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ARTICLE

A critical evaluation of the conformational requirements of fusogenic peptides in membranes

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Abstract It is generally assumed that fusogenic peptides would require a certain conformation, which triggers or participates in the rate-determining step of membrane fusion. Previous structure analyses of the viral fusion peptide from gp41 of HIV-1 have yielded contradictory results, showing either an α-helical or a β-stranded conformation under different conditions. To find out whether either of these conformations is relevant in the actual fusion process, we have placed sterically demanding substitutions into the fusion peptide FP23 to prevent or partially inhibit folding and self-assembly. A single substitution of either *D*- or *L*-CF_3-phenylglycine was introduced in different positions of the sequence, and the capability of these peptide analogues to fuse large unilamellar vesicles was monitored by lipid mixing and dynamic light scattering. If fusion proceeds via a β-stranded oligomer, then the *D*- and *L*-epimers are expected to differ systematically in their activity, since the *D*-epimers should be unable to form β-structures due to sterical hindrance. If an α-helical conformation is relevant for fusion, then the *D*-epimers would be slightly disfavoured compared to the *L*-forms, hence a small systematic difference in fusion activity should be observed. Interestingly, we find that (1) all *D*- and *L*-epimers are fusogenically active, though to different extents compared to the wild type, and – most importantly – (ii) there is no systematic preference for either the *D*- or *L*-forms. We therefore suggest that a well-structured α-helical peptide conformation or a β-stranded oligomeric assembly can be excluded as the rate-determining state. Instead, fusion appears to involve conformationally disordered peptides with a pronounced structural plasticity.

Keywords Fusogenic peptides · gp41 · HIV-1 · α-helical conformation · β-stranded self-assembly · *D*-/*L*-CF_3-phenylglycine · Peptide epimers · Lipid mixing assay · Fluorescence dequenching · Vesicle size · Dynamic light scattering

Introduction

Infection of cells by enveloped viruses such as HIV requires fusion of the viral and the target cell membranes, so that at the end of the fusion process the viral nucleocapsid is inside the host cell and can initiate viral replication. A key role in membrane fusion is attributed to envelope proteins in the viral membrane, which contain apolar fusion peptide (FP) domains that interact with the target cell membrane (Durell et al. 1997; Dutch et al. 2000). The N-terminal stretch of about 20 amino acids in the ectodomain of the envelope glycoprotein gp41 of HIV-1 has been identified as the sequence, which induces fusion between large unilamellar vesicles (LUVs) in vitro (Freed et al. 1990;

Dedicated to Prof. K. Arnold on the occasion of this 65th birthday.

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Pereira et al. 1997) or between red blood cells (Mobley et al. 1995). Further evidence for the role of such peptides in triggering membrane fusion comes from mutagenesis studies on intact envelope proteins of several viruses (Bosch et al. 1989; Freed et al. 1990; Delahunty et al. 1996), and in particular from studies with synthetic fusion peptides. These include fusion peptides derived from influenza virus (Hoekstra 1990; White 1990**;** Pecheur et al. 1999**),** hepatitis virus (Pecheur et al. 1999; Rojo et al. 2003)**,** Sendai virus (Hoekstra 1990; Martin et al. 1994; Pecheur et al. 1999), and HIV (Pritsker et al 1998, 1999; Yang et al. 2001, 2003, 2004a; Buzón et al 2005). A fundamental step in the fusion process is the interaction of the fusion peptide with the target membrane (Binder et al. 2000; Ohki et al. 1994; Ulrich et al. 1998, 1999; Glaser et al. 1999). It has been shown that the fusogenic activity of the fusion peptide of gp41 is highly dependent on its exact sequence (Pereira et al. 1997), e.g. peptide length (Sackett and Shai 2002), hydrophobicity (Mobley et al. 1999) or flexibility (Delahunty et al. 1996; Morris et al. 2004). Many mutations, even of only a single amino acid within the fusion peptide of gp41 have reduced or abolished its fusogenicity (Buchschacher et al. 1995; Kliger et al. 1997). Since the fusion activities depend on mutations in a similar manner for both peptide-induced fusion and virus-induced fusion (Durell et al. 1997; Freed et al. 1992; Pereira et al. 1995; Kliger et al. 1997) it has been suggested that peptide-induced fusion of LUVs may serve as a useful model to study certain aspects of viral/target cell fusion. However, the structural requirements, which are necessary for fusion peptides to induce membrane fusion are poorly understood.

