage of calcein release is calculated as: $(I(t) - I_0/I_{tot} - I_0) \times 100$, where I_0 = initial fluorescence, I_{tot} = total fluorescence observed after addition of Triton X-100, and $I(t)$ = fluorescence at time t. In our experimental conditions, in the absence of peptide, the spontaneous leakage rate was less than 1%/10 min. All experiments were with constant stirring at 25°C.

3. Results

3.1. Characteristics of the peptides

The sequence, mean hydrophobicity, and net charge of the Aβ(29–40), PBP and PBP-Aβ(29–40) peptides are summarized in Table 1. The 12-residue long Aβ(29–40) peptide is highly hydrophobic, with a mean hydrophobicity close to that of a transmembrane peptide on the Eisenberg's scale [34], and devoid of charges. The 17-residue long PBP peptide is a highly polar and charged peptide, containing six ionized residues at physiological pH, a cluster of three Lys and three Arg distributed along the sequence. The PBP-Aβ(29–40) peptide consists of the

Table 1
Characteristics of PBP-Aβ(29–40), Aβ(29–40) and PBP peptides

Peptide	Sequence	Mean hydrophobicity[a]	Net charge at pH 7.5
PBP-Aβ(29-40)	YRTRLLHLKGKKHIRVHGAIIGLMVGGVV + + + ++ +	0.13	+6
Aβ(29-40)	GAIIGLMVGGVV	0.86	0
PBP	YRTRLLHLKGKKHIRVH + + + ++ +	-0.39	+6

[a]Mean hydrophobicity was calculated using the Eisenberg consensus scale [34].

A β(29–40) peptide linked to the PBP peptide at the N-terminal end. This composite peptide thus contains 29 residues, and combines the hydrophobic and hydrophilic properties of the A β(29–40) and PBP peptides, respectively.

3.2. Conformational properties of the PBP-Aβ(29–40) and PBP peptides

Using circular dichroism spectroscopy, we first examined the conformation of the peptides in a mixture of TFE and buffer (10 mM phosphate, pH 7.5) as a function of the TFE concentration. Fig. 1 displays the CD spectra of the PBP-A β(29–40) and PBP peptides in various solvent conditions. In an aqueous buffer, the two peptides were totally soluble, and displayed CD spectra typical of a random peptide structure with a negative band at 197 nm and no positive signal peak. Upon increasing the TFE concentration, a strong helix-promoting solvent, similar spectral changes were observed for both peptides.

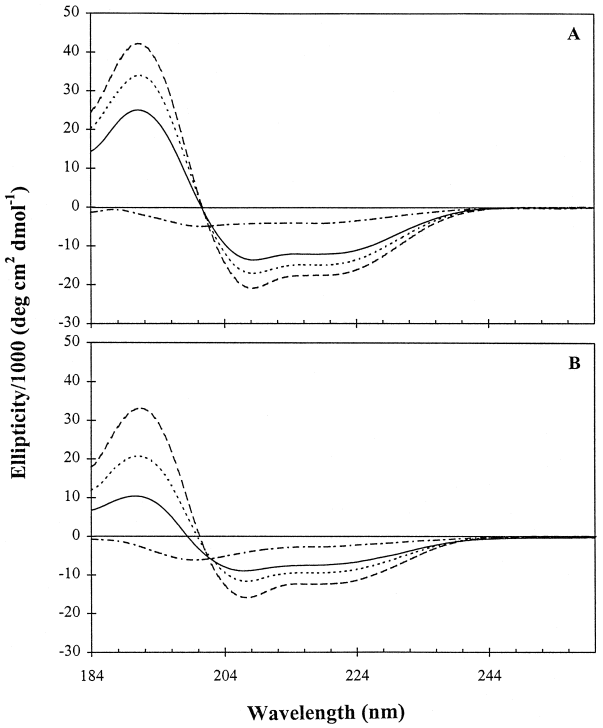

Fig. 1. Circular dichroism spectra of PBP-A β(29–40) (A) and PBP (B) peptides. The peptides were dissolved at 0.1 mg/ml, in phosphate buffered water solution pH 7.5 (— - —), 20% TFE (————), 50% TFE (---) and 100% TFE (— — —).

The intensity of the ellipticity minimum at 197 nm decreased and became positive, while two negative absorption peaks at 208 and 222 nm, characteristic of an α-helical conformation, appeared at increasing TFE concentrations. Comparison of the amplitudes of the peak minima suggests that the PBP-A β(29–40) peptide has a higher propensity to form an α-helix than the short PBP peptide. Indeed, the α-helical content calculated for the PBP-A β(29–40) peptide amounted to 41, 49 and 57% in 20, 50 and 100% TFE, respectively, compared to 29, 37 and 47% α-helix for PBP under the same conditions. When the secondary structure is followed as a function of the TFE concentration, an isodichroic point is observed at 201 nm, suggesting a simple equilibrium between helical and random coil structures for these two peptides. Moreover, the absence of any significant absorption at 218 nm suggests that β-sheet structures do not contribute to the spectra.

