The Effect of Peptide/Lipid Hydrophobic Mismatch on the Phase Behavior of Model Membranes Mimicking the Lipid Composition in *Escherichia coli* Membranes

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ABSTRACT The effect of hydrophobic peptides on the lipid phase behavior of an aqueous dispersion of dioleoylphosphatidylethanolamine and dioleoylphosphatidylglycerol (7:3 molar ratio) was studied by ^{31}P NMR spectroscopy. The peptides (WALP*n* peptides, where *n* is the total number of amino acid residues) are designed as models for transmembrane parts of integral membrane proteins and consist of a hydrophobic sequence of alternating leucines and alanines, of variable length, that is flanked on both ends by tryptophans. The pure lipid dispersion was shown to undergo a lamellar-to-isotropic phase transition at ~60°C. Small-angle x-ray scattering showed that at a lower water content a cubic phase belonging to the space group *Pn3m* is formed, suggesting also that the isotropic phase in the lipid dispersion represents a cubic liquid crystalline phase. It was found that the WALP peptides very efficiently promote formation of nonlamellar phases in this lipid system. At a peptide-to-lipid (P/L) molar ratio of 1:1000, the shortest peptide used, WALP16, lowered the lamellar-to-isotropic phase transition by ~15°C. This effect was less for longer peptides. For all of the WALP peptides used, an increase in peptide concentration led to a further lowering of the phase transition temperature. At the highest P/L ratio (1:25) studied, WALP16 induced a reversed hexagonal liquid crystalline (H_{II}) phase, while the longer peptides still promoted the formation of an isotropic phase. Peptides with a hydrophobic length larger than the bilayer thickness were found to be unable to inhibit formation of the isotropic phase. The results are discussed in terms of mismatch between the hydrophobic length of the peptide and the hydrophobic thickness of the lipid bilayer and its consequences for lipid-protein interactions in membranes.

INTRODUCTION

The interactions between proteins and lipids are essential for membrane function and structure. Hydrophobic matching, i.e., the matching between the hydrophobic length of an integral membrane protein and the hydrophobic thickness of the lipid bilayer, can be an important factor in protein-lipid interactions (Mouritsen and Bloom, 1984; Killian, 1998). This may be illustrated by the observed correlation between the length of membrane-spanning parts of proteins in different membranes and the hydrophobic thickness of these membranes (Bretscher and Munro, 1993; Pelham and Munro, 1993), as well as by results of reconstitution studies of proteins in lipid bilayers of varying thickness (Caffrey and Feigenson, 1981; Cornea and Thomas, 1994; Riegler and Möhwald, 1986; Dumas et al., 1997). So far, little is known about the molecular interactions underlying the effects of hydrophobic mismatch, which among other factors is due to the complexity of the systems studied. To get a better understanding of the lipid-protein interactions involved, we and others use simple designed peptides as models for the membrane-spanning parts of integral membrane proteins (de Planque et al., 1998, 1999; Killian et al., 1996; Morein et al., 1997; Huschilt et al., 1985; Morrow et al., 1985; Zhang et al., 1992, 1995). Examples of such model peptides are WALP peptides, which have a hydrophobic core of alternating leucines and alanines of varying lengths that is flanked on both ends by two tryptophans (Table 1). These peptides were shown to form transmembrane α-helices in bilayers of diacylphosphatidylcholine (de Planque et al., 1999; Killian et al., 1996; Morein et al., 1997). The choice of tryptophans as "anchoring residues" was rationalized by the fact that these amino acid residues are often found close to the polar/apolar membrane interface in integral membrane proteins (Landolt-Marticorena et al., 1993; von Heijne, 1994; Reithmeier, 1995) and that they appear to have a preferred interaction with the interfacial region of the lipid bilayer (Persson et al., 1998; Yau et al., 1998). Previously it was shown that WALP peptides can strongly affect the lipid phase behavior in diacylphosphatidylcholines in a mismatch-dependent manner (de Planque et al., 1999; Killian et al., 1996; Morein et al., 1997). Peptides that are much too short to span the hydrophobic part of the bilayer induce a reversed hexagonal (H_{II}) phase, whereas intermediate length peptides induce an isotropic phase. In the presence of even longer peptides the lipids remain in a bilayer organization.

While diacylphosphatidylcholines normally form stable bilayers in water over a large temperature range, lipid extracts from cell membranes often show temperature-dependent lamellar-to-nonlamellar phase transitions (Morein et al., 1996; Rietveld et al., 1993; Lindblom et al., 1986). To study the effect of hydrophobic mismatch on a lipid system that more resembles a biological cell membrane, we chose

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TABLE 1 Amino acid sequence of the peptides used and their estimated total length

Peptide	Sequence	Length (Å)
WALP16	Acetyl-GWW$(LA)_5$WWA-ethanolamine	24
WALP17	Acetyl-GWW$(LA)_5$LWWA-ethanolamine	25.5
WALP19	Acetyl-GWW$(LA)_6$LWWA-ethanolamine	28.5
WALP21	Acetyl-GWW$(LA)_7$LWWA-ethanolamine	31.5
WALP23	Acetyl-GWW$(LA)_8$LWWA-ethanolamine	34.5
WALP31	Acetyl-GWW$(LA)_{12}$LWWA-ethanolamine	46.5

It is assumed that the WALP peptides have an α-helical conformation and that each amino acid contributes 1.5 Å to the length of the α-helix.

to investigate the effect of WALP analogs of different lengths on the phase behavior of mixtures of dioleoylphosphatidylethanolamine (DOPE)/dioleoylphosphatidylglycerol (DOPG) (7:3 molar ratio). This lipid mixture was chosen for several reasons: 1) it mimics the lipid composition in *Escherichia coli* cell membranes (Kadner, 1996) but is much better defined with only one type of acyl chain present; 2) like *E. coli* lipids at excess water content, phosphatidylethanolamine/phosphatidylglycerol mixtures undergo a lamellar-to-isotropic phase transition at elevated temperatures (Killian et al., 1990, 1992); 3) effects on phase behavior are functionally relevant because in *E. coli* the lipid composition is regulated so that the propensity of the membrane lipids to form nonlamellar phases is kept within strict limits (Morein et al., 1996; Rietveld et al., 1993).

