Lipid Dependence of Membrane Anchoring Properties and Snorkeling Behavior of Aromatic and Charged Residues in Transmembrane Peptides †

Articles

Lipid Dependence of Membrane Anchoring Properties and Snorkeling Behavior of Aromatic and Charged Residues in Transmembrane Peptides[†]

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ABSTRACT: ^{31}P NMR spectroscopy was used to investigate the effects of transmembrane α-helical peptides with different flanking residues on the phase behavior of phosphatidylethanolamine and phosphatidyl-ethanolamine/phosphatidylglycerol (molar ratio 7:3) model membranes. It was found that tryptophan-flanked (WALP) peptides and lysine-flanked (KALP) peptides both promote formation of nonlamellar phases in these lipid systems in a mismatch-dependent manner. Based on this mismatch dependence, it was concluded that the effective hydrophobic length of KALP peptides is considerably shorter than that of the corresponding WALP peptides. Peptides with other positively charged residues showed very similar effects as KALP. The results suggest that the peptides have a well-defined effective hydrophobic length, which is different for charged and aromatic flanking residues, but which is independent of the precise chemical nature of the side chain. Strikingly, the effective length of KALP peptides in the lipid systems investigated here is much smaller than that previously found for the same peptides in phosphatidylcholine. This suggests that snorkeling of lysine side chains, as proposed to occur in phosphatidylcholine, does not occur in lipid systems that are prone to form nonlamellar phases by themselves. This suggestion was supported by using peptides with shortened lysine side chains and by investigating the effects of mixtures of WALP and KALP peptides. The lipid dependency of the snorkeling behavior is explained by considering the free energy cost of snorkeling in relation to the free energy cost of the formation of nonlamellar phases.

Structure and function of biological membranes are to a large extent governed by interactions between proteins embedded in the lipid bilayer and surrounding lipids. For a transmembrane protein to be inserted in a stable way into the membrane, it needs to have a hydrophobic surface exposed to the hydrophobic interior of the membrane. In the energetically most stable situation, there will be a matching between the hydrophobic length of the membrane-spanning part of the protein and the hydrophobic thickness of the lipid bilayer. The extent of matching, or the degree of hydrophobic mismatch, is believed to be an important factor in determining the organization of proteins as well as lipids in membranes (*1−3*).

In addition to the hydrophobic interior, a large part of a lipid bilayer consists of the chemically heterogeneous and more polar headgroup, or interfacial, region (*4*). Therefore, also the amino acids in the region flanking the hydrophobic transmembrane part of a protein or peptide will be important for the way a protein or peptide interacts with its host membrane.

Much understanding of the roles of hydrophobic mismatch and flanking residues on peptide and lipid organization has been obtained in model membranes using designed model peptides (*5−14*). Examples of such artificial peptides are WALP and KALP peptides (Table 1). These peptides have a hydrophobic core of varying length consisting of alternating leucines and alanines, that is flanked on both ends by either two tryptophans (WALP peptides) or two lysines (KALP peptides). Both these flanking residues are frequently found near the polar/apolar membrane interface in membrane proteins (*15−17*).

The effects of (mis)matching WALP and KALP peptides have been well characterized in PC[1] model membranes (*6−8, 18, 19*). Under physiological conditions, these lipids

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Table 1: Amino Acid Sequence of the Peptides Used

peptide	sequence
WALP16	acetyl-GWW(LA)$_5$WWA-ethanolamine
WALP19	acetyl-GWW(LA)$_6$LWWA-ethanolamine
WALP23	acetyl-GWW(LA)$_8$LWWA-ethanolamine
KALP23	acetyl-GKK(LA)$_8$LKKA-amide
KALP31	acetyl-GKK(LA)$_{12}$LKKA-amide
RALP23	acetyl-GRR(LA)$_8$LRRA-amide
HALP23	acetyl-GHH(LA)$_8$LHHA-amide
DALP23	acetyl-GDD(LA)$_8$LDDA-amide
K′ALP23[a]	acetyl-GK′K′(LA)$_8$LK′K′A-amide

[a] K′ represents a residue with a shortened lysine side chain, derived from L-2,3-diaminopropionic acid, with only one methylene group in the side chain. See Figure 3 for the chemical structure of the side chains for all flanking residues.

normally only form bilayers, organizing into a lamellar liquid-crystalline (L$_\alpha$) phase. Remarkably, it was found that incorporation of relatively short WALP and KALP peptides could completely alter the lipid organization, provided that they were present at sufficiently high concentration. Moreover, the effects on phase behavior were found to be dependent on the extent of hydrophobic mismatch. When the hydrophobic length of the peptide was slightly smaller than the hydrophobic thickness of the lipids, so-called negative mismatch, both types of peptides induced an isotropic, probably cubic phase, but when the peptides were even shorter, they induced a reversed hexagonal (H$_{II}$) phase (6−8, 20). One striking difference was observed between WALP and KALP peptides in PC: KALP peptides behaved as if they had a slightly shorter hydrophobic length than WALP peptides with the same total number of amino acid residues (6). This was ascribed to a difference in the preferred positioning of the flanking residues at the membrane/water interface, with the tryptophan side chains in the WALP peptides mostly being localized near the carbonyl region of the lipids, and the lysine side chains in the KALP peptides "snorkeling", i.e., stretching toward the aqueous phase so that the positively charged amino groups become localized in a more polar environment. From these studies, it is clear that the interaction of flanking residues with the lipid/water interface can be an important factor in determining the effective hydrophobic length of transmembrane peptides.