3.3. Influence of the secondary structure of the peptide upon its lipid mixing properties

We first investigated the influence of the secondary structure of the PBP-A β(29–40) peptide on its fusogenic activity. For this purpose, the PBP-A β(29–40) peptides dissolved either in buffer, in 100% TFE, or in buffer containing 20 and 50% TFE, respectively, were added to PC/PE/PIP_2 SUV. The extent of intervesicular lipid mixing induced by the peptides, was followed as a measure of their fusogenic activity using a probe dilution assay [35,36]. SUV labeled with fluorescent Pyr-PC were mixed with unlabeled vesicles, and the excimer to monomer intensity ratio of the pyrene probe was monitored as a function of time. Vesicle fusion results in a decrease of the excimer intensity and an increase of the monomer intensity, due to dilution of the probe into the fused vesicles. The overall decrease in the E/M ratio as a result of fusion, is expressed as percentage of the initial E/M value. As shown in Fig. 2A, the PBP-A β(29–40) peptides was in a random conformation when dissolved in aqueous buffer, and did not induce any detectable lipid mixing. In contrast, solubilizing the PBP-A β(29–40) peptide in 20% TFE increased the fusogenic activity of the peptide, as 36% of lipid mixing was observed 30 min after

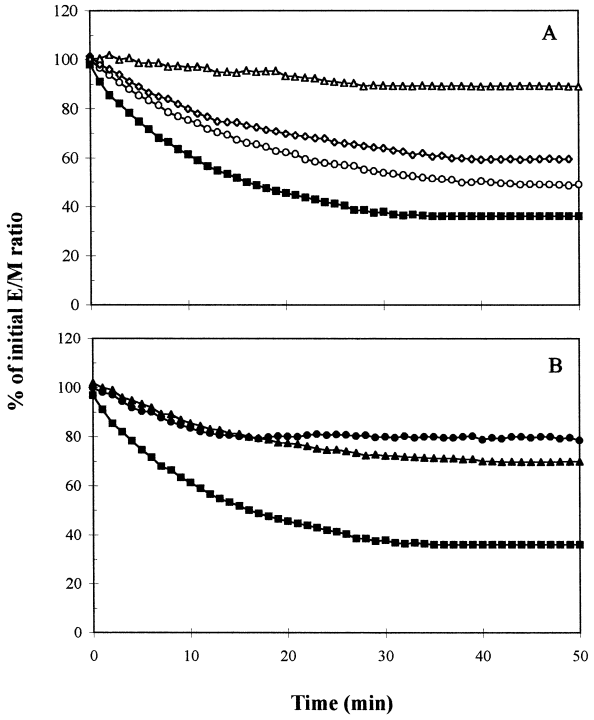

Fig. 2. Influence of the peptide secondary structure and of the PIP_2 concentration on the rate of lipid mixing of PC/PE/PIP_2 SUV induced by 5 μM of PBP-A β(29–40) peptides. Aliquots of PBP-A β(29–40) peptides were added to a mixture of labeled SUV containing 2.5 mol% of Pyr-PC and of unlabeled SUV (42 μM total phospholipids) in a 10 mM Tris–HCl buffer pH 7.5, containing 150 mM NaCl, 1 mM NaN_3, and 0.01 g/l Na-EDTA. The Pyr-PC excimer/monomer ratio was monitored at 25°C and is plotted as a percentage of the initial value vs. time. (A) Peptides dissolved in a 10 mM Tris–HCl buffer containing 150 mM NaCl, 1 mM NaN_3, and 0.01 g/l Na-EDTA (△), in a mixture of buffer and TFE containing 20% TFE (◇), 50% TFE (○), or in pure TFE (■) were added to a mixture of labeled and unlabeled PC/PE SUV containing 3 mol% PIP_2. (B) Peptides dissolved in 100% TFE were added to a mixture of labeled and unlabeled PC/PE SUV (●), PC/PE SUV containing 1.5 mol% PIP_2 (▲), and 3 mol% PIP_2 (■).

addition of the peptide to SUV. Increasing the amount of TFE in the buffer further increased lipid mixing, up to 62% after 30 min when the PBP-A β(29–40) peptide was dissolved in pure TFE. As shown in Fig. 1, the α-helical content of the peptide is maximal in 100% TFE, suggesting that the fusogenic properties of the PBP-A β(29–40) peptide increase when the peptide is in a helical conformation. Peptides were dissolved in 100% TFE in all subsequent experiments.