Previous structural investigations on the conformation of fusion peptides and in particular on the gp41 fusion peptide have yielded contradictory results. Depending on the peptide-to-lipid ratio and on the lipid composition, either α-helical or β-stranded structures were observed (Rafalski et al. 1990; Mobley et al. 1999). An α-helical structure that is inserted at an oblique angle into the lipid bilayer has been proposed (Brasseur et al. 1990; Martin et al. 1993, 1996), but on the other hand it was shown that a predominantly α-helical mutant of the gp41 fusion peptide is inactive in membrane fusion (Pereira et al. 1995). Therefore, one might expect a β-sheet structure to represent the actual fusion-active conformation, as it has been described by Weliky et al. using solid state NMR (Yang et al. 2001, 2003, 2004a), consisting of a mixture of parallel and antiparallel β-strands (Yang and Weliky 2003). Using complementary spectroscopic methods, Shai and coworkers have reported a predominantly β-stranded structure of the gp41 fusion peptide when bound to membranes (Pritsker et al 1998, 1999; Sackett and Shai 2003, Gerber et al. 2004). From these controversial results a common picture has emerged over the recent years, namely that many fusion peptides exhibit a characteristic conformational plasticity. These glycine-rich sequences tend to be able to rearrange readily between α-helical and β-stranded conformations. It has even been suggested that the conversion of an α-helical structure to a more extended β-stranded conformation could be the trigger for membrane fusion (Durell et al. 1997; Epand 1998; Afonin et al. 2003). As a functional model, one could imagine that the peptides are first inserted as α-helical monomers into the membrane, and above a threshold concentration they might then re-align on the membrane surface to oligomerize into a β-conformation and form a "fusion intermediate" or "transition state", which finally triggers membrane fusion by allowing lipid mixing and access to the trans-monolayer of the target membrane. Finally, it may also be conceivable that neither conformation corresponds to the actual fusogenic state in the moment of action.

In the present study we focus on the structure of the N-terminal fusion peptide FP23 of gp41, asking which conformation is really consistent with the proposed intermediate/transition state at the moment when the actual bilayer fusion occurs. Is this fusogenic state formed by an arrangement of α-helices, by extended β-strands or any other ordered oligomeric structure? To this aim we have examined the fusion activity of the FP23 and compared it with a series of analogues in which a single bulky amino acid is replaced by the non-natural *L*- or *D*-4-trifluoromethyl-phenylglycine (CF_3-Phg) at different positions. A conservative substitution by this rigid *L*-amino acid is expected to have less of an influence on the fusogenic activity than the incorporation of the sterically obstructive *D*-enantiomer of CF_3-Phg. Figure 1 illustrates the arguments used here to deduce structural information about the rate-determining step of fusion. If the critical step involves a β-stranded oligomer, then the *L*-epimers should be readily able to engage in fusion, since the geometry of the rigidly connected *L*-phenyl-group will tolerate an oligomeric arrangement of parallel or antiparallel β-strands. In contrast, for the *D*-epimers a tight hydrogen-bonded arrangement of β-strands is implausible due the spacious requirement of the *D*-phenyl-group which "sticks out" the wrong way, i.e. which lies in the plane of a putative β-sheet. Likewise, if fusion proceeds via a well-ordered α-helical conformation, folding will be somewhat impaired in the *D*-analogues but not in the *L*-epimers. Whether a specific peptide conformation in the membrane is required for fusion can thus be deduced by checking for any systematic differences in