To date, effects of WALP peptides, but not of KALP peptides, have been investigated in two other lipid systems besides PC. These are 18:1$_t$-PE, which undergoes an L$_\alpha$ to reversed hexagonal (H$_{II}$) phase transition at elevated temperatures (21), and a 18:1$_c$-PE/18:1$_c$-PG (7:3 molar ratio) mixture, which undergoes an L$_\alpha$ to cubic phase transition and mimics the lipid composition in *Escherichia coli* cell membranes (22). In both lipid systems, it was shown that WALP peptides, at sufficiently high concentration, can affect phase behavior in a qualitatively similar manner as described above for PC systems (21−23), suggesting that these effects

are general. However, WALP peptides may behave similarly in different lipid systems, simply because the tryptophan side chain may "anchor" near the carbonyl region and not interact with the headgroup. The same does not necessarily hold for transmembrane peptides with other, more polar or charged flanking residues, because (i) these residues are likely to interact with the headgroup region itself, (ii) the interaction of charged side chains with the interface may be influenced by electrostatic interactions in the presence of anionic lipids, and (iii) the flexibility of long side chains such as lysine and their ability to "snorkel" may be sensitive to the nature of the lipid headgroup.

In this study, we use mismatch-dependent effects on the lipid phase behavior as a tool to obtain information on the behavior of different flanking residues at the lipid/water interface and on how this behavior depends on the lipid system. For this purpose, we investigated the effects of peptides with a leucine/alanine helical core and a range of different flanking residues on phase behavior in PE, and PE/PG, model membranes using ^{31}P NMR spectroscopy. The results indicate that the different peptides all promote formation of nonlamellar phases in these lipid systems in a mismatch-dependent manner. The effective hydrophobic length of the peptides was different for charged and aromatic residues, but, within these categories, appeared to be independent of the precise chemical nature of the side chain. Surprisingly, the effective hydrophobic length of the KALP peptides was found to be much smaller in PE and PE/PG systems, than that previously found in PC. These results are discussed with regard to the energy cost of lysine snorkeling in relation to the energy cost of the formation of nonlamellar phases in different lipids.

MATERIALS AND METHODS

Materials

Peptides were synthesized using 9-fluorenylmethoxycarbonyl (Fmoc) tBu solid-phase peptide synthesis and purified as described elsewhere (6, 7, 20). The sequences of the peptides used are shown in Table 1.

1,2-Dielaidoyl-*sn*-glycero-3-phosphoethanolamine (18:1$_t$-PE), 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphoethanolamine (16:1$_c$-PE), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (18:1$_c$-PE), and 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (18:1$_c$-PG) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Trifluoroacetic acid (TFA) and 2,2,2-trifluoroethanol (TFE) were obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical grade. Water was deionized and filtered with a Milli-Q Water purification system from Millipore (Bedford, MA). Different buffers were used for different pH values of the samples. The buffer with pH 5.4 contained 25 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES), buffers with pH 7.4 contained 25 mM Tris or 10 mM PIPES, and the buffer with pH 8.9 contained 25 mM Tris. For pH 7.4 samples, no differences were noted between samples prepared with buffer containing Tris or PIPES. All buffers contained 100 mM NaCl and 0.2 mM ethylenedinitrilotetraacetic acid (EDTA).

Methods

Sample Preparation. The peptide−lipid samples for NMR were prepared by a mixed-film method as follows. Stock

solutions were prepared of the lipids in chloroform, and the amount of lipid was checked by a phosphate assay. The concentration of stock solutions was usually approximately 5 mM. Typically 20 μmol of lipid was used for each sample. The peptide was dissolved in TFA and dried to a film under a stream of nitrogen and subsequently dissolved in 0.5−1 mL of TFE. The sample was dried to a film in a rotavapor and resolubilized in TFE. This procedure was repeated twice, to ensure that as much TFA as possible was removed from the sample. The concentration of WALP peptide in stock solution was determined by the absorbance at 280 nm using an extinction coefficient of 21 300 M^{-1} cm^{-1} (*7*), and the concentration of other peptides was determined on weight basis. Appropriate volumes of fresh peptide solution and TFE, adding up to a volume of 1 mL, 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 1.5−2 mL of buffer and transferred to thick-walled 8 mm glass tubes. In some cases, samples with $16:1_c$-PE or $18:1_t$-PE were prepared using a lyophilizing method described previously (*21*). No difference was seen between samples prepared using the mixed film or the lyophilization method.

The samples were then centrifuged 3 times for 20 min at 15 000 rpm in an SS34-rotor with a Sorvall RC-2B centrifuge between 4 and 20 °C. Between the 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. The samples were freeze−thawed, by subsequent freezing in an ethanol/CO_2(s) bath and thawing in a water bath at approximately 50 °C, at least 10 times before measurements. Samples were sealed under a nitrogen atmosphere and stored at −20 °C.