3.4. Influence of the PIP_2 concentration on the lipid mixing of PC/PE/PIP_2 vesicles induced by the PBP-A β(29–40) peptide

In order to determine the contribution of the specific PIP_2-binding of the PBP fragment to the fusogenic activity of the PBP-A β(29–40) peptide, lipid mixing experiments were performed with both PC/PE SUV, and with PC/PE SUV containing 1.5 or 3 mol% PIP_2. Fig. 2B depicts the variation of the E/M ratio as a function of time, when 5 μM of PBP-A β(29–40) peptide was added to a fixed amount of SUV (42 μM of phospholipids). The PBP-A β(29–40) peptide interacted with both PC/PE SUV and PC/PE SUV containing PIP_2. The extent of lipid mixing was enhanced by the presence of increasing amounts of PIP_2 in the mixed vesicles. Indeed, the maximal decrease of the E/M ratio was around 60% when 3 mol% PIP_2 were incorporated in the vesicles, compared to only 30% at 1.5 mol% PIP_2, and 20% for SUV without PIP_2. In order to assess the increase of the fusogenic potential of the PBP-A β(29–40) peptide on PIP_2-containing SUV, we followed the extent and kinetics of lipid mixing as a function of the peptide concentration. Using similar experimental conditions, the activity of the A β(29–40) peptide was compared to that of the PBP-A β(29–40) peptide. Increasing amounts of PBP-A β(29–40) and A β(29–40) peptides were added to a fixed amount of SUV, and the decrease of the E/M ratio was measured after 30 min incubation time. Control experiments were performed with SUV and the PBP peptide. All results are depicted in Fig. 3, where the lipid mixing activity, expressed as the total percentage decrease in E/M ratio, is plotted as a function of the peptide concentration. The change in the E/M ratio as a function of time at increasing amounts of the PBP-A β(29–40), A β(29–40), and PBP peptides, shows that for all peptides tested, the extent of lipid mixing is concentration-dependent. Significant fusogenic activity was measured when the PBP-A β(29–40) peptide was added to PC/PE SUV containing 3 mol% of PIP_2 (Fig. 3A). The activity of the chimeric peptide was enhanced compared to that of the A β(29–40) peptide, since 7.5 μM of this latter were required to reach 30% of lipid mixing—i.e., at approximately half the maximal lipid mixing capacity of the peptides—compared to only 1.5 μM, i.e.,

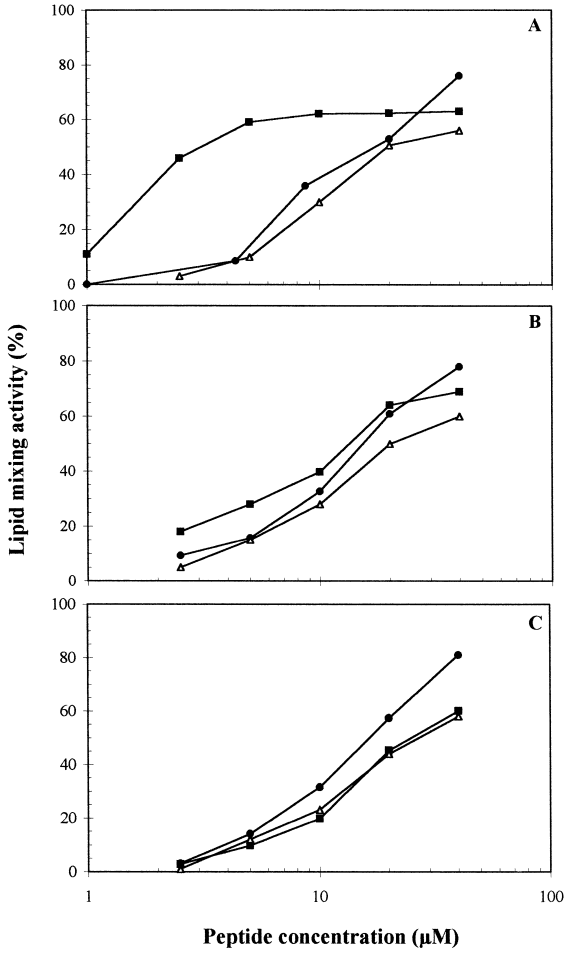

Fig. 3. Effect of the incorporation of PIP_2 in PC/PE SUV on the activity of PBP-Aβ(29–40) (■), Aβ(29–40) (●) and PBP (△) peptides. The activity of the different peptides was determined as their ability to induce intervesicular lipid mixing. Aliquots of peptides dissolved in 100% TFE were added to a mixture of labeled and unlabeled (A) PC/PE SUV containing 3 mol% of PIP_2, (B) PC/PE SUV containing 1.5 mol% of PIP_2, (C) and PC/PE SUV without PIP_2. The percentual decrease of the excimer/monomer ratio, expressed as percent lipid mixing, after 30 min incubation at 25°C of SUV (42 μM total phospholipids) and peptides, is reported as a function of the peptide concentration.