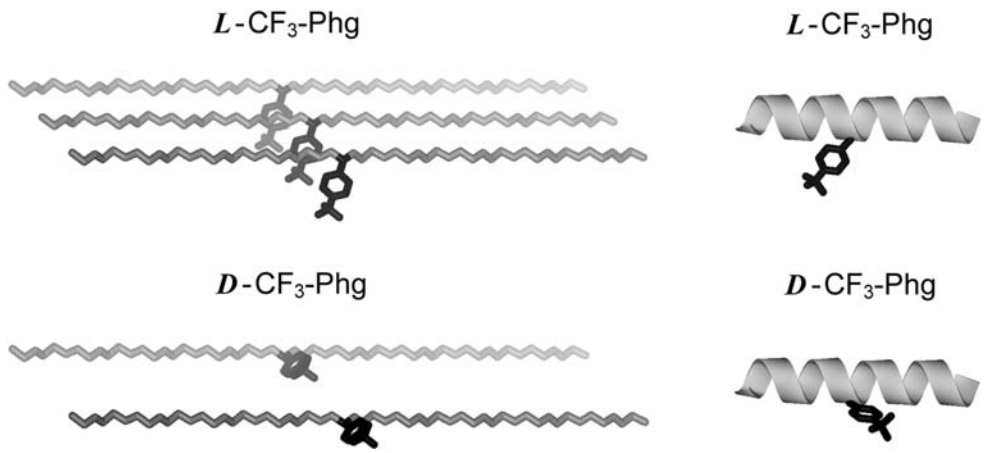

Fig. 1 Schematic illustration of plausible conformations of fusogenic peptides containing a rigid and sterically demanding CF_3-Phe substitution. Peptides containing the *L*-amino acid (*top left*) can readily self-assemble as β-sheets, whereas the sterically obstructive phenyl group in the *D*-configuration (*bottom left*) will prevent such arrangement. An α-helical fold is somewhat more favourable for peptides containing the *L*-amino acid (*top right*) than for the *D*-epimers (*bottom right*)

the fusion activities of the *D*- and *L*-epimers of FP23. If the *D*-epimers are nearly inactive, an oligomeric β-stranded structure will be the most likely fusogenic conformation. If the *D*-epimers are systematically less active than the *L*-epimers, an α-helical structure can be considered as a plausible conformation, and finally, if there is no systematic preference for either the *D*- or the *L*-epimers, then an ordered or oligomeric structure does not seem to be necessary for membrane fusion.

Labelling of peptides with CF_3-Phg is well established in our laboratory, as it is usually applied for solid state ^{19}F-NMR structure analysis (Ulrich et al. 1999; Afonin et al. 2003, 2004; Glaser et al. 2005; Ulrich 2005; Ulrich et al. 2005; Strandberg et al. 2006; Tremouilhac et al. 2006). For the present functional analysis of FP23 the positions to be labelled were chosen in the central stretch of the peptide, so that there would be no concern about fraying termini. To maintain the hydrophobicity of the peptide and to minimize any distortions, only the bulky amino acids Leu (at position 7, 9 and 12) or Phe (at position 8 and 11) were replaced by CF_3-Phg (Grasnick 2006). The small side chain of Ala was retained at positions 6, 14 and 15, and also Gly at positions 10 and 13 was preserved to maintain the intrinsic flexibility of FP23 (Table 1). The peptides listed in Table 1 were synthesized as *D*- as well as *L*-epimers (abbreviated as 7D, 7L, ..., 12D, 12L) and their fusion activities were monitored by lipid mixing assays and light scattering.

Materials and methods

Chemicals

Fmoc-protected amino acids were purchased from IRIS Biotech (Marktredwitz, Germany) or Novabiochem (Merck Biosciences, Darmstadt, Germany). 6-Chloro-1-hydroxybenzotriazole (Cl-HOBt), *O*-(1H-6-chlorobezoltriazol-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate (HCTU), diisopropylethanolamine (DIPEA) and 1-fluoro-2,4-dinitrophenyl-5-L-alanine-amide (Marfey's reagent) were also obtained from IRIS, and 4-trifluoromethyl-phenylglycine (CF_3-Phg) was from ABCR (Karlsruhe, Germany). N-terminal Fmoc-protection of racemic *D*/*L*-CF_3-Phg was performed with Fmoc-Cl as previously reported (Carpino and Han 1972). 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), triisopropylsilane (TIS), trifluoroethanol (TFE) and trifluoroacetic acid (TFA) were obtained from Sigma-Aldrich (Taufkirchen,

Table 1 Peptides and CF_3Phg-substituted peptide analogues

Wild type (WT)	AVGIG^{5}ALFLG^{10}FLGAA^{15}GSTMG^{20}ARS-$CONH_2$
L7CF_3Phg	AVGIG^{5}A-CF_3Phg-FLG^{10}FLGAA^{15}GSTMG^{20}ARS-$CONH_2$
F8CF_3Phg	AVGIG^{5}AL-CF_3Phg-LG^{10}FLGAA^{15}GSTMG^{20}ARS-$CONH_2$
L9CF_3Phg	AVGIG^{5}ALF-CF_3Phg-G^{10}FLGAA^{15}GSTMG^{20}ARS-$CONH_2$
F11CF_3Phg	AVGIG^{5}ALFLG^{10}-CF_3Phg-LGAA^{15}GSTMG^{20}ARS-$CONH_2$
L12CF_3Phg	AVGIG^{5}ALFLG^{10}F-CF_3Phg-GAA^{15}GSTMG^{20}ARS-$CONH_2$