^{31}P NMR Spectroscopy. ^{31}P NMR experiments were carried out on a Bruker MSL 300 NMR spectrometer at 121.4 MHz. The sample temperature was regulated using a Bruker B-VT1000 temperature controller. Experiments were performed using a one-pulse experiment with a 17 μs 90° pulse, a 1.3 s relaxation delay time, a 25 kHz spectral width, 1024 data points, and gated proton-noise decoupling. Between 1500 and 6000 scans were collected. Spectra were processed using the WIN-NMR software from Bruker on a personal computer by DC offset correction, zero-filling to 2048 data points. Some ^{31}P NMR experiments were performed on a wide-bore Bruker Avance 500 NMR spectrometer at 202.5 MHz, using a 13.4 μs 90° pulse, a 2 s relaxation delay time, a 100 kHz spectral width, 2048 data points, and gated proton-noise decoupling. A total of 3000 scans were collected. Spectra were processed on the spectrometer, zero-filled to 16 384 data points. For all NMR spectra, an exponential window function corresponding to a line broadening of 100 Hz was applied prior to Fourier transformation. Before the actual NMR measurements, the $18:1_t$-PE samples were further equilibrated by slowly cycling them 3 times over the temperature range between 20 and 60 °C, over a period of approximately 1 h per cycle. Unless stated otherwise, the temperature for measurements was 30 °C for $18:1_c$-PE/ $18:1_c$-PG or $16:1_c$-PE samples and 45 °C for $18:1_t$-PE samples to avoid any gel phase present in the samples. Samples were allowed to equilibrate at the measurement temperature for at least 30 min before measurements.

Sucrose Gradient Density Centrifugation. Sucrose gradients were prepared from a stock solution of 17.5% (w/v) sucrose in Milli-Q water. Gradients were prepared with sucrose concentrations from 5.5 to 17.5% in steps of 1.5%, and from 10.5 to 14.5% in steps of 0.5%. Centrifugation was performed using an SW41 Ti rotor on a Beckman L5− 65 ultracentrifuge at 150000*g* for 22 h.

Determination of Peptide−Lipid Association. After sucrose gradient density centrifugation, the bands were isolated. To minimize the amount of sucrose in the samples, each band was dispersed in about 25 mL of Milli-Q-water and centrifuged in a 60 Ti rotor in a Beckman L5-65 ultracentrifuge at 40000*g* for 30 min. The pellet was then dissolved in 3 mL of TFE. After further dilution (3:20) in TFE, a CD spectrum was measured using a Jasco J-810 spectropolarimeter. The spectra were baseline-corrected using the CD spectrum of a sample of a similar amount of lipids in TFE. The peptide concentration was estimated from a calibration sample of KALP23 and lipid with known peptide concentration, dissolved in TFE.

RESULTS

$18:1_t$-PE and $18:1_c$-PE/$18:1_c$-PG lipid systems undergo a transition from a liquid-crystalline L_α phase to an H_{II} phase or an isotropic phase, respectively, at elevated temperatures (*24, 25*). Previously it was shown that incorporation of low concentrations of WALP peptides in either of these lipid systems results in a lowering of the phase transition temperature, while at high peptide concentrations the type of phase that is induced is determined by the extent of mismatch between peptides and lipids (*20, 21*). Therefore, in the present study, a high peptide to lipid molar ratio (P/L) of 1:10 was used for all peptide−lipid systems investigated to ensure that mismatch-induced effects on lipid phase behavior dominate over the effect of lowering the phase transition temperature. All measurements were performed at temperatures at which the lipids by themselves form lamellar (L_α) phases. The phase behavior of the systems was studied using ^{31}P NMR.

WALP and KALP Peptides. The ^{31}P NMR spectra of $18:1_c$-PE/$18:1_c$-PG lipids with and without peptide are shown in Figure 1. Without peptide, the spectrum shows the characteristics of phospholipids in the lamellar liquid-crystalline (L_α) state, with a high-field peak and low-field shoulder (*26, 27*). The intensity of the shoulder is lower than for a randomly oriented lipid dispersion, which indicates a macroscopic alignment of the lipid phase in the magnetic field as previously described (*22, 28*). The ^{31}P NMR spectrum of $18:1_c$-PE/$18:1_c$-PG with WALP16 has a reversed asymmetry, with a high-field shoulder and a low-field peak, typical for lipids in an inverted hexagonal (H_{II}) phase (*26, 27*). The longer peptides WALP19 and WALP23 induce a phase giving rise to an isotropic line shape. This line shape can be induced by a number of different phases where the lipids undergo isotropic motions causing an averaging of the NMR signal. It is likely that the isotropic phases found in this study are cubic phases, as was shown for the isotropic phases formed by pure $18:1_c$-PE/$18:1_c$-PG and induced by WALP peptides in DEPE (*21, 22*). The even longer peptide WALP31, as used in a previous study, was not included in the present study because it does not fully associate with the lipids (*21, 22*). The results indicate that introduction of
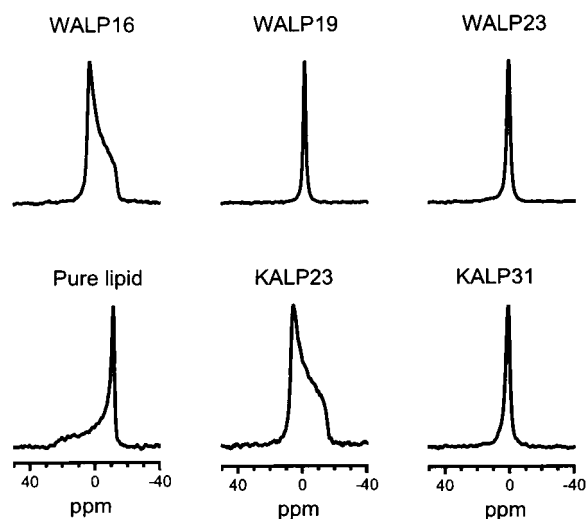
FIGURE 1: ^{31}P NMR spectra from 18:1$_c$-PE/18:1$_c$-PG (7:3 molar ratio) samples without and with incorporated peptide. All samples contain excess buffer, and the peptide/lipid molar ratio is 1:10. The identity of the added peptide is indicated above the spectra. The spectra are recorded at 25 °C.