5-fold less, for the PBP-Aβ(29–40) peptide. In agreement with the fusogenic activity measured for the PBP-Aβ(29–40) peptide, the PBP peptide alone had no significant effect in the concentration range where maximal activity is observed with the PBP-Aβ(29–40) peptide. Indeed, seven times more PBP peptide (10 μM) is needed to reach 30% of lipid mixing when compared to PBP-Aβ(29–40). Fig. 3A shows that the PBP peptide is active in the same concentration range as the Aβ(29–40) peptide. However, in contrast to the Aβ(29–40) peptide, the PBP peptide is too hydrophilic to induce vesicle fusion. The lipid destabilization properties of the PBP peptide can therefore be attributed to specific interactions occurring between the PBP peptides which are soluble in aqueous buffer and SUV at high peptide concentrations. Due to their high hydrophobicity, the Aβ(29–40) peptides have a limited solubility in aqueous solvent, thereby requiring high concentrations to induce fusion. A two-fold decrease of the PIP_2 concentration in vesicles did not significantly affect either the fusogenic activity of Aβ(29–40), or the lipid destabilisation properties of the PBP peptide (Fig. 3B). In contrast the fusogenic advantage of PBP-Aβ(29–40) peptide almost disappeared, as the activity of this composite peptide became comparable to that of the Aβ(29–40) peptide alone. The PBP-Aβ(29–40) peptide showed a slightly increased effect compared to the PBP peptide, which can be attributed to a residual fusogenic activity of the peptide driven by specific PIP_2-peptide interactions, as this differential effect disappeared when PIP_2-free SUV were used (Fig. 3C). It is noteworthy that the lipid mixing effect of the Aβ(29–40) and PBP peptides did not depend on the lipid composition of the vesicles tested. Similar results were obtained with LUV (data not shown).

3.5. Effect of the charged phospholipid on the vesicular mixing induced by the PBP-Aβ(29–40) peptide

Incorporation of PIP_2 in SUV membranes significantly increased the fusogenic activity of the PBP-Aβ(29–40) peptide. The specificity of the interactions between PIP_2 and the PBP-Aβ(29–40) peptide was investigated by comparing the efficiency towards another negatively charged phospholipid, phosphatidylserine, PS, when incorporated into SUV membranes. PS and PIP_2 differ both by the number of negative charges in their polar headgroup and by their molecular shape. The cross sectional area of the two acyl chains of PS is similar to that of the polar headgroup region, resulting in a cylindrical geometry for this phospholipid. In contrast, PIP_2 whose cross sectional area of the aliphatic chains is smaller than that of the large bulky phosphoinositide polar headgroup, presents a conical shape [37]. Consequently,

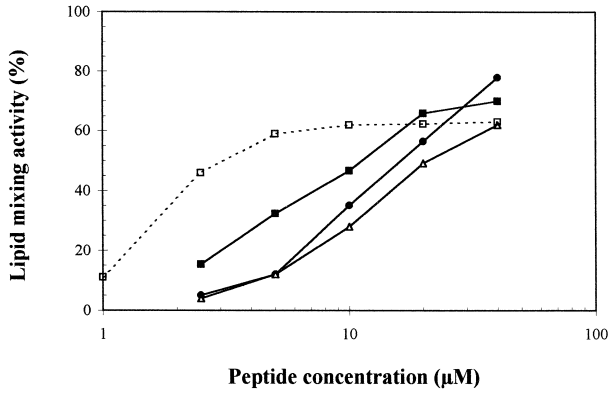

Fig. 4. Compared effect of PS on the activity of the PBP-Aβ(29–40) (■), Aβ(29–40) (●) and PBP (△) peptides. The activity of the different peptides was determined as their ability to induce intervesicular lipid mixing. Peptide aliquots dissolved in 100% TFE were added to a mixture of labeled and unlabeled PC/PE/PS (18 mol%). The activity of the PBP-Aβ(29–40) peptide on PC/PE/PIP_2 (3 mol%) SUV is shown for comparison (□, broken line). The percentual decrease of the excimer/monomer ratio, expressed as percent lipid mixing, after 30 min incubation at 25°C, of SUV (42 μM total phospholipids) and peptides is plotted as a function of the peptide concentration.

due to the high curvature of SUV vesicles, the PIP_2 molecules do not distribute symmetrically between the internal and external leaflet of lipid bilayers such as PS does. In mixed phosphatidylcholine SUV containing 2.5 mol% PIP_2, 75% distributes to the outer layer, the remainder goes to the inner layer [38]. Moreover, PIP_2 undergoes charge neutralization in the physiological pH range resulting in approximately four negative charges at pH 7.5 [38], while under these conditions, PS contains one negative charge. On the basis of these data, and in order to compare the effect of an equal number of negative charges in the external membrane leaflet, SUV were prepared with 18 mol% PS, i.e., 9 mol% of negative charges in the outer layer which is approximately the number of charges present in the outer leaflet when using SUV prepared with 3 mol% PIP_2. The effect of PS on the activity of the Aβ(29–40) and PBP peptides, was also investigated under the same conditions. The results of this comparative study are depicted in Fig. 4. When PC/PE SUV containing 18 mol% PS were tested, the activity of the PBP-Aβ(29–40) peptide was slightly increased as the concentration of the PBP-Aβ(29–40) peptide inducing 30% lipid mixing decreased by a factor 2. However, the high fusogenic activity of the PBP-Aβ(29–40) peptide observed on PC/PE/PIP_2(3%) vesicles at a three times lower concentration could not be obtained with PS charged SUV. The presence of PS in SUV membranes did not significantly affect the activities of the Aβ(29–40) and PBP peptides, which were similar to those observed with PC/PE and PC/PE/PIP_2 SUV.