Germany). Piperidine, dimethylsulfoxide (DMSO), and acetanhydride were from Roth (Karlsruhe Germany), *N*-methyl-2-pyrrolidone (NMP) and Triton X-100 from Merck (Darmstadt, Germany), and ethanedithiol (EDT) and acylase I from pig kidney (EC 3.5.1.14) were from Fluka (Taufkirchen, Germany). The lipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPS), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-(phospho-L-serine) (POPS), sphingomyelin, phosphatidylinositol (PI), and cholesterol (Chol), as well as the fluorescent labelled lipids L-α-phosphatidylethanolamine-*N*-(4-nitrobenzo-2-oxa-1,3-diazole (NBD-PE) and L-α-phosphatidylethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Rh-PE) were obtained from Avanti Polar Lipids (Alabaster, AL, USA).

Peptide synthesis

Peptides were synthesised on an ABI 433A instrument (Applied Biosystems, Foster City, CA, USA) using standard solid-phase Fmoc protocols (Chang and Meienhofer 1978; Fields and Noble 1990). Fmoc-deprotection was performed using 20% piperidine in NMP, and Cl-HOBt/HCTU/DIPEA was used for coupling reactions. The protected peptides were cleaved from the resin with TFA/H_2O/TIS/EDT in a 94:2.5:2.5:1 volume ratio. The crude peptides were precipitated with cold diethyl ether, taken up in acetonitrile/water (1:1) and lyophilized. Peptides were purified with reversed-phase HPLC (Jasco, Tokyo, Japan) equipped with a diode array detector on a preparative C18 column (22 mm × 150 mm, Vydac, Hesperia, CA, USA) with a flow rate of 15 ml min^{-1} at 35°C. Standard acetonitrile/water buffers containing 5 mM HCl instead of 0.1% TFA were employed as previously described (Afonin et al. 2003). All the labelled peptides eluted in the range of 30–50% acetonitrile, and the epimeric peptides containing the *D*- and *L*-forms of CF_3-Phg could be separated. To identify *D*- and *L*-CF_3-Phg, enantiomerically pure samples of the purified peptides (0.3 mg) were hydrolyzed with 6 M HCI (1.0 ml) for 16 h at 105°C, followed by derivatization with Marfey's reagent (Marfey 1984). For assignment, the commercially available racemic mixture of *D*/*L*-CF_3-Phg first had to be acetylated using acetanhydride/LiOH in quantitative yields, and subsequently deacylated by a stereospecific enzymatic reaction using Acylase I (EC 3.5.1.14) from pig kidney as previously described (Morgan et al. 1997; Chenault et al. 1989; Grasnick 2006). Combining the results from the enzymatic analysis and Marfey's reactions, the peptides containing the *D*- and *L*-forms of CF_3-Phg were assigned. The masses of the peptides were verified with MALDI-TOF spectrometry on a Bruker Biflex IV instrument (Bruker Daltronic, Bremen, Germany).

Preparation of large unilamellar vesicles (LUVs)

For vesicle preparation a lipid/cholesterol mixture was used similar to "LM-3" (Yang et al. 2003) which reflects approximately the lipid headgroup and cholesterol composition of the HIV-1 virus and its target T-cell membranes (Aloia et al. 1993). The mixture contained POPC, POPE, POPS, sphingomyelin and cholesterol in a 10:5:2:2:10 molar ratio. Lipid and cholesterol powders were dissolved together in chloroform, and the chloroform was removed under a gentle stream of nitrogen, followed by overnight vacuum pumping. Lipid dispersions were formed by addition of 5 mM pH 7.4 HEPES, followed by vigorously vortexing (10 × 1 min) and homogenizing with ten freeze-thaw cycles. Finally, the LUVs were prepared by extrusion through a polycarbonate filter with 100 nm diameter pores (Avanti Mini Extruder). The temperature during extrusion was kept at ≥40°C by heating the extruder block to approximately 50°C. The formation of the vesicles was monitored by dynamic light scattering (see below). Starting from multilamellar vesicles with an average diameter of approximately 500 nm and a broad size distribution, it was observed already after 5–7 passes that the vesicle diameter and size distribution parameter became constant. Therefore, eleven passes were sufficient to yield LUVs with a sharp size distribution and were applied for all vesicle preparations.