FIGURE 2: ^{31}P NMR spectra from 18:1$_t$-PE samples without and with incorporated peptide. All samples contain excess buffer, and the peptide/lipid molar ratio is 1:10. The spectra are recorded at 40 °C.

different length WALP peptides into 18:1$_c$-PE/18:1$_c$-PG results in the formation of nonlamellar phases in a peptide length dependent way, consistent with previous results at lower peptide concentrations (*22*).

KALP peptides are similar to WALP peptides, but the residues flanking the hydrophobic core of the peptide are positively charged lysines instead of aromatic tryptophans (Table 1). Because the shorter KALP16 and KALP19 peptides are water-soluble and have been found not to incorporate into lipid bilayers (*6*), they were not included in the present study. As shown in Figure 1, when KALP23 is incorporated in 18:1$_c$-PE/18:1$_c$-PG, it induces formation of an H$_{II}$ phase, while KALP31 induces an isotropic phase. This indicates that also for KALP peptides there is a mismatch-dependent effect on lipid phase behavior. However, the effective hydrophobic length of KALP peptides appears to be significantly shorter than that of corresponding WALP peptides, since KALP23 has a similar effect as WALP16 and the effect of KALP31 is similar to that of WALP19 or WALP23.

When WALP and KALP peptides are incorporated into 18:1$_t$-PE lipids at P/L = 1:10, a nearly identical effect on lipid phase behavior is seen as in the PE/PG mixture. As shown in Figure 2, pure 18:1$_t$-PE lipids form a lamellar (L$_\alpha$) phase, while an H$_{II}$ phase is induced by WALP16 and an isotropic phase by WALP23. WALP19 induces mainly an isotropic phase although an H$_{II}$ phase component is also observed in the NMR spectrum. These results are consistent with previous results obtained at lower peptide concentrations (*21*). As also shown in Figure 2, KALP23 induces an H$_{II}$ phase and KALP31 an isotropic phase. These results indicate that for both the WALP and the KALP peptides their effective hydrophobic length in 18:1$_t$-PE is similar to that in 18:1$_c$-PE/18:1$_c$-PG. For KALP peptides, this implies that in 18:1$_c$-PE/18:1$_c$-PG the behavior of the peptide−lipid systems is dominated by mismatch effects rather than by electrostatic interactions between the positively charged lysines and the negatively charged PG. Furthermore, it can be concluded that in both lipid systems KALP peptides have
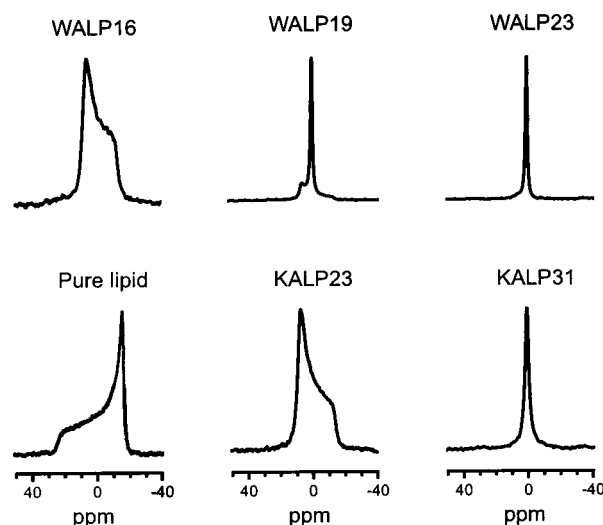
a significantly shorter effective hydrophobic length than WALP peptides with the same total length.

For the KALP peptides, the effective hydrophobic length may be influenced by the ability of the long and flexible lysine side chains to stretch out and snorkel (*6, 29*). To investigate this potential role of snorkeling, it is possible to use peptides in which the lysine side chains are replaced by a shorter analogue, with only one methylene group instead of four in lysine (*12*). This shorter side chain should not be able to snorkel. In Figure 3 , the ^{31}P NMR spectra are shown of KALP23 and the modified peptide K′ALP23 (Table 1) in 18:1$_c$-PE/18:1$_c$-PG (Figure 3A,B) and 18:1$_t$-PE (Figure 3F,G). At the top of the figure, the chemical structures of the normal and shortened lysine side chains are shown. No difference in the phase behavior induced by the two peptides was found in these lipid systems. This suggests either that the lysine side chains do not snorkel, or that the effective length of KALP23, even in the case of snorkeling, is still short enough to induce an H$_{II}$ phase. To further compare the effective hydrophobic length of the two peptides, we therefore used a PE lipid with a slightly shorter acyl chain, 16:1$_c$-PE, which has a phase transition from an L$_\alpha$ phase to an H$_{II}$ phase around 40 °C (*24*). At 30 °C, where the pure lipid is in the L$_\alpha$ phase (not shown), incorporation of both KALP23 and K′ALP23 peptides leads to the formation of an isotropic phase, as shown in Figure 3K,L. As a control, the short WALP16 peptide was mixed with 16:1$_c$-PE, and the ^{31}P NMR spectrum gave a line shape typical of an H$_{II}$ phase (data not shown), demonstrating the capability of 16:1$_c$-PE to form H$_{II}$ phases when short peptides are incorporated. Since both KALP23 and K′ALP23 induce an H$_{II}$ phase in the slightly longer 18:1$_t$-PE, the mismatch-dependent effects on phase behavior are sensitive to a very small change in acyl chain length. Together, the results indicate that the effective hydrophobic lengths of both peptides are similar and therefore that the lysine side chains of KALP23 are not snorkeling in PE or in PE/PG lipid systems.