The aim of our studies was to answer the following questions: Are relatively short transmembrane peptides able to promote the formation of nonlamellar phases as a function of hydrophobic mismatch in this lipid system? If so, how sensitive is this system, i.e., what peptide concentrations are required to induce changes in phase behavior? Does the type of nonlamellar phase formed depend on the extent of mismatch, as found in diacylphosphatidylcholines? If short peptides destabilize the bilayer, can relatively long peptides stabilize the bilayer and raise the lamellar-to-nonlamellar phase transition temperature? To address these questions we first characterized the lipid/water mixture in the absence of peptides, i.e., we determined the phase behavior and studied the nature of the nonlamellar phase formed. Next, we investigated the effect of different WALP peptides on the phase behavior of the lipids, both as a function of peptide length and as function of peptide concentration. It was found that very low peptide concentrations are sufficient to significantly affect lipid phase behavior and that they do so in a mismatch-dependent manner. No evidence for bilayer stabilization was found, even for the longest peptides used.

MATERIALS AND METHODS

Materials

The WALP peptides were synthesized and purified as described elsewhere (de Planque et al., 1999; Killian et al., 1996). 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-sn-glycero-3-phosphoglycerol (DOPG) were purchased from Avanti Polar Lipids (Alabaster, AL). The purity was checked by thin-layer chromatography (TLC), and the lipids were used without further purification. Trifluoroacetic acid (TFA) and 2,2,2-trifluoroethanol (TFE) were obtained from Merck (Darmstadt, Germany).

Methods

Sample preparation

The peptide-lipid samples for NMR were prepared by a mixed-film method as follows. The phospholipids (typically 20 μmol of lipid) were dissolved in 1.5 ml chloroform. The peptide was dissolved in TFA and dried to a film under a stream of nitrogen and subsequently dissolved in 0.5 ml TFE. The peptide concentration in stock solution was determined by the absorbance at 280 nm, using an extinction coefficient of 21,300 M^{-1} cm^{-1} (Killian et al., 1996). Appropriate volumes of peptide stock solution and TFE, adding up to a volume of 150 μl, were added to the lipid solution. The mixture was vortexed and dried to a film in a rotavapor. The samples were further dried overnight under vacuum. The dry lipid film was hydrated with 2 ml buffer (10 mM piperazine-*N,N′*-bis(2-ethanesulfonic) acid , 100 mM NaCl, 0.2 mM EDTA (pH 7.4)) and transferred to thick-walled 8-mm glass tubes. The samples were then centrifuged three times for 20 min at 15,000 rpm in a SS34-rotor with a Sorvall RC-2B centrifuge at 10°C. Between centrifugation steps the supernatant was removed, and the peptide-lipid pellet was again dispersed in buffer. After the final centrifugation step enough supernatant was left to ensure that the NMR samples contained excess water, i.e., enough buffer was left so that the water surface was ~2 mm above the lipid pellet. The samples were freeze-thawed, by subsequent freezing in an ethanol/CO_2(s) bath and thawing at ~50°C, at least 10 times before measurements.

^{31}P-NMR spectroscopy

^{31}P-NMR experiments were carried out on a Bruker MSL 300 NMR spectrometer at 121.4 MHz, with a 17-μs 90° pulse, a 1-s interpulse time, and gated proton-noise decoupling. A 100-Hz line broadening was applied before Fourier transformation. Temperature-dependent measurements were recorded in the range of 20–65°C in steps of 10°C. The samples were allowed to equilibrate for 2 h at each temperature before the spectra were recorded. To check reproducibility, the temperature dependence of each sample was measured at least twice, and in some cases by measurement of duplicate samples. The percentages of the different phases present were estimated from the resulting ^{31}P NMR spectra by integration, and subtraction of spectra containing a single type of phase.

Small-angle x-ray diffraction

X-ray diffraction measurements were performed at station 8.2 at the Daresbury Laboratory (Cheshire, England), using a monochromatic beam of wavelength 0.15 nm. X-ray data were calibrated against a sample of wet rat-tail collagen. The samples were first incubated overnight at 60°C and then kept at room temperature before measurements were taken. Immediately before the diffraction experiment the sample was taken out of flame-sealed glass tubes and placed between mica sheets held by a copper spacer. The sample temperature was controlled by mounting the sample on a modified microscope cryostat (Linkham, England) and monitoring the temperature with a thermocouple embedded in the sample adjacent to the beam.

Sucrose density gradient centrifugation

Samples first used for NMR measurements were dispersed in 1 ml of water and placed at the top of a linear sucrose gradient (5.5–17.5%). The samples

were centrifuged for 22 h at 150,000 × *g* at 20°C in a Beckman SW41 rotor. Separate bands were isolated, and the peptide content in each band was determined by UV absorbance at 280 nm, as described above, after dilution of the sample in TFE. The phospholipid content was determined according to the method of Rouser et al. (1970).

RESULTS

Phase behavior of the pure lipids

In this work we studied the effect of WALP peptides of different lengths (Table 1) on the phase behavior of a mixture of DOPE and DOPG with a lipid molar ratio of 7:3. The temperature-dependent phase behavior of the lipid mixture, in the absence of peptides, at excess water (buffer) concentration can be inferred from the ^{31}P NMR spectra shown in Fig. 1 *A*.

At 20°C the ^{31}P NMR spectra show the characteristics of phospholipids in the lamellar liquid crystalline (L_α) state with a high-field peak and a low-field shoulder (Lindblom, 1996). The intensity of the shoulder is lower than for a randomly oriented lipid dispersion (powder spectrum), which indicates a macroscopic alignment of the lipid phase in the magnetic field, as described previously for lipid extracts from *E. coli* (Seelig et al., 1985). The L_α phase is stable up to at least 50°C, and at ~60°C an isotropic component appears in the ^{31}P NMR spectra. At 65°C the spectrum shows a single narrow symmetrical peak. This isotropic phase was optically transparent and remained stable for a long time after the temperature was lowered to 20°C, suggesting that it might represent a cubic phase (Lindblom and Rilfors, 1989). Because the formation of the isotropic phase (both in the pure lipid samples and in the presence of peptide) was found to be rather slow (data not shown), a relatively long incubation time of 2 h was used at each temperature, before the spectrum was recorded. Control measurements in which the samples were further left to equilibrate overnight at selected temperatures yielded qualitatively similar results but usually resulted in a slightly higher fraction of the isotropic phase. It should also be mentioned that in the cases where a single L_α phase was observed after 2 h of incubation, a longer incubation at the same temperature did not result in the formation of an isotropic phase.