Lipid mixing measurements

Lipid mixing experiments were carried out according to Struck et al. (1981) by measuring the fluorescence intensity change resulting from the fluorescence resonance energy transfer (FRET) between NBD-PE and Rh-PE inserted in the LUV bilayer. Vesicles containing both dyes at 2 mol% each were prepared as described above and mixed with dye-free vesicles at a 1:9 molar ratio, to give a final lipid concentration of 150 μM in 5 mM HEPES buffer, pH 7.4. Fluorescence was monitored using a Yobin Yvon FluoroMax2 spectrofluorimeter and recorded with excitation set at 450 nm (6 mm slit) and emission at 530 nm (6 mm slit). To avoid cross-talk and scattering artefacts, the emission monochromator was shielded with two cut-off filters (GG470 and GG495, 2 mm each, Schott, Mainz, Germany). During measurements the samples (final volume = 1.5 ml) were continuously stirred and the temperature was controlled with a thermostatic bath at

37°C. The initial fluorescence of the labelled/unlabelled vesicle suspension was taken as 0% lipid mixing, and the 100% scale was determined by adding Triton X-100 at a final concentration of 0.5% (v/v). Peptides were always added from stock solutions of 1 mg/ml (≈470 μM) in DMSO prepared by weighing to give a final concentration of 6 μM (4 mol%). Their individual concentrations were additionally calibrated by UV-spectra. Adding an aliquot of pure DMSO to the vesicles as a control caused only a small deviation of the baseline (≤2%) as expected from sample dilution.

Vesicle size measurements

The size of the lipid vesicles was determined by dynamic light scattering (Zetasizer Nano S, Malvern Instruments Ltd.). According to the Cumulants analysis the mean size (z-average diameter) of the vesicles was derived from the slope of the linearized form of the autocorrelation function (first Cumulant), and the second Cumulant was used to calculate the polydispersity index (PdI), a parameter, which yields an estimate of the width of the size distribution. During the preparation of LUVs, small amounts of the lipid suspension (20 μl in 5 mM HEPES, pH 7.4) were collected after every second extrusion pass in order to measure the vesicles size. An z-average diameter of 120 ± 24 nm was observed after 5–7 passes, with a PdI of 0.085 ± 0.029, indicating a narrow, nearly monomodal distribution. The sizes of fused vesicles were determined 30 min after addition of the peptides at 6 μM (4 mol%). Aliquots (300 μl) of the samples in which lipid mixing had been induced at 37°C were transferred to the Zetasizer instrument, and the vesicles mean size was characterized at 25°C. With increasing average diameter also the PdI was observed to increase (up to 0.35), indicating a broader size distribution as expected for fused vesicles.

Circular Dichroism (CD) spectroscopy

Circular Dichroism (CD) spectra were recorded on a Jasco J-810 spectropolarimeter (Jasco Corp., Tokyo, Japan). Samples were prepared with 0.2 mg peptide in 1 ml TFE. Spectra were recorded in quartz cuvettes (0.1 cm path length) at 20°C, three scans were averaged, and the TFE baseline was subtracted. Since the high hydrophobicity of the peptides could lead to losses due to aggregation no mean residue ellipticity values were calculated, given that the peptide concentrations could not be accurately determined. Instead, the ellipticity data (mdeg) measured by the instrument were normalized to the ellipticity of the FP23 wild type at 206 nm. The spectra are thus presented as normalized ellipticities, with the intention to emphasize changes in the line shape rather than showing absolute (and inaccurate) intensities.

Results

Fusogenic activity according to lipid mixing

The fusogenic activity of the FP23 wild type peptide and its analogues substituted with D- or L-CF_3-Phg was characterized by their ability to induce lipid mixing in LUVs. FRET experiments were carried out with LUVs containing NBD- and Rhodamine-labelled lipids (Struck et al. 1981), which were mixed with a ninefold excess of dye-free vesicles. When dye dilution is induced by a fusogenic peptide, the NBD fluorescence at 530 nm is increased. Once fusion has been completed, the percentage of fusion is determined relative to the 100% level achieved by the addition of Triton, and relative to the 0% baseline before the induction of fusion. Figure 2 shows that 4 mol% of the wild type peptide (grey bar, left column) induced about 50% fusion (dashed line), which was complete after 5–10 min. This extent of lipid mixing is comparable to the values in the literature (Peisajovich et al. 2000; Yang et al. 2001, 2003, 2004b; Buzón et al. 2005).