Other Charged Peptides. To further investigate the importance of the flanking residues, peptides with other charged
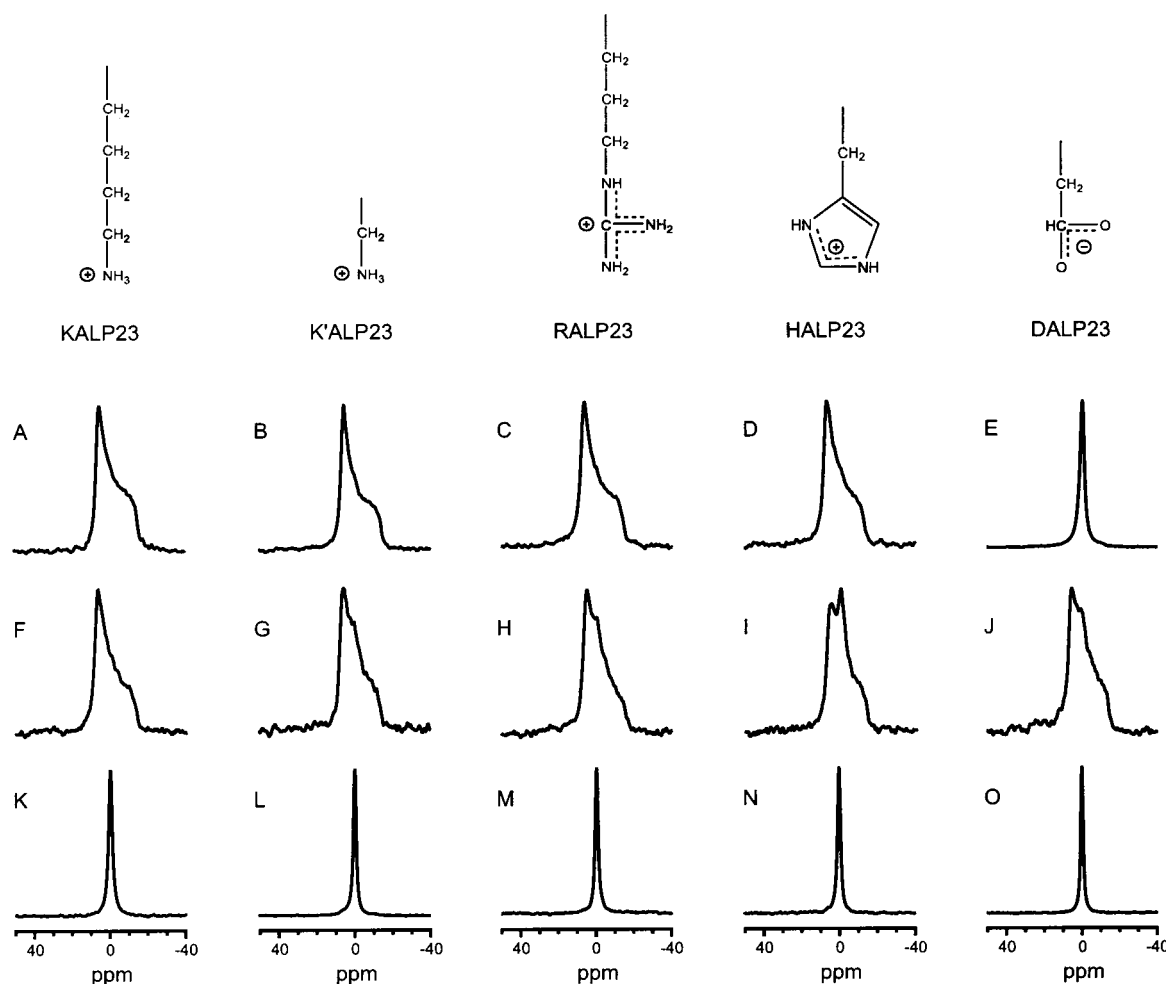
FIGURE 3: ^{31}P NMR spectra of mixtures of lipids and charged peptides. Upper row: $18:1_c$-PE/$18:1_c$-PG mixed with (A) KALP23, (B) K′ALP23, (C) RALP23, (D) HALP23, (E) DALP23. Middle row: $18:1_t$-PE mixed with (F) KALP23, (G) K′ALP23, (H) RALP23, (I) HALP23, (J) DALP23. Lower row: $16:1_c$-PE mixed with (K) KALP23, (L) K′ALP23, (M) RALP23, (N) HALP23, (O) DALP23. The peptide/lipid molar ratio is 1:10 in all samples. The $18:1_c$-PE/$18:1_c$-PG and $16:1_c$-PE samples were measured at 30 °C, while the $18:1_t$-PE samples were measured at 45 °C. The chemical structures of the side chains of the flanking residues are shown above the spectra.

residues were synthesized. The amino acid sequences of the peptides are shown in Table 1. Peptides using as flanking residues arginine (RALP23 peptide), histidine (HALP23), and aspartic acid (DALP23) were introduced into the lipid systems, and the spectra are shown in Figure 3. Strikingly, the spectra in Figure 3F−J and 3K−O demonstrate that in PE lipids, all charged peptides induce the same phase behavior. Therefore, they all appear to have the same effective length, and the chemical structure of the flanking residue side chains seems not to be important for the effective hydrophobic length in PE systems. In $18:1_c$-PE/$18:1_c$-PG, all the positively charged peptides also induce an H_{II} phase (Figure 3A−D). However, in this lipid, the negatively charged DALP induces an isotropic phase (Figure 3E).