To shed further light on the nature of this isotropic phase, small-angle x-ray diffraction measurements were performed. However, no well-resolved diffraction peaks were observed. It is possible that the high water concentration favors formation of very small microcrystallites (ordered small domains of the cubic liquid crystalline phase) result-

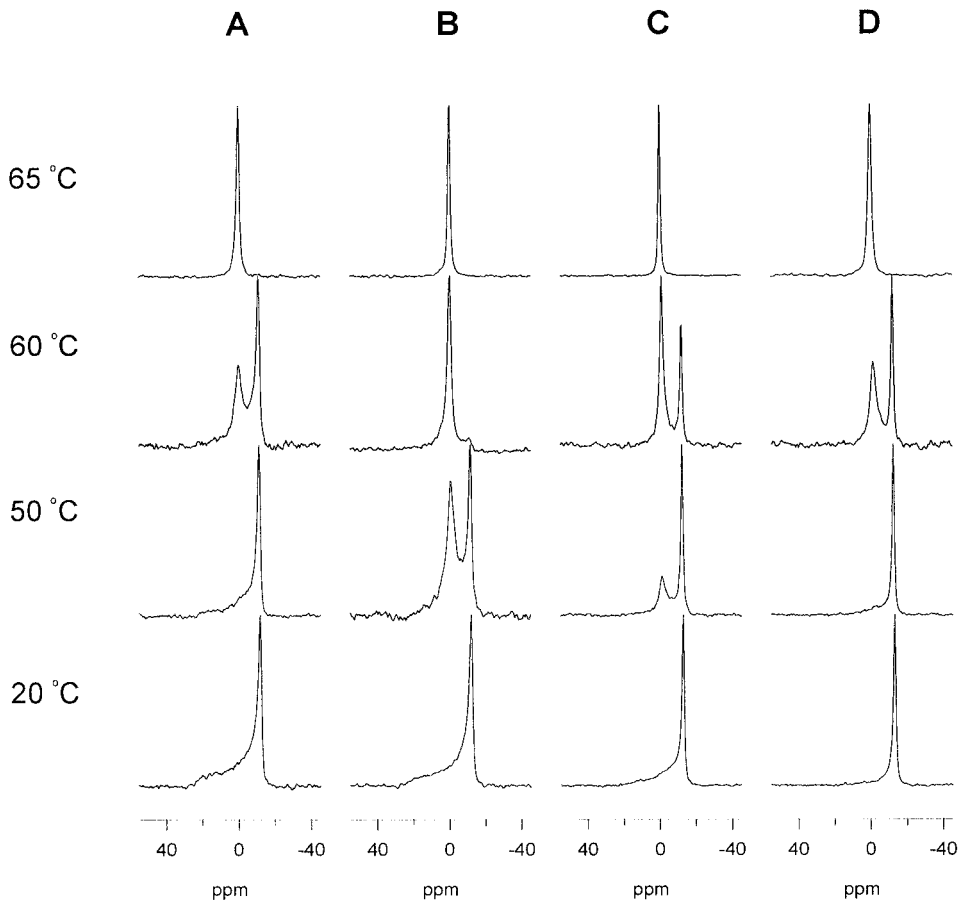

FIGURE 1 ^{31}P-NMR spectra showing the phase behavior as a function of temperature for DOPE/DOPG (7:3 molar ratio) without (*A*) and with (*B–D*) incorporated peptide. All samples contain excess water (i.e., buffer), and the P/L molar ratio is 1:1000. (*B*) WALP16; (*C*) WALP19; (*D*) WALP23.

ing in an intensity of the peaks in the diffraction experiments that is too low, or that formation of a well-ordered cubic phase at these high water concentrations would require much longer equilibration times. Therefore a lipid dispersion was prepared that contained only 60 wt% water. This system was shown by ^{31}P NMR to undergo a lamellar-to-isotropic phase transition at ~60°C, similar to the phase transition for the lipids in excess buffer (results not shown).

As shown in Fig. 2 *A*, several x-ray diffraction peaks were detected from this sample. The observed reflections are consistent with the presence of a cubic phase and a small amount of H_{II} phase. The x-ray peaks from the cubic phase were indexed according to the space group *Pn3m*, and, from the plot of the inverse spacing ($1/d$) against $(h^2 + k^2 + l^2)^{1/2}$, the unit cell dimension was calculated to be 154 Å. In the same way the lattice spacing for the H_{II} phase was calculated to be 75 Å, resulting in a tube diameter of 87 Å. The presence of an H_{II} phase is not surprising because at slightly lower water content and at the same temperature, this lipid system forms an H_{II} phase (results not shown). The dimensions of the H_{II} phase are larger than the values of 66 and 70 Å found for H_{II} phases formed in pure DOPE and DOPE/dioleoylphosphatidylcholine mixtures, respectively, at a similar temperature and water content (Tate and Gruner, 1989). This is explained by the presence of the anionic DOPG, which makes the lipid phase take up more water and thereby increase the tube diameter. It has been well documented, both experimentally and theoretically, that a charged lipid will increase the water uptake of a liquid crystalline phase (see, e.g., Lindblom et al., 1991).

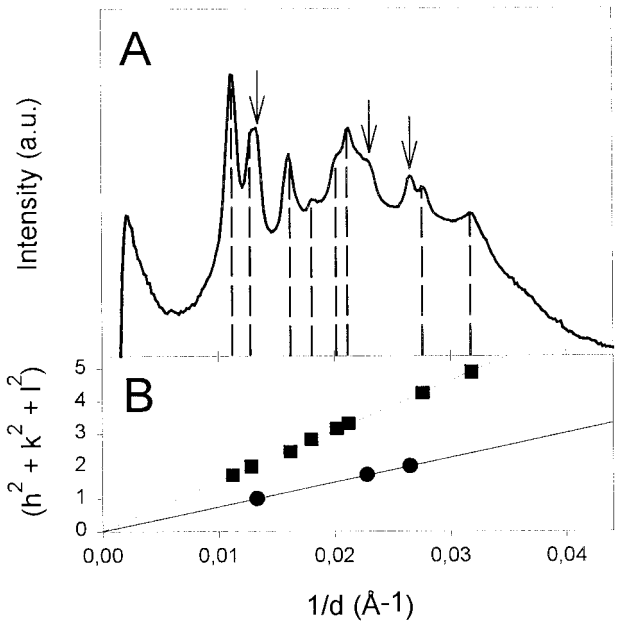

FIGURE 2 (*A*) X-ray powder diffraction pattern of DOPE/DOPG (7:3 molar ratio) hydrated with 60 wt% water recorded at 65°C. The reflections assigned to the cubic phase are marked with dashed lines, and the reflections assigned to the H_{II} phase are marked with arrows. (*B*) The inverse spacing ($1/d$) plotted against $(h^2 + k^2 + l^2)^{1/2}$ for the observed reflections for the cubic phase (■) and the H_{II} phase (●). The reflections from the cubic phase were indexed according to the space group Pn3m, and the unit cell dimension was calculated to be 154 Å. The lattice spacing for the H_{II} phase was calculated to be 75 Å, resulting in a tube diameter of 87 Å.