The fusogenic activities of the different D- and L-epimers are compared with that of the FP23 wild type

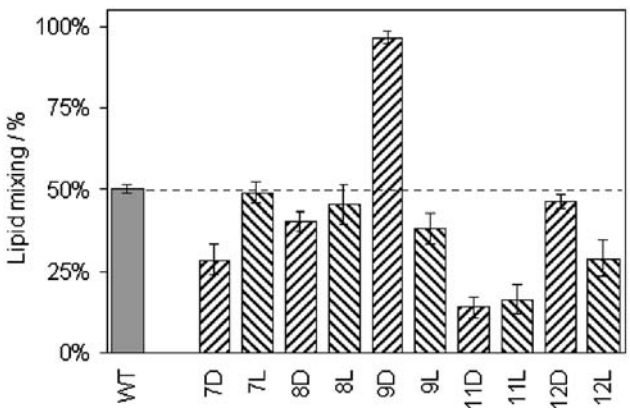

Fig. 2 Lipid mixing assay of the fusogenic peptide FP23 from gp41 of HIV-1, and of its analogues carrying a single D- or L-CF_3-Phg substitution in different positions (*as numbered*). The extent of lipid mixing induced by the wild type peptide (WT, *grey column*, relative to 100% achieved by the addition of Triton) is compared to the mixing levels induced by equivalent amounts of the corresponding D- or L-epimers (*hatched columns*). Error bars indicate the standard deviation over four series of experiments. No systematic difference is observed between the L- and D-epimers

peptide in Fig. 2. The respective percentage of fusion reached after 20 min was determined as a mean value from four separate series of experiments, giving reliable results. Almost all peptide analogues (hatched columns) retained the fusogenic activity of FP23 at levels between 30 and 50%. Only the epimers 11D and 11L show smaller activities (12 and 17%, respectively), and compared to the wild type the activity of epimer 9D is nearly doubled, as its signal reached the 100%. Overall, the most important and definite conclusion to be drawn from Fig. 2 is that there is no systematic loss of activity for either the *D*- or *L*-epimers.

Vesicle size distribution after fusion

To validate the lipid mixing signals as a reliable monitor of the fusion process, a second series of measurements was carried out to determine the vesicle sizes before and 30 min after addition of the fusogenic peptides. Using dynamic light scattering the diameter and size distribution of the vesicles was measured on an absolute scale, and the increase in average diameter is displayed in Fig. 3.

As seen in Fig. 3, each of the FP23 analogues (hatched columns) induced a significant increase in vesicle diameter. Starting from an average diameter of 120 nm, the diameter of the vesicles nearly doubled for the wild type peptide (→ 230 nm), and for the various *D*- and *L*-epimers the diameters increased to different extents (→ 135–220 nm). The observed patterns of the lipid mixing assay (Fig. 2) and the vesicle size increase (Fig. 3) display a nearly perfect match. Even for sample 9D the vast increase in diameter is consistent with the approximately doubled lipid mixing signal of this sample. The observed increase in diameter by approximately 420 nm would correspond to a fusion of approximately 20 vesicles, which is consistent with the observed level of 100% fusion representing complete dilution of the fluorescent-labelled lipids. In summary, it can be stated that the patterns of the fusogenic activity of the different peptides obtained by lipid mixing and by vesicle size measurements agree very well, and there is no systematic loss of activity for either the *D*- or *L*-epimeric peptides.

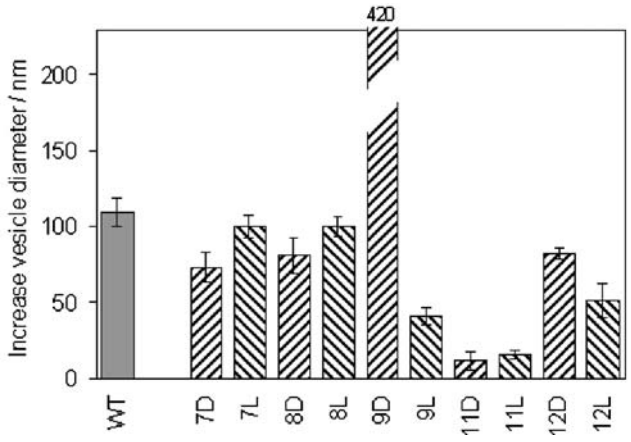

Fig. 3 Measurement of vesicle size by dynamic light scattering, to monitor membrane fusion induced by FP23 and its analogues carrying a single *D*- or *L*-CF_3-Phg substitution in different positions (*as numbered*). The numerical increase in vesicle diameter induced by the wild type peptide (WT, *grey column*) is compared to the increase observed after adding an equivalent amount of the corresponding *D*- or *L*-epimers (*hatched columns*). The average diameter of LUV before fusion was 120 ± 24 nm. No systematic difference is observed between the *L*- and *D*-epimers