Previously it has been observed that DALP23 has a low affinity for *E. coli* lipid vesicles, in contrast to WALP23 or KALP23 (*9*). We therefore investigated the association of DALP23 with the *E. coli* mimicking system $18:1_c$-PE/$18:1_c$-PG using sucrose density gradient centrifugation. This resulted in one diffuse upper band and a pellet (data not shown). The upper band was localized at the position of pure lipids, and was found to contain less than 10% of the total amount of peptide, based on analysis of the spectral intensity from CD measurements. The rest of the peptide was found in the pellet. In contrast, for all positively charged peptides,

only one band was found, at a position expected for lipids and bound peptides (*7*) and containing, within the margin of error, the total amount of peptides used in the samples. The results indicate that specifically the DALP23 peptides do not incorporate well into the lipids but form some kind of aggregates. Thus, the peptide may be present in too low a concentration in the lipid system to induce H_{II} phase formation and instead may induce an isotropic phase by simply lowering the phase transition temperature (*22*). The lack of membrane association of DALP23 is likely to be a result of repulsive electrostatic forces between the negatively charged aspartic acid residues and the negatively charged PG lipids. This is in agreement with the observation that in PE lipid systems DALP23 does behave similar to the positively charged peptides.

The potential importance of charge on the flanking residues was further investigated by monitoring the effect on phase behavior of HALP23. Histidine has a pK_a value of about 6.5, and it is therefore possible to change the charge of the HALP peptide by a change of the pH of the buffer used in the lipid−peptide sample. Samples were prepared with KALP23 and HALP23 in $18:1_c$-PE/$18:1_c$-PG at a pH of 5.4 and 8.9, and the ^{31}P NMR results are shown in Figure 4. Without peptides, the lipids form a lamellar phase at both high and low pH (Figure 4A,B). The intensity of the shoulder
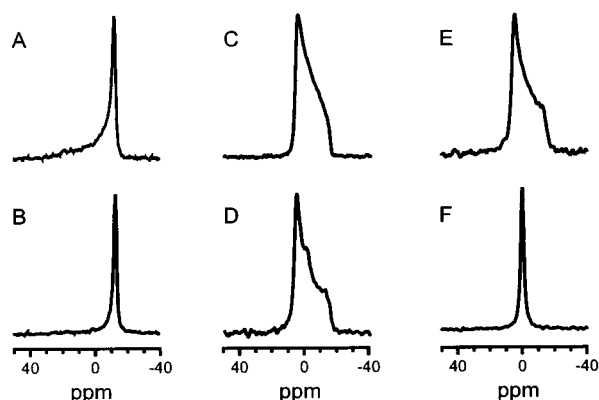
FIGURE 4: ^{31}P NMR spectra of $18:1_c$-PE/$18:1_c$-PG (A) without peptides at pH 5.4, (B) without peptides at pH 8.9, (C) with KALP23 at pH 5.4, (D) with KALP23 at pH 8.9, (E) with HALP23 at pH 5.4, and (F) with HALP23 at pH 8.9. The spectra are recorded at 30 °C. In samples with peptide, the peptide/lipid molar ratio is 1:10.

FIGURE 5: ^{31}P NMR spectra at 30 °C of two peptides incorporated in the same system of $18:1_c$-PE/$18:1_c$-PG (molar ratio 7:3) with a total peptide/lipid molar ratio of 1:10. (A) WALP16/WALP23 1:1 (mol/mol), (B) WALP23/KALP23 1:1 (mol/mol), (C) WALP23/K'ALP23 1:1 (mol/mol), (D) WALP23/K'ALP23 1:3 (mol/mol).

is low due to a macroscopic alignment in the magnetic field. KALP23, which is positively charged at both these pH values, induces an H_{II} phase at both high and low pH (Figure 4C,D). In contrast, HALP23 induces an H_{II} phase at pH 5.4, where it is positively charged (Figure 4E), but an isotropic phase at pH 8.9, where it is uncharged (Figure 4F). These results indicate that the presence of a charge on the flanking residues can indeed be important for the phase behavior induced by the peptides in $18:1_c$-PE/$18:1_c$-PG.

Phase Separation and Snorkeling. The results show a striking difference between the effects on phase behavior, and hence the effective hydrophobic length, of peptides with charged flanking residues and the effects of WALP peptides, which have aromatic amino acids as flanking residues. This raises the possibility that a mismatch-dependent macroscopic phase separation can be induced by combining two peptides with different lengths in one type of lipid system. To investigate this possibility, experiments were performed on mixtures of peptides in $18:1_c$-PE/$18:1_c$-PG. When WALP16 and WALP23 were mixed in this lipid system, a superposition of an H_{II} phase and an isotropic phase was seen in the ^{31}P NMR spectrum (Figure 5A). Since WALP16 by itself induces an H_{II} phase in $18:1_c$-PE/$18:1_c$-PG, while WALP23 induces an isotropic phase (Figure 1), this suggests that indeed phase separation occurs with WALP16 being enriched in the H_{II} phase and WALP23 being enriched in the isotropic phase.