Phase behavior of peptide-lipid samples

Next, the effect of incorporated peptides on the phase behavior of the DOPE-DOPG dispersed in excess buffer was investigated. Fig. 1, *B–D*, shows ^{31}P NMR spectra of samples with the peptides WALP16, 19, and 23 incorporated into DOPE/DOPG bilayers at a low peptide-to-lipid (P/L) molar ratio of 1:1000. This low P/L ratio was used to test the sensitivity of the system for mismatch-dependent changes in lipid organization. From the spectra it can be seen that even the addition of such small amounts of relatively short peptides lowers the L_α to nonlamellar phase transition temperature (T_{LN}) substantially. It can also be seen that the effect on T_{LN} is correlated with peptide length. Incorporation of WALP16 into DOPE/DOPG lowers the onset of the L_α to isotropic phase transition with ~10–15°C. Incorporation of WALP19 lowers the onset slightly less, while samples containing WALP23 show the same phase behavior as observed for the pure lipids. The same systematic effect, but with a smaller change in T_{LN}, was also observed for samples with a lower P/L ratio (= 1:1500) (results not shown).

This mismatch dependence is further illustrated in Fig. 3, where the effect of peptide length on the lipid phase behavior is quantified as the transition midpoint ($T_{50\%}$) for the lamellar-to-isotropic phase transition. $T_{50\%}$ is taken as the temperature where there is 50% isotropic phase present in the sample. Fig. 3 also shows the results obtained at a higher P/L ratio of 1:500. Here the effects on phase behavior are more pronounced, and the value of $T_{50\%}$ is lower for all peptides, as compared to $T_{50\%}$ for samples with a P/L ratio

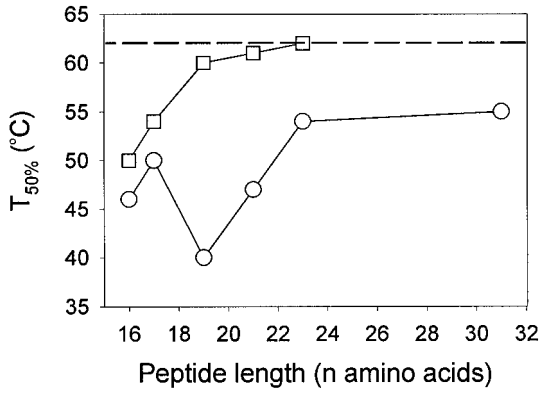

FIGURE 3 The transition midpoint, taken as the temperature where there is 50% isotropic phase as estimated from the ^{31}P NMR spectra, as a function of peptide length for samples with a P/L molar ratio of 1:1000 (□) or 1/500 (○). The dashed line marks the transition midpoint for the pure lipid.

of 1:1000. A striking difference is that, at a P/L ratio of 1:500, the shorter peptides, WALP16 and WALP17, are now less effective in lowering $T_{50\%}$ than the intermediate-length peptides, WALP19 and WALP21. In Fig. 3 it can also be seen that even the longest peptide used, WALP31, reduces $T_{50\%}$ and thus does not stabilize the bilayer.

In Fig. 4 the temperature dependence of the effects of each of the different peptides used in this study is presented at P/L ratios in the range from 1:1000 to 1:200. It can be seen that for all of the peptides used the effect on T_{LN} increases with peptide concentration. Furthermore, for the pure lipid and all of the samples with a low P/L ratio of 1:1000, the phase transition is relatively sharp and completed within a range of ~20°C above the onset of the phase transition. At higher P/L ratios (≥1:500) the same is also true for the shortest (WALP16 and WALP17) and longest (WALP31) peptides used, but not for samples with intermediate long peptides (WALP19, WALP21, and WALP23). For the latter samples the temperature range where there are coexisting L_α and isotropic phases is larger and extends from below 20°C to ~60°C.

The lower efficiency of the shortest peptides in inducing the isotropic phase, as compared to the intermediate length peptides at a P/L ratio of 1:500 (see also Fig. 3) is even more evident at higher peptide concentrations. At a P/L ratio of 1:200, and at 20°C, the WALP16-containing sample is fully in the L_α state, while there is 95% isotropic phase present in the WALP19-containing sample.

In diacylphosphatidylcholines it was found that intermediate-length WALP peptides induced isotropic phases, but that shorter peptides induced an H_{II} phase (Killian et al., 1996). This is not observed in the DOPE/DOPG system for