CD spectroscopy

The question whether FP23 and its *D*- or *L*-CF_3-Phg substituted analogues have a preference for a certain secondary structure was examined by CD spectroscopy. In Fig. 4 representative spectra of FP23 wild type, the 9*D/L*- and the 12*D/L*-epimers in TFE are presented, all of which show a lineshape that is typical of an α-helical conformation with a minor contribution of random coil structure. The *L*-form of CF_3-Phg does not seem to induce any significant perturbation in the peptide backbone, as the spectra of the *L*-epimers are virtually superimposable on that of FP23 wild type. The *D*-form, on the other hand, is expected to cause more perturbation in the helix backbone and indeed, their spectra differ more from the wild type than those of the *L*-form in showing a slightly reduced α-helical content.

Discussion

The aim of this study was to examine whether membrane fusion requires a well-ordered peptide structure, such as an α-helical conformation or a β-stranded oligomer. If such structured intermediate has to be formed, then the rate-determining step of fusion would be slowed down if the folding or self-assembly process is hindered. For that purpose we have introduced a single sterically obstructive *D*-CF_3-Phg side chain into various positions (Leu7, Phe8, Leu9, Phe11 and Leu12) of the fusogenic sequence FP23 from the gp41 protein of HIV-1. Generally, the *L*-form of CF_3-Phg can be readily accommodated in an oligomeric β-sheet as well as an α-helix without much impact on the backbone conformation. The *D*-form, on the other hand, will prevent the assembly of oligomeric β-sheets due to steric hindrance, which is immediately obvious for the case of parallel β-strands (Fig. 1). Even the formation of an

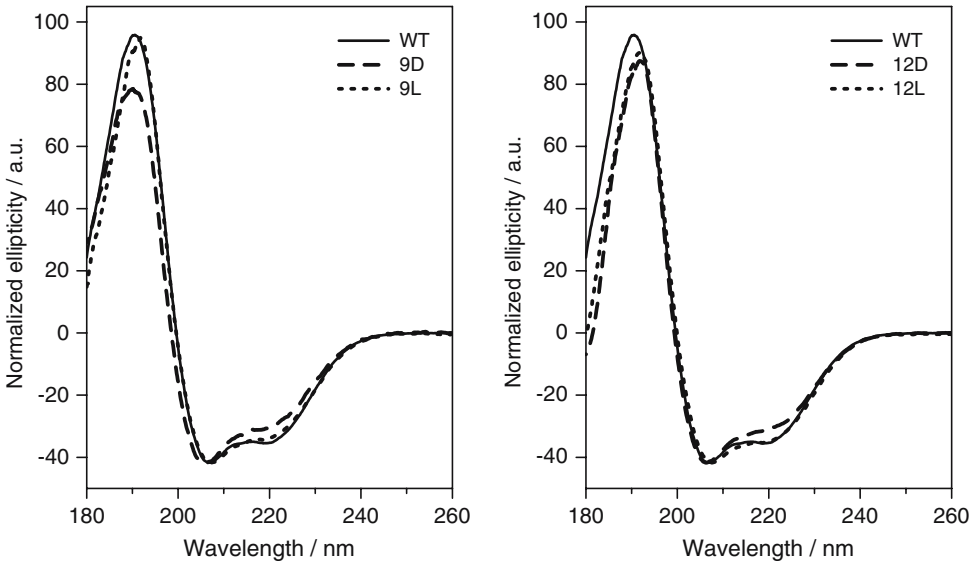

Fig. 4 Representative CD spectra of FP23 wild type peptide (*WT*) in TFE solution and its analogues carrying a single *D*- or *L*-CF_3-Phg substitution in position 9 (*left panel*) or 12 (*right panel*), respectively. All lineshapes are typical of an α-helical conformation. The spectra of the *L*-forms are virtually the same as of the wild type, whereas the *D*-forms show a slight decrease in helicity

antiparallel, out-of-register assembly is under examination here, since the labelled positions in FP23 cover more than half the length of the peptide. If we consider the possibility of an α-helix, however, its formation will not be prevented by incorporation of CF_3-Phg, although the *D*-form will cause more perturbation in the local backbone torsion angles than the *L*-form. Indeed, this effect is demonstrated by the CD data of FP23 shown in Fig. 4. Based on these arguments, here we compare the fusion activities of the *D*-analogues with the corresponding *L*-epimers and the wild type peptide, using lipid mixing assays and vesicle size measurements. Significant and systematic differences in the activities of the *D*- and *L*-epimers would be expected if an α-helical or β-stranded structure was necessary for fusion. Basically, the activity of the *D*-epimeric peptides should be nearly suppressed if fusion proceeds via oligomeric β-sheets, whereas as small but significant reduction in activity would point to an α-helical conformation. Only if the rate-determining step of fusion proceeds independently of the peptide conformation, then we would expect no systematic differences between *D*- and *L*-forms. Interestingly, both assays of fusion activity (lipid mixing, vesicle size) showed no preference for either the *D*- or the *L*-epimers. We therefore conclude that the fusogenic state of FP23 does not require any particular well-structured conformation.