3.6. Interaction of the PBP-Aβ(29–40) and PBP peptides with PC / PE / PIP_2 vesicles. Effect on the vesicle size

The changes in the size of the PC/PE/PIP_2(3%) vesicles due to the events of fusion were monitored

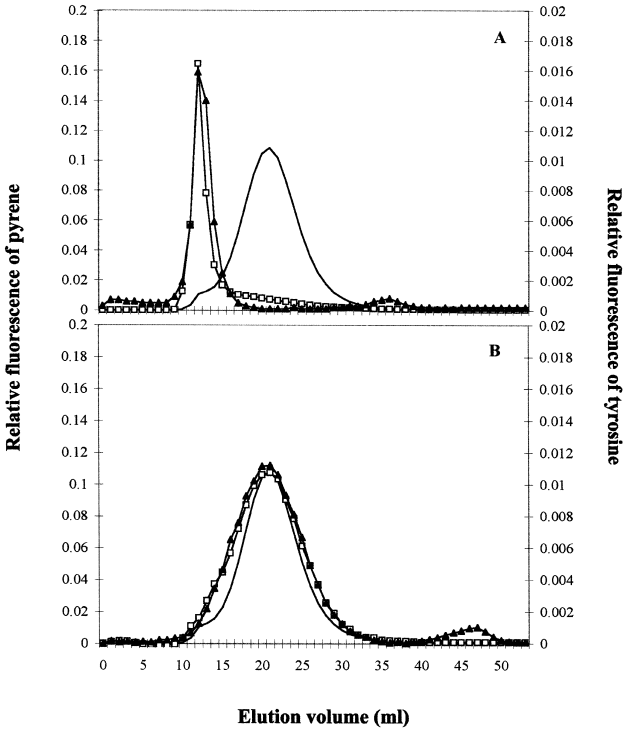

Fig. 5. Effect of PIP_2 on the gel filtration behavior of SUV in the absence (————) and in the presence (□-□) of PBP-Aβ(29–40) (A) and PBP (B) peptides. PBP-Aβ(29–40) and PBP peptides dissolved in 100% TFE were added at a 5 μM final concentration to 75 μM of PC/PE/PIP_2 (3 mol%) SUV labeled with 2.5 mol% of Pyr-PC. After 15 min incubation in the presence of the peptides, the mixture was applied to a Sepharose CL-4B column, and the elution profile was recorded by monitoring the Pyr-PC fluorescence emission at 398 nm. The localization of the PBP-Aβ(29–40) and the PBP peptide in the chromatogram was determined by following the Tyrosine fluorescence emission at 305 nm (▲-▲). The void volume of the Sepharose column corresponds to an elution volume of 12 ml.

by size exclusion gel chromatography on a Sepharose CL-4B column before and after mixing with the PBP-A β(29–40) and PBP peptides, by following the pyrene fluorescence monome of the labeled particles. Fig. 5A shows that the homogenous population of pyrene-labeled PC/PE/PIP_2 SUV containing 3 mol% PIP_2, incubated in the presence of 5 μM of the PBP-A β(29–40) peptides, is transformed into larger particles eluting in the void volume of the column, consistent with vesicle aggregation and/or fusion. Pyrene fluorescence intensity was maximal in the void volume (12 ml). In the absence of peptides, the labeled vesicles eluted at 21 ml. Direct monitoring of the fluorescence emission of the tyrosine residues of the PBP-A β(29–40) peptides was performed in parallel. As shown in Fig. 5A, the peptides eluted together with the fused vesicles, in the void volume of the column. Control experiments carried out with 5 μM of the PBP peptide showed no significant change in the vesicle size, consistent with the absence of fusogenic activity of this peptide (Fig. 5B). However, in agreement with its lipid destabilisation properties at higher concentration, the PBP peptide associated with lipid vesicles, as it co-eluted with the lipids (Fig. 5B). Changes in the vesicular size could not be detected at any concentration of the PBP peptide.