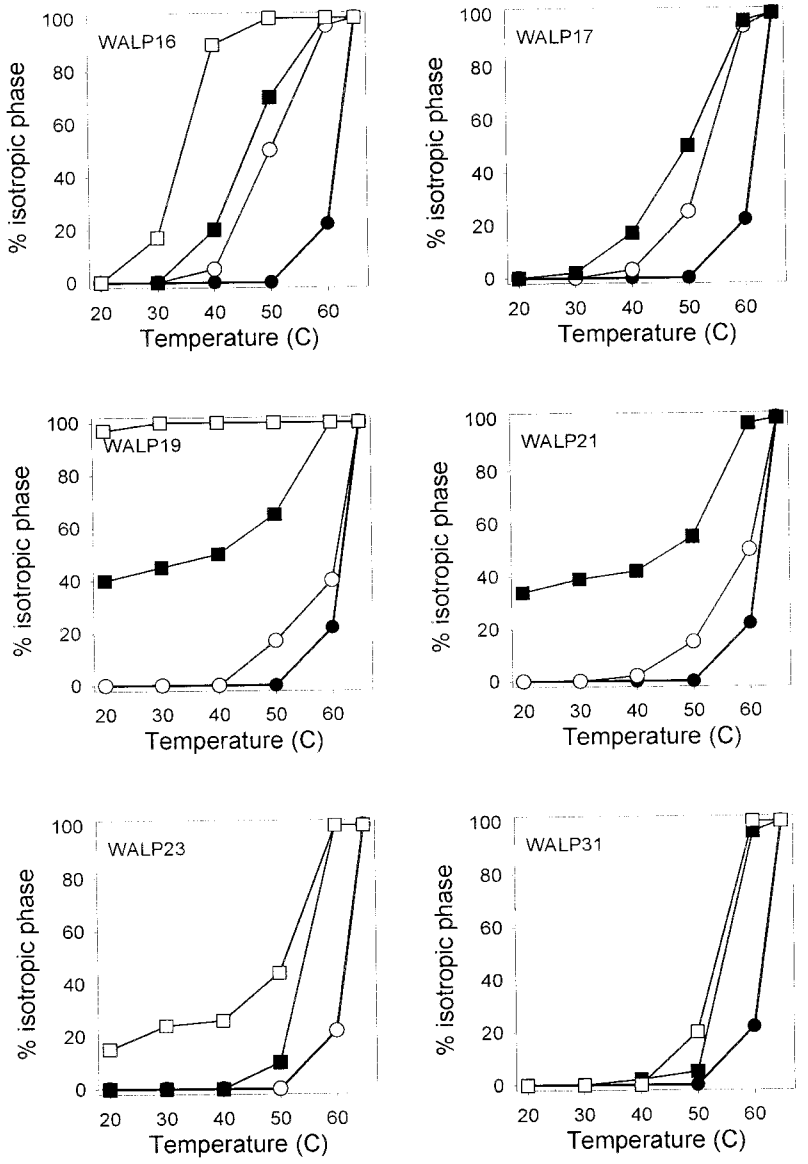

FIGURE 4 The percentage isotropic phase as a function of temperature for WALP-peptide/lipid samples with different P/L ratios. The actual peptide is indicated in each graph. The P/L ratios are 1:1000 (○), 1:500 (■), and 1:200 (□). For comparison the phase behavior of the pure lipid is also indicated (●).

P/L molar ratios up to 1:200. Because in diacylphosphatidylcholines relatively high amounts of peptide were needed to induce nonlamellar phases, it may be possible that a mismatch-induced H_{II} phase can only be expressed at high P/L ratios. It may also be that a bilayer stabilization by long peptides can only be expressed at high peptide concentrations. To investigate these possibilities, the phase behavior of samples with P/L ratios equal to 1:25 were also examined.

Fig. 5 *A* shows a ^{31}P NMR spectrum of a WALP16/DOPE-DOPG sample recorded at 20°C. The lineshape in the spectrum shows the coexistence of an L_α and an H_{II} phase. Thus, at this high P/L ratio, the relatively short peptide WALP16 is indeed able to induce an H_{II} phase in DOPE/DOPG. The major feature in the spectrum of the WALP19-containing sample (Fig. 5 *B*) is a narrow isotropic peak, but there is also a small component that could be due to the presence of a small fraction of H_{II} phase in this sample. The spectrum of the sample containing WALP23 is dominated by a single narrow peak (Fig. 5 *C*). Thus, at a high P/L ratio the type of phase that is formed depends on the extent of mismatch. For the longest peptide used in this study, WALP31, the spectra recorded at 20°C show the coexistence of an L_α phase and a broad symmetrical peak at 0 ppm (Fig. 5 *D*), indicating also that this relatively long peptide destabilizes the bilayer.

Fig. 5, *E–H*, contains ^{31}P NMR spectra of samples recorded at 65°C, which is above T_{LN} for the pure lipids. In the WALP16-containing sample there are coexisting H_{II} and isotropic phases. The fraction of H_{II} phase present is roughly the same at 20 and 65°C (65–70%), suggesting that the peptide preferentially partitions into the H_{II} phase. Furthermore, at 65°C, the spectrum of the sample containing WALP19 shows a large isotropic peak and a small fraction of H_{II} phase, while the samples with the longer peptides all are fully isotropic. Hence none of the peptides are able to inhibit the formation of nonlamellar structures of these lipids at higher temperature, not even the longest peptide used, WALP31.

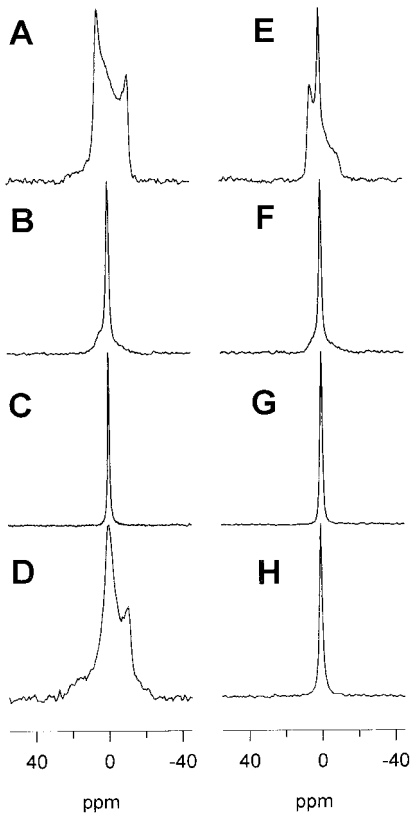

FIGURE 5 ^{31}P NMR spectra of WALP-peptide containing samples with a P/L molar ratio of 1:25. The spectra were recorded at 20°C (*A–D*) and 65°C (*E–H*), and the samples contain WALP16 (*A* and *E*), WALP19 (*B* and *F*), WALP23 (*C* and *G*), or WALP31 (*D* and *H*).

Sucrose density gradient centrifugations

To gain more information about the distribution of peptide and lipid between the lamellar and nonlamellar phases, sucrose density gradient centrifugation experiments were performed. All peptide-containing samples had a P/L ratio of 1:25. A schematic presentation of the results is shown in Fig. 6, *A–D*. The pure DOPE/DOPG mixture (Fig. 6 *A*) gave a single band close to the top of gradient. A sample containing WALP16 separated into two bands: one upper low-density band, with a P/L ratio of ~1:400, that gave an NMR spectrum indicative of a pure bilayer, and a lower high-density band, with a P/L ratio close to 1:7, that gave an ^{31}P NMR spectrum indicative of an H_{II} phase. This suggests that the DOPE/DOPG bilayer can accommodate only a small amount of this relatively short peptide and that indeed