In a similar way, we can expect a phase separation when KALP23 and WALP23 are mixed in $18:1_c$-PE/$18:1_c$-PG, since KALP23 on its own induces an H_{II} phase in this lipid system (Figure 1). However, only one isotropic phase is observed in this mixture (Figure 5B). Thus, it appears that KALP23 is incorporated into the isotropic phase, therefore acting as if it is effectively longer than when it is alone in $18:1_c$-PE/$18:1_c$-PG. A likely explanation is that the length of this peptide is not rigid but that it can be increased by snorkeling of lysine side chains to match the hydrophobic thickness of the lipid environment in the isotropic phase (6). If snorkeling were responsible, then it would be more difficult or even impossible for the K'ALP23 peptide to fit in the isotropic phase. Indeed, a mixture of K'ALP23 and WALP23 in $18:1_c$-PE/$18:1_c$-PG again shows two phases (Figure 5C),
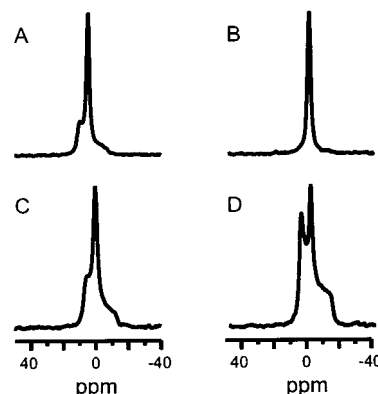
suggesting that the K'ALP peptide cannot adapt its effective hydrophobic length to fit in an isotropic phase, and therefore causes an H_{II} phase component. Thus, the hydrophobic length seems to be more or less fixed for WALP and K'ALP, but flexible in KALP, most likely due to the possibility of snorkeling.

Unfortunately, it turned out to be impossible to physically separate the hexagonal and isotropic phases of the mixed peptide samples by sucrose density gradient centrifugation, and therefore the peptide content of these phases could not be determined. However, the fact that they could not be separated suggests that the peptide concentration is similar in both phases. In an attempt to estimate the peptide distribution between the different phases, samples using K'ALP23/WALP23 molar ratios of 1:1 (Figure 5C) and 3:1 (Figure 5D) were used. The spectra in Figure 5C and 5D are consistent with an approximately 3-fold increase of the H_{II} phase/isotropic phase ratio in the 3:1 mixture as compared to the 1:1 mixture. This supports the interpretation that combining peptides of different effective length can result in a phase separation, where the shorter peptide is located in the H_{II} phase and the longer peptide in the isotropic phase.

DISCUSSION

In this study, we investigated the effect of different flanking residues in transmembrane model peptides on the phase behavior of systems with non-bilayer-forming lipids. First, we will compare our results on the effects of aromatic and charged flanking residues with those previously found in PC systems to obtain a general picture of the lipid dependency of the interactions of flanking residues with the lipid−water interface. Based on these results, we will discuss the role of snorkeling of lysine side chains.

Effects of WALP Peptides. Previously it was shown in PC systems, using both WALP peptides of different lengths and lipids with different acyl chain lengths, that nonlamellar phases can be induced in a mismatch-dependent way at P/L = 1:10 (7). Here we show that at the same P/L ratio WALP peptides induce similar nonlamellar phases in PE systems and in PE/PG systems. In Table 2 the nonlamellar phases induced by WALP peptides in the different lipid systems are summarized.

Table 2: Schematic Overview of the Type of Nonlamellar Phase Induced by Peptide−Lipid Hydrophobic Mismatch in Phospholipid Systems, Found in Different Studies[a]

lipid	WALP-16	WALP-19	WALP-21	WALP-23	KALP-23	KALP-31
$18:1_c$-PC	H_{II}[b]	I[b]	−	−	−	−
$20:1_c$-PC	−	I[c]	I[c]	I[c]	I[c]	−
$22:1_c$-PC	H_{II}[b]	H_{II}[b]	I[c]	I[c]	I[c]	−
$24:1_c$-PC	H_{II}[b]	H_{II}[c]	H_{II}[c]	I[c]	H_{II}[c]	−
$16:1_c$-PE[d]	H_{II}	−	−	−	I	I
$18:1_t$-PE[d]	H_{II}	I/H_{II}	−	I	H_{II}	I
$18:1_c$-PE/PG[d]	H_{II}	I	−	I	H_{II}	I

[a] H_{II} denotes the reversed hexagonal phase, and I denote phases that give rise to isotropic peaks as observed by ^{31}P NMR. Not investigated systems are marked with (−). [b] Data from (7). [c] Data from (6). [d] This study.

In PC systems, a clear mismatch-dependent effect of the peptides on phase behavior is seen: the longer the peptide, the longer the lipid acyl chain length required to form an H_{II} phase. For example, WALP16 induces an H_{II} phase when there are 18 carbons or more in the acyl chains. For WALP19, an acyl chain length of 22 carbons is required for H_{II} phase formation and for WALP21 a chain length of 24 carbons, while in this same lipid WALP23 still induces an isotropic phase.

The results in the present study suggest that these effects are independent of lipid headgroup composition. As shown in Table 2, WALP16 induces an H_{II} phase in $18:1_c$-PC, $18:1_t$-PE, and $18:1_c$-PE/$18:1_c$-PG, while WALP19 induces an isotropic phase in all three lipid systems. However, in $18:1_t$-PE, WALP19 also induces the formation of a small amount of H_{II} phase. A possible explanation for this effect is that the trans double bond increases the length of the acyl chains compared to a cis double bond, thereby increasing the mismatch. Alternatively, it may be related to the fact that PE has a higher propensity to form an H_{II} phase by itself.

The results are consistent with the proposed location of tryptophan residues of WALP peptides approximately at the lipid carbonyl groups (6), and with the proposed model of WALP-induced formation of an H_{II} phase. In this model (7), the peptides span the lipid bilayer between water tubes, but they do not extend to the lipid headgroup region. In this way, the available area per lipid headgroup increases, allowing the lipids to form an inverted hexagonal phase where the curvature is high and the area per lipid headgroup is relatively small.