3.7. Core mixing of PC / PE / PIP_2 vesicles induced by the PBP-Aβ(29–40) peptides

In order to confirm the fusogenic properties of the PBP-A β(29–40) peptides on PC/PE SUV containing 3 mol% of PIP_2, core-mixing experiments were carried out on two populations of PIP_2-containing SUV, labeled separately. Control experiments were performed with the A β(29–40) peptide and with the PBP peptide. When the A β(29–40)-PBP and A β(29–40) peptides were added to a mixture of calcein and Co^{2+}- and of EDTA-containing SUV, an increase of the calcein fluorescence was observed (data not shown). This fluorescence increase, due to release of free calcein, might result from the EDTA–Co^{2+} complex formation. On the other hand, dilution and subsequent dissociation of the calcein–Co^{2+} complex might also occur under leakage of the vesicle content, resulting in an increase of the emission intensity of free calcein. Fluorescence increase due to leakage of vesicle contents was prevented by addition of 0.4 mM Co^{2+} (chelated in a 1:1 molar ratio with citrate) in the aqueous buffer. Fig. 6 illustrates the effect of increasing concentrations of the PBP-A β(29–40) and A β(29–40) peptides, on the core mixing of calcein and Co^{2+}- and of EDTA-containing SUV, after 30 min incubation in the presence of Co^{2+} in the aqueous phase. These results show that, as previously demonstrated for the interaction of the A β(29–40) peptide with PC/PE SUV, the PBP-A β(29–40) and A β(29–40) peptides both induce core mixing of the two populations of PC/PE/PIP_2 vesicles containing 3 mol% PIP_2. In agreement with the results of the lipid mixing assay, the PBP-A β(29–40) peptide was active at lower concentrations than the A β(29–40) peptide, since half maximal core mixing was observed at 1.5 μM with the PBP-A β(29–40) peptide, a concentration where the A β(29–40) peptide had no activity. Control experiments demonstrated that the PBP peptide did not induce core mixing of the two populations of vesicles. Similar results were obtained with LUV (data not shown).

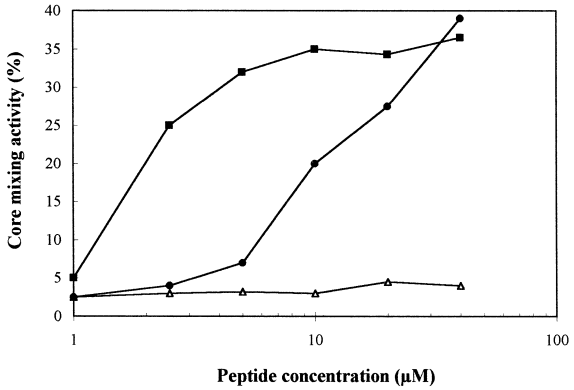

Fig. 6. Core-mixing of PC/PE/PIP_2 (3 mol%) SUV induced by PBP-A β(29–40) (■), A β(29–40) (●) and PBP peptides (△). Peptides dissolved in 100% TFE were added to a mixture of calcein and Co^{2+}-, and of EDTA- containing SUV (1:1 molar ratio, 42 μM total phospholipids), in a 10 mM Tris–HCl pH 7.5 buffer, containing 150 mM NaCl, 1 mM NaN_3, and 0.4 mM Co^{2+} chelated with 0.4 mM citrate. Core mixing of the two populations of vesicles as described under Section 2, and the percentage of the maximum calcein fluorescence after 30 min incubation was plotted as a function of the peptide concentration. One hundred percent of the calcein fluorescence was established by lysing the vesicles with Triton X-100 (0.5% w/v) in the presence of 10 mM EDTA.

3.8. Leakage of PC / PE / PIP_2 vesicle contents induced by the Aβ(29–40)-PBP and PBP peptides

Previous results showed that the Aβ(29–40) peptides did not only cause vesicle fusion, but also affect the permeability of the lipid vesicles [10]. This effect was further investigated for the PBP-Aβ(29–40) peptides by following leakage of encapsulated calcein from PC/PE SUV containing 3 mol% of PIP_2. The addition of the PBP-Aβ(29–40) peptides to these SUV induced membrane destabilization as measured by the calcein release from the vesicles. Fig. 7 illustrates the concentration-dependent release of calcein from SUV, caused by the addition of the PBP-Aβ(29–40), Aβ(29–40) and PBP peptides. The amount of calcein leakage induced by the three peptides varied with the peptide concentration in the same way as the lipid mixing measured with the same PIP_2-containing vesicles. The PBP-Aβ(29–40) peptide was more active than Aβ(29–40), inducing 100% calcein release at a 5 μM concentration compared to an 8-fold higher concentration of Aβ(29–40). Control experiments demonstrated that a 5 μM concentration of PBP peptide induced only 5% calcein release. These data then suggest that the PBP peptide neither contributes to vesicle fusion nor to any changes in membrane properties of the lipid vesicles. Similar results were obtained with LUV (data not shown).