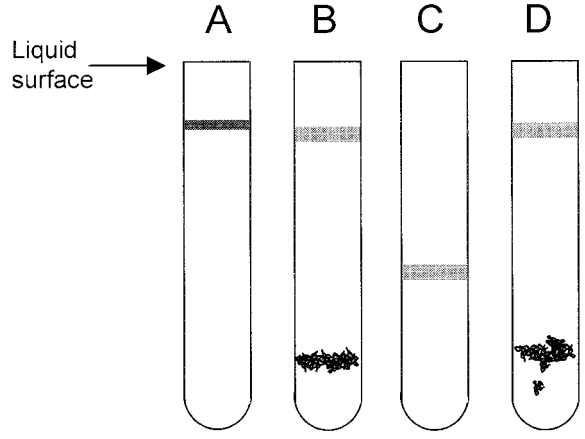

FIGURE 6 Schematic drawing showing the positions of the separated bands in sucrose density gradients after centrifugation (see Materials and Methods for details). The sucrose density gradients where loaded with pure DOPE/DOPG (*A*), WALP16: DOPE/DOPG (*B*), WALP23: DOPE/DOPG (*C*), and WALP31: DOPE/DOPG (*D*). The peptide-to-lipid molar ratio was 1:25 in all peptide-containing samples, and the sucrose density gradients were centrifuged at 20°C.

a high peptide concentration is required to form the H_{II} phase, as found in PC systems (Killian et al., 1996). In contrast, a sample containing WALP23 in DOPE/DOPG contained only one band, with a P/L ratio close to 1:25, indicating that this intermediate-length peptide is well incorporated into the DOPE-DOPG mixture. A WALP31-containing NMR sample (P/L = 1:25) again separated into two bands in the sucrose density centrifugation experiment (Fig. 6 *D*): one upper low-density band with a P/L ratio of ~1:400 that gave an NMR spectrum indicative of a pure bilayer, and a lower high-density band with a P/L ratio close to 1:7 that gave an ^{31}P NMR spectrum with a broad peak around 0 ppm, similar to the broad isotropic component in the spectrum in Fig. 5 *D*. These results suggest that this long peptide does not incorporate into the bilayer and forms less defined peptide-lipid aggregates, together with only a part of the lipid in this sample.

DISCUSSION

In this study we investigated the effect of peptides with different hydrophobic lengths on the phase behavior of a mixture of DOPE/DOPG (7:3 molar ratio). The results showed that 1) the hydrophobic WALP peptides can very efficiently promote the formation of nonlamellar phases in this *E. coli* membrane-mimicking lipid system when there is a mismatch between the hydrophobic length of the transmembrane peptides and the hydrophobic thickness of the surrounding lipid bilayer; 2) the system is extremely sensitive and that even very low amounts of peptide can have a large effect on the lamellar-to-nonlamellar phase transition temperature; 3) at high P/L ratios, the nonlamellar phase formed depends on the extent of mismatch; and 4) no bilayer stabilization occurs in the presence of peptides that are longer than the bilayer thickness.

Below we will discuss the results of our study in detail, starting with the pure lipid system, and we will discuss the implications of our findings for more biological systems.

Characterization of the DOPE/DOPG lipid system

At excess water concentration this lipid mixture undergoes an L_α-to-isotropic phase transition at elevated temperatures, which is similar to the behavior of *E. coli* membrane lipid extracts. The structure of this isotropic phase could not be fully resolved. There are, however, several indications that it could be a cubic phase or a cubic-like phase (a so-called L_3 phase or melted cubic phase) (Lindblom and Rilfors, 1989). First, this isotropic phase shows some characteristics often found for bicontinuous cubic phases, i.e., hysteretic phase behavior and optical transparency (Lindblom and Rilfors, 1989). Second, DOPE forms an H_{II} phase, and DOPG forms bilayers at all temperatures used in this study (Lindblom et al., 1991; Gruner et al., 1988). Therefore, a mixture of these two lipids could be expected to form a phase with intermediate curvature, such as a reversed bicontinuous cubic phase. Third, x-ray diffraction results obtained from a DOPE/DOPG sample with lower water content (60 wt% water) are consistent with the presence of a cubic phase of the space group *Pn3m* with a unit cell of 154 Å. The dimensions of the cubic phase are close to the value of 148 Å found for the bicontinuous cubic phase formed by *E. coli* lipids with a similar lipid composition (Morein et al., 1996).

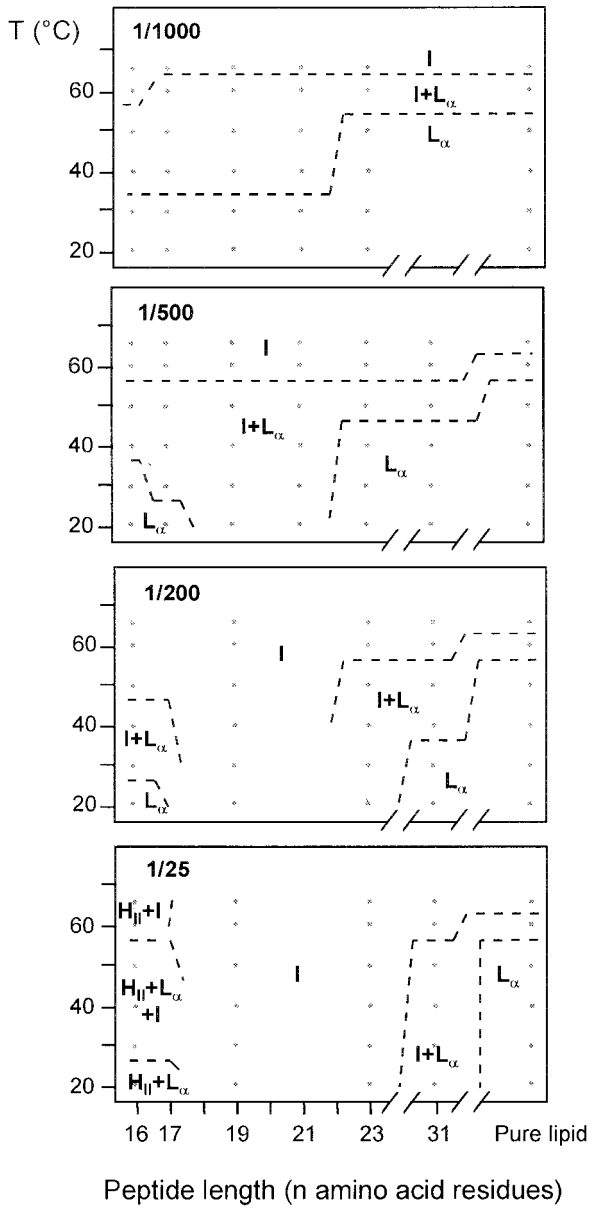

FIGURE 7 Schematic overview of the phases formed by the pure lipid and peptide-lipid samples, as a function of peptide concentration, temperature, and mismatch conditions. Each point represents a measurement on at least one sample.