This finding is in good agreement with results from our earlier study on the fusogenic peptide B18 from the sea urchin fertilization protein bindin (Afonin et al. 2003). In that investigation we had tested the fusion rates of a series of B18 analogues, each of which carried a single 4-fluoro-phenylglycine (4F-Phg) substitution at different positions for a comprehensive solid state ^{19}F-NMR structure analysis (Afonin et al. 2003). In this system we also did not observe any systematic preference for either the *D*- or the *L*-epimers, as illustrated in Fig. 4. The fusion rates had been determined less accurately, but the overall conclusion is the same as from the present FP23 data. The results of these investigations on two different types of fusogenic peptides with different sequences, i.e. the terminal fusion peptide FP23 of gp41 and the internal sequence of B18 of bindin, suggest that membrane fusion in vitro does not require a specific peptide conformation in the rate-determining step.

At this point one might argue that the peptide structure may be irrelevant if the critical step of fusion is determined only by the rearrangements of the lipid molecules. However, it is obvious that the peptide sequence plays a major role in the fusion kinetics, as seen in Figs. 2 and 5 from the variations between the different substituted positions. Hence it is a surprising outcome of this study that the secondary structure plays much less of a role than the primary peptide sequence. This finding sheds new light on many previous studies concerned with the structure analysis of fusogenic peptides, which seem to have yielded conflicting and contradictory results. Namely, in previous attempts to determine the structure of FP23 and other fusogenic peptides, either α-helical or β-stranded conformations had been reported, depending on the experimental conditions such as lipid composition and peptide concentration (Mobley et al. 1999; Ulrich et al. 1999; Afonin et al. 2004). It was thus suggested that fusogenic peptides tend to be conformationally rather variable, i.e. they possess a high structural plasticity (Del Angel et al. 2002; Vaccaro et al. 2005). Our new data of FP23' fusion activity has now demonstrated that this conformational plasticity described in previous

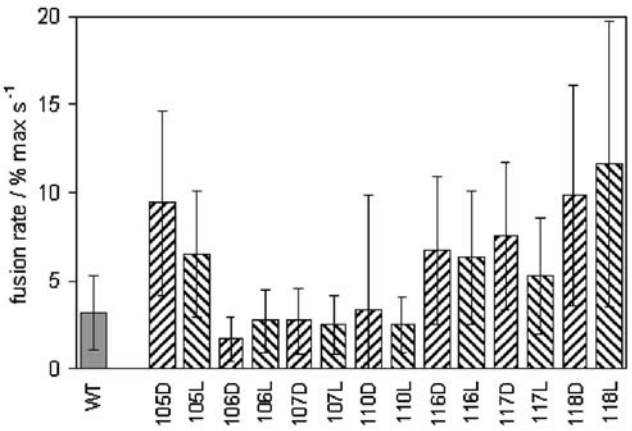

Fig. 5 Lipid mixing assay of the fusogenic peptide B18 from the sea urchin protein bindin, and of its analogues carrying a single *D*- or *L*-4F-Phg substitution in different positions (numbered according to the sequence of *S. purpuratus*), as reported previously by Afonin et al. (2003). For the fusion assay LUVs of bovine brain sphingomyelin/cholesterol (80:20 molar ratio) were used, with and without 0.8 mol% each of the fluorescence-labelled lipids NBD-PE and Rh-PE. The labelled vesicles were suspended in 2 ml buffer together with a threefold excess of unlabelled vesicles, giving a total lipid concentration of 200 μM. The measurements were performed at 30°C, and the fusion rate was determined from an exponential fit to the initial increase in fluorescence intensity after addition of 2 μM peptide, and was compared with the total increase after addition of Triton (% max/s). No systematic difference in the rates of fusion is observed between the *L*- and *D*-epimers

static pictures is closely connected to the conformational tolerance displayed here in the dynamic function of such peptide.

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