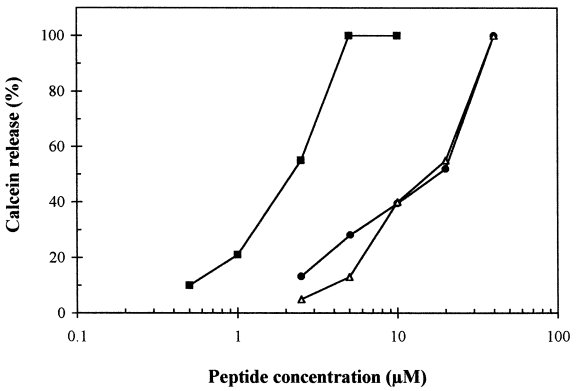

Fig. 7. Leakage of calcein from PC/PE/PIP_2 (3 mol%) SUV induced by PBP-Aβ(29–40) (■), Aβ(29–40) (●) and PBP (△) peptides. Peptides were dissolved in 100% TFE, and peptide aliquots were added to calcein SUV (42 μM total phospholipids) in a 10 mM Tris–HCl pH 7.5 buffer, containing 150 mM NaCl, 1 mM NaN_3, and 0.01 g/l Na-EDTA. Calcein release from the vesicles was followed as described under Section 2, and calcein release after 30 min of incubation with increased concentration of peptides was plotted as a function of the peptide concentration. One hundred percent of leakage was established by lysing the vesicles with Triton X-100 (0.5% w/v).

4. Discussion

The purpose of this work was to design new composite peptides with increased fusogenic efficiency compared to that of natural hydrophobic fusion peptides, whose solubility in aqueous buffers is mostly limited. The chimeric peptide described here, consists of the fusion peptide of the beta-amyloid peptide (Aβ) [10], and of a positively-charged peptide (PBP) which binds specifically to a negatively-charged phospholipid PIP_2 [23,24]. The fusion peptide identified at residues 29–40 of the Aβ peptide involved in the Alzheimer's disease [15], is referred to as Aβ(29–40). It was shown to induce vesicular fusion with a concomitant alteration of the lipid bilayer permeability [10]. PIP_2, a natural phospholipid present at less than 3 mol% in cellular membranes, was reported to promote the membrane binding of the Aβ peptide [22]. The targeting PIP_2-binding peptide was therefore linked to the N-terminal end of the Aβ(29–40) peptide in order to mimic the positively charged N-terminal domain of the Aβ peptide [18].

Our results show that specific membrane targeting can significantly increase the fusogenic properties of the Aβ(29–40) peptide. Indeed, the chimeric peptide PBP-Aβ(29–40) promoted the fusion of PIP_2-containing SUV at significantly lower concentrations than the Aβ(29–40) peptide. When 3 mol% PIP_2 were added to the phospholipid vesicles, the mid-point for maximal fusion was observed at a six-fold lower concentration of the PBP-Aβ(29–40) peptide compared to the Aβ(29–40) peptide. Fusion of SUV vesicles was evidenced by the formation of larger vesicular aggregates, significant intervesicular lipid mixing and core mixing. In analogy to the Aβ(29–40) peptide, the PBP-Aβ(29–40) peptide was further able to perturb the membrane bilayer, thus promoting calcein release. Moreover, as observed for lipid mixing, lower concentrations of the composite peptide were required for calcein leakage compared to the

Aβ(29–40) peptide. In agreement with the fusion and leakage experiments, gel filtration of a mixture of PIP_2-containing SUV and of the PBP-Aβ(29–40) peptide, confirmed the binding of the PBP-Aβ(29–40) peptides to fused vesicles. The hydrophilic PBP peptide alone could not induce vesicle fusion, as demonstrated by the absence of core mixing and aggregation of vesicles. Lipid mixing and calcein release were negligible at concentrations corresponding to the optimal activity of the chimeric PBP-Aβ(29–40) peptide, suggesting that the PBP peptide has no direct effect on vesicle fusion. During lipid mixing and calcein release experiments, changes in the pyrene E/M ratio and leakage of vesicular contents were observed at peptide/lipid molar ratios above 1/4. The lipid destabilization properties of the PBP peptide occurring at concentrations of this water-soluble peptide around 15 μM, probably result from non-specific interactions between the peptide and SUV. Concentrations of the Aβ(29–40) peptide similar to those of the PBP peptide were required for membrane destabilization. However, the low aqueous solubility of the hydrophobic Aβ(29–40) peptide probably accounts for the high peptide concentrations necessary to induce fusion.

The extent of fusion induced by the PBP-Aβ(29–40) peptide measured on SUV containing variable amounts of PIP_2, decreased between 3 and 1.5 mol% PIP_2, and disappeared when PIP_2 was not included in the vesicle preparation. This suggests that the absence of ionic interactions between the peptide and the charged lipids abolished the ability of the peptide to disrupt lipid vesicles. The differences in the fusogenic activity of the PBP-Aβ(29–40) peptides on vesicles containing variable amounts of PIP_2, was not due to a higher stability of the vesicles with decreased charge. All SUV preparations had similar diameters, as estimated from gel filtration experiments. Moreover, the neutral Aβ(29–40) peptides had comparable fusogenic activity on charged and on neutral vesicles, consistent with a similar internal strain and overall stability of the vesicles. The presence of PIP_2 in PC/PE vesicles did not affect the lipid mixing activity of the PBP peptides, in agreement with a non-specific effect of these peptides and with comparable stability of the lipid vesicles.