Effects of peptide/lipid hydrophobic mismatch on the phase behavior of the DOPE/DOPG mixture

Before discussing these effects we will first consider some general aspects of the lamellar-to-reversed nonlamellar phase transition.

The phase behavior of a phospholipid mixture may be qualitatively understood by a consideration of the molecular shape and packing of lipid molecules or the monolayer spontaneous radius of curvature of a lipid mixture (Gruner, 1985; Lindblom and Rilfors, 1989, and references therein). Amphiphilic lipid molecules for which the cross-sectional area of the headgroup and the acyl chains are almost equal to each other will organize into a lamellar phase. If the lipid headgroup cross-sectional area is smaller than that of the hydrophobic part, the lipids will tend to curve toward the water region (negative curvature), favoring the formation of reversed nonlamellar phases, such as H_{II} phases or reversed bicontinuous cubic phases. A transition from a lamellar to a reversed nonlamellar liquid crystalline phase can be achieved in two qualitatively different ways. One is by changing the monolayer spontaneous curvature of the system, e.g., by increasing the hydrophobic cross-sectional area relative to the headgroup area. Another way is by relieving the hydrocarbon packing constraints that hinder the lipids from organizing into nonlamellar phases (see e.g., Sjölund et al., 1987).

In principle, incorporation of transmembrane peptides may influence the lamellar-to-reversed nonlamellar phase transition by either of these two ways. Short transmembrane peptides could increase the spontaneous negative curvature, because they will only fill the hydrophobic part of the lipid bilayer. Relatively long peptides, on the other hand, might even inhibit formation of nonlamellar structures, because they will also penetrate into the headgroup region of the bilayer. Alternatively, short transmembrane peptides could relieve lipid-packing constraints and in this way promote the formation of, for instance, H_{II} phases, as previously proposed for gramicidin and WALP peptides in diacylphosphatidylcholines (Killian, 1998; Killian et al., 1996). Hydrocarbon packing constraints in the H_{II} phase arise from the requirement for a nonuniform effective length of the acyl chains around the tubes of the H_{II} phase. It was postulated that relatively short transmembrane peptides, at high P/L ratios, could induce an H_{II} phase by spanning the hydrophobic part of this phase where the intertube distance is the shortest, thus partly relieving these packing constraints. In addition, because these short peptides only fill the hydrophobic volume, the available area per headgroup will increase, thereby relieving steric constraints between the headgroups and allowing even bulky headgroups, as in diacylphosphatidylcholines, to be accommodated at the highly curved lipid/water interface of the H_{II} phase.

A third alternative for peptide-induced formation of nonlamellar phases may be related to the transition mechanism itself. Siegel and Epand (1997) suggested that transition intermediates, in an L_α-to-H_{II} or L_α-to-reversed cubic phase transition, can already form far below T_{LN}, but that they are unstable. The presence of transmembrane peptides in the lipid bilayer could lower the bilayer rupture tension (Evans and Needham, 1987) and thereby promote the formation of such transition intermediates, even at very low peptide mole fractions. This would facilitate the formation of nonlamellar phases. This effect is also likely to be mismatch dependent.

From the above, it is clear that the relative hydrophobic lengths of the peptides may play an important role in determining their effects on lipid phase behavior. Therefore, one needs to make some estimates of the bilayer thickness in the L_α state relative to the hydrophobic length of the peptides used in this study. The hydrophobic thickness of the lipid bilayer is estimated to be ~27 Å, as found for dioleoylphosphatidylethanolamine and dioleoylphosphatidylcholine (Gruner et al., 1988; Rand and Fuller, 1994). Although the precise hydrophobic length of the peptides is not known, it is a good assumption that the peptides used in this study cover a range of lengths from shorter to longer than the hydrophobic thickness of the lipid bilayer. From Table 1 it can be seen that the shortest peptide used, WALP16, when present in an α-helix, has a total length that is less than the hydrophobic thickness of the lipid bilayer. For the longest peptide, WALP31, a minimum hydrophobic length can be defined as the length of the stretch of leucine and alanine residues between the tryptophans. This corresponds to 37.5 Å in an assumed α-helix, which is considerably longer than the hydrophobic thickness of the lipid bilayer.

With this background information in mind, we will now examine the effects of the WALP peptides on the lipid phase behavior of DOPE/DOPG mixtures in more detail. In Fig. 7 an overview of the dominant features of the lipid phase behavior is shown for samples containing different length analogs and P/L ratios of WALP peptides. The effect of the peptides on the phase behavior of the lipids can be divided schematically into three "concentration regions," low (P/L $\approx$ 1:1000), intermediate (P/L $\approx$ 1:500 to 1:200), and high (P/L $\approx$ 1:25) fractions of peptide. We will now discuss each of these concentration regions.

It should be realized that from the ^{31}P-NMR spectra it is not possible to define the exact nature of the structures giving rise to the narrow symmetrical NMR peak. Therefore, we will simply refer to these structures as "the isotropic phase."

Low concentration region (P/L $\approx$ 1:1000)

Already at these low concentrations, the peptides have a significant effect on lipid phase behavior, in that they promote the formation of the isotropic phase. This effect is clearly mismatch correlated (Fig. 3), the shorter peptides being more effective in lowering T_{LN} than the longer ones.

At this low P/L ratio it is unlikely that there is sufficient peptide present to affect the spontaneous curvature or to help relieve the steric packing constraints of the lipids and thereby promote the formation of nonlamellar phases. A more probable explanation for the peptide-induced phase transitions in this concentration region is that the peptides lower the bilayer rupture tension (Evans and Needham, 1987) and thereby induce or stabilize transition intermediates, as suggested by Siegel and Epand (1997) and discussed above. These transition intermediates are highly curved structures similar to those found in bicontinuous cubic phases. Shorter peptides could be expected to have a progressively larger perturbing influence on the surrounding lipids than longer peptides (Morein et al., 1997; May and Ben-Shaul, 1999), and therefore they may have a stronger effect on the formation and/or stabilization of the transition intermediates. This would explain the mismatch dependence of the induction of isotropic phases in this low concentration region.