Fusion of PIP_2-containing SUV induced by the PBP-Aβ(29–40) peptide results from the insertion of the fusogenic peptide into the lipid bilayer. This occurred only after ionic interactions between the positively-charged PBP peptide and the PIP_2-negative headgroups, suggesting that peptide-targeting enhances the fusogenic activity of the peptide. Our data moreover suggest that, although the presence of PIP_2 target sites on the membrane surface is required, it is not sufficient to promote vesicular fusion. The membrane destabilization properties of the PBP-Aβ(29–40) peptides are further dependent upon the secondary structure of the peptides. CD measurements show that, when the peptides are dissolved at increasing concentrations of a strong helix-promoting solvent such as TFE, they undergo a coil to α-helix α-transition. This is accompanied by a significant increase of the fusogenic activity of the peptides on SUV. In most studies on viral fusion proteins, an α-helical structure has been suggested to represent the fusion active conformation of lipid-bound peptides [9,14,39–42], although other studies suggest that peptides in a β-sheet conformation could also be fusogenic [43,44]. The entire Aβ peptide for example was shown by circular dichroism to adopt mostly a β-sheet conformation, when located in a lipid environment [18,22]. These apparent discrepancies might be related to differences between assays used to quantify peptide–lipid interaction. Structural measurements for example are performed under equilibrium conditions, after fusion is complete. This does not exclude the possibility that an α-helical conformation of the peptide is involved in the first stage of the fusion process, prior to the formation and stabilization of β-sheet structures through hydrophobic interactions with the core of the lipid bilayer. Comparable peptide concentrations of either PBP-Aβ(29–40) or Aβ(29–40) are required for vesicular fusion and for calcein release on PIP_2-containing PC/PE SUV. This observation supports the hypothesis that, membrane fusion involving insertion of the peptides under an α-helical form into the lipid membrane might be followed by peptide self-association within the lipid phase. This would result in the formation of holes in the lipid membrane and leakage of liposomal contents. Brasseur et al. [7,8] proposed that fusogenic peptides behave as oblique-orientated helices triggering membrane fusion. Such helices are characterized by an asymmetric distribution of the bulky hydrophobic residues along the axis of the peptide [7]. The

more hydrophobic extremity of the helix inserts into the lipid membrane at an oblique angle with respect to the lipid–water interface [9,14], thus inducing a local disorganisation of the bilayer structure [12,45].

It had been previously described that actin binding of either villin or fragmin containing the PIP_2-binding PBP peptide, cannot be completely inhibited by other negatively charged phospholipids such as phosphatidylserine [23,27]. Liposomal membranes containing enough PS to ensure an equal number of negative charges in the outer layer could not restore the high fusogenic efficiency observed with PIP_2. The activity of the PBP-Aβ(29–40) peptide on PIP_2-containing vesicles was three-fold higher than on PS-containing SUV. This difference might be related to the topology of the negative charges in the membrane. The cylindrical shape of phosphatidylserine, presenting one net negative charge at physiological pH, forms a steric barrier, preventing a close proximity of two negatively charged phospholipid headgroups. As a result, the formation of small clusters of negative charges in PS-enriched membranes, is not favourable at low molar PS/(PC + PE) ratios. Thus, clusters of negative charges in lipid membranes, as those displayed by one highly charged PIP_2 molecule, probably favor stronger electrostatic lipid–peptide interactions. The binding of PIP_2 to the PBP sequence in actin-binding proteins was attributed to the particular lysine-rich sequence KXKK [23]. Arg and Lys residues might both contribute to the electrostatic interaction of the Aβ(29–40)-PBP peptides with negatively-charged vesicles. However, clusters of negative charges as those displayed by one PIP_2 molecule might favor stronger interactions with Lys residues that are close neighbours in the peptide sequence. This hypothesis is supported by data showing respectively higher and lower efficiency of PIP_3 and PIP for the binding of a PIP_2-binding domain identified in the N-terminal region of ezrin [46]. As the lysine ε-NH_2 group has a unique flexibility, this amino acid residue might match more closely the acidic headgroups of PIP_2, and thereby promote a strong association with PIP_2.

In conclusion, our results demonstrate that, both the ability of the Aβ(29–40) amyloid peptide to induce fusion and alter the membrane permeability of vesicles, and that of the PBP peptide to bind specifically to PIP_2, are conserved within the chimeric PBP-Aβ(29–40) peptide. In addition to the requirement for an α-helical conformation, the presence of PIP_2 lipid targets was found essential for optimal fusogenic activity of the PBP-Aβ(29–40) peptide. This suggests that the fusion process is initiated by an interaction between the PBP moiety and the PIP_2 headgroups, followed by the insertion of the peptide in the lipid phase. It is obvious that this composite peptide can only mimic the complex fusogenic properties of a viral protein to a limited extent. Nevertheless, the properties of the peptide that were identified as critical in the present study, such as specific membrane binding, helical secondary structure and membrane destabilisation were also proposed as requirements for fusion induced by viral proteins [9–11,14,36], and could be applied to the design of other chimeric fusogenic peptides.

Acknowledgements

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