Intermediate concentration region (P/L ≈ 1:500 to 1:200)

For all peptides, the effects in this higher concentration are more pronounced. A striking difference, in comparison with the low P/L ratios, is that there is no clear mismatch dependence, but that the intermediate-length peptides are now most efficient in lowering T_{LN}. In this concentration region one could expect that there may be sufficient peptide present to affect the spontaneous curvature or to partly relieve the hydrocarbon packing constraint. If the major effect of the peptides on the phase behavior of the lipids is to change the spontaneous curvature, one would expect the shorter peptides to be the most efficient ones in affecting the lipid phase behavior and longer peptides to inhibit the formation of nonlamellar phases, as discussed above. Neither of these effects is observed. This would suggest that the peptides promote formation of the isotropic phase, mainly by relieving lipid-packing constraints. In the isotropic phase the average bilayer thickness is expected to be slightly smaller than in the L_α phase. Therefore, a possible explanation is that the intermediate-length peptides are still relatively short as compared to the bilayer thickness of the L_α phase and that they fit best into the isotropic phase. For this reason they could be more efficient in inducing this phase than either the shortest peptide, which would be too short to fit well into the isotropic phase, or the longer peptides, which could be accommodated better into the L_α phase.

However, the results do not exclude the alternative or additional possibility that effects on spontaneous curvature play a role. The shortest peptide, WALP16, would be much too short to fit into the L_α phase. As a consequence, the peptide may aggregate or be incorporated to a lesser extent into the membrane (see e.g., Killian, 1998). This would lead to a reduced effective peptide concentration, which would also explain the reduced efficiency of WALP16 in affecting lipid phase behavior. Similarly, the longest peptide, WALP31, may be too long to fit into the L_α phase, again resulting in a reduced effective concentration in the membrane and thereby in a loss of the ability to significantly inhibit the formation of nonlamellar phases.

Studies of the high concentration regions provide more insight into the interaction of the different peptides with the lipids.

High concentration region (P/L ≈ 1:25)

At these high P/L ratios it was possible to analyze the extent of membrane incorporation of the peptides by sucrose density gradient centrifugation. These experiments (Fig. 6) indicated that intermediate-length peptides quantitatively associate with the lipids, while indeed both the longest peptide, WALP31, and the shortest peptide, WALP16, segregate out of the bilayer, together with a small amount of lipid. In the case of WALP16 this peptide-rich phase could be characterized as an H_{II} phase. This suggests that WALP16 is sufficiently short to match the length of the hydrophobic part between the tubes of the H_{II} phase and that, at these high concentrations, it can induce the formation of this phase by a relief of lipid-packing constraints, similar to the situation proposed for gramicidin and WALP16 in DOPC (Killian et al., 1996; Morein et al., 1997). In the case of WALP31 the peptide-rich phase gave rise to a broad isotropic ^{31}P NMR signal, suggesting that it does not represent a well-defined lipid organization. Most likely this phase consists of amorphous aggregates. Thus, at least for the peptide/lipid system studied here, the preferred reaction to a large positive hydrophobic mismatch appears to be segregation out of the bilayer. Very similar mismatch-dependent incorporation behavior of the WALP peptides was recently observed in bilayers of diacylphosphatidylcholine (de Planque et al., manuscript in preparation).

Unfortunately, it is very difficult or even impossible to perform this same analysis at a low P/L ratio. This is mainly due to the fact that the differences in density between peptide-free and peptide-containing lipid dispersions will be too small to get well-separated bands in a sucrose density gradient, and to the fact that the absolute amount of peptide will be too low for accurate quantification by UV-absorbance measurements. Therefore, the extent to which the peptides are associated with lipids under mismatch conditions at lower P/L ratios is not known. However, extrapolation of the results of the centrifugation experiments at high P/L ratios indicates that intermediate-length peptides at lower concentrations should also be quantitatively incorporated into the lipid system investigated here.

To summarize, we suggest that the extent of hydrophobic matching between peptide and lipid plays a crucial role in determining effects on lipid phase behavior. At very low peptide concentrations the peptides act by inducing the formation of transition intermediates in a mismatch-depen-

dent way. At intermediate and high peptide concentrations additional factors are involved. It is proposed that the peptides in this concentration range can stabilize or induce the formation of nonlamellar phases by relieving lipid-packing constraints in these phases. Alternatively, or additionally, it is possible that they modulate lipid phase behavior by affecting the spontaneous curvature of the system. Deviating behavior of the shortest and longest peptides in this case may be explained by a lowering of the effective peptide concentration in the bilayer when the mismatch becomes too large. This is not unlikely, because at high P/L ratios it could be shown that these peptides segregate out of the bilayer.

Biological relevance

In this study it has been shown that hydrophobic mismatch can have a large effect on the phase behavior of the lipids in a system that mimics the lipid composition in living organisms. It was shown that, even with very low peptide concentrations and under slight mismatch conditions, it is possible to promote the formation of isotropic phases in lipid dispersions that by themselves are able to form such structures. We propose that hydrophobic mismatch could be an important factor in determining the morphology of highly curved membrane structures and the so-called cubic membranes that have been proposed to exist in eukaryotic cells (Lindblom and Rilfors, 1989, and references therein; Landh, 1995). In this respect the secretory pathway in eukaryotic cells is of particular interest (Bretscher and Munro, 1993; Pelham and Munro, 1993). This pathway has to cope with a large variety of integral membrane proteins with different hydrophobic lengths that are all initially inserted into the thin endoplasmic membrane. Subsequently, the proteins are sorted such that the proteins with the longest membrane spanners finally end up in the plasma membrane that is the thickest membrane in the pathway. The endoplasmic reticulum and Golgi membranes have highly curved regions and contain large fractions of lipids prone to forming nonlamellar phases. Our observation that long WALP peptides segregate into structures separate from the original bilayer support a sorting mechanism based on mismatch.

Hydrophobic mismatch may also play an important role in other membrane processes, in addition to protein sorting, that include protein-lipid interactions. One example is protein translocation in *E. coli*, where the presence of lipids prone to forming nonlamellar phases is essential (Rietveld et al., 1995). It has been proposed that mismatch may play an important role in local protein (or peptide)-induced reorganization of the lipids in this process, as well as in lateral interactions of transmembrane segments of integral proteins of the translocation complex and processing system (de Kruijff et al., 1997). Other examples are the insertion and folding of integral membrane proteins (Lemmon and Engelman, 1994; Popot et al., 1994; Hunt et al., 1997) and membrane fusion (Epand, 1998).

Another intriguing finding of our studies is that none of the hydrophobic peptides studied stabilize the lamellar phase. Yet it is the paradigm that integral membrane proteins stabilize the bilayer. This could imply that bilayer stabilization is brought about by multispanning membrane proteins.

Finally, we would like to note that our finding that a slight mismatch has a large effect on stabilizing an isotropic, probably cubic or L_3 lipid phase could also have important implications for the crystallization of integral membrane proteins for high-resolution structural studies (Landau and Rosenbusch, 1996).

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