Effects of KALP Peptides As Compared to WALP Peptides. For KALP peptides, the effect on the phase behavior strongly depends on the lipid headgroup composition, in contrast to the situation for WALP peptides. As shown in Table 2, in PC these charged model peptides also induce nonlamellar phases in a mismatch-dependent way. For example, KALP23 induces an isotropic phase in $20:1_c$-PC and $22:1_c$-PC but an H_{II} phase in $24:1_c$-PC. Thus, KALP23 induces the same nonlamellar phases as WALP21, indicating that in PC systems the effective hydrophobic length of a KALP peptide is slightly shorter than that of a corresponding WALP peptide. Surprisingly, in the present study, it is shown that both in $18:1_c$-PE/$18:1_c$-PG and in $18:1_t$-PE, KALP23 induces the same phases as WALP16. In $16:1_c$-PE, however, KALP23 induces an isotropic phase and thus seems to have a longer effective hydrophobic length than WALP16 that induces an
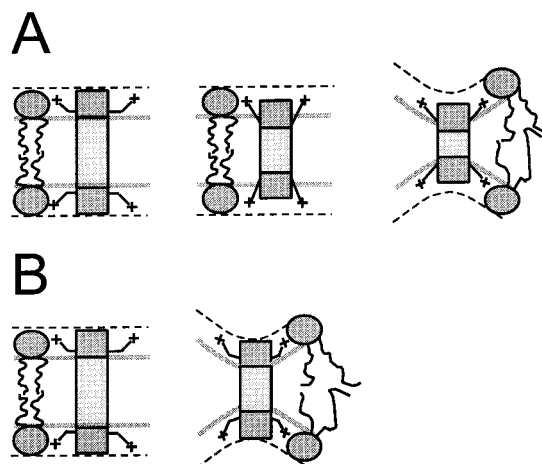

FIGURE 6: Lipid/KALP peptide system can compensate for a mismatch in different ways. When the hydrophobic length of the peptide matches the hydrophobic thickness of the lipids, the peptides can be incorporated into a lamellar phase in both PC and PE systems. In a PC system (A), changing to a nonlamellar phase is difficult. Therefore, when the peptide gets shorter, snorkeling of lysine side chains is favored and will be the first response of the system. Only if the peptide gets even shorter will PC go to a nonlamellar phase. For the PE system (B), it is much easier to organize in a nonlamellar phase. Therefore, there is no snorkeling, and instead a phase transition takes place already at a small mismatch.

H_{II} phase. Based on its mismatch-dependent effects on phase behavior, it can be stated that KALP23 behaves as if it has an effective hydrophobic length between that of WALP16 and WALP19.

To explain the different apparent hydrophobic length of KALP peptides in PC as compared to PE or PE/PG, we propose the snorkeling model illustrated in Figure 6. In Figure 6A, the PC case is depicted as proposed previously (6). When the KALP peptide length matches the hydrophobic thickness of the lipid bilayer, a lamellar phase is formed. When a slightly shorter peptide is used, the lysine side chains will snorkel to increase the effective peptide length. The alternative way of dealing with a mismatch would be the formation of nonlamellar phases. However, in PC, as a typical bilayer preferring lipid, it is not easy to form such phases. Therefore, a nonlamellar phase will be formed only if the peptide becomes too short and snorkeling does not suffice any longer. In contrast, in PE or PE/PG (Figure 6B), the lipids easily form nonlamellar phases. When the peptide length matches the hydrophobic thickness of the lipid bilayer, a lamellar phase is formed, as in PC systems. However, for shorter peptides, even at a small mismatch, instead of snorkeling a nonlamellar phase is formed. Apparently, in these lipid systems, snorkeling is less favorable than formation of nonlamellar phases to relieve the mismatch. This model is supported by experiments using K′ALP23. In this peptide, the flanking residues have a shortened side chain compared to lysine that is not able to snorkel. In PC, K′ALP23 behaves as if it is shorter than KALP23 (20). In contrast, the results of the present study show no difference in effects on phase behavior between KALP23 and K′ALP23 in PE or PE/PG, which both are lipids that are prone to form nonlamellar phases.

There is a free energy cost of snorkeling, since the stretching should decrease the conformational entropy of the

side chain by a considerable amount. We propose that in PC snorkeling takes place because of the high cost of forming an H_{II} phase in this lipid system. Furthermore, it appears that in PE or PE/PG lipid systems with a mixture of WALP and KALP peptides, snorkeling is favorable compared to the entropy loss involved in phase separation. Although in some cases snorkeling thus may be the most favorable way of solving a mismatch situation, it should be stressed that this will take place only when the energetic cost of snorkeling is lower than that of alternative ways of relieving mismatch.

If the side chains are not snorkeling, then the effective hydrophobic length of the different peptides may be determined by the average position of the backbone atoms of the flanking residues at the lipid/water interface. If it is assumed that the C_α of tryptophan in WALP peptides are located close to the carbonyl groups of the lipids, while C_α of lysine residues in KALP are located close to the phosphate group, then one might expect a difference in the effective length between WALP and KALP peptides of approximately 8 Å, because the phosphate groups are approximately 8 Å further apart than carbonyls (*30*). One residue in an α-helix corresponds to 1.5 Å in peptide length, and therefore 8 Å would correspond to five or six amino acids. In this view, it can be expected that KALP23 in the absence of snorkeling has an effective hydrophobic length corresponding to WALP17 or WALP18, in agreement with the experimental observations.

Effects of Peptides with Other Charged Flanking Residues. Our results show that all charged model peptides (KALP23, K'ALP23, RALP23, HALP23, DALP23) behave identically in PE systems, in that they all induce an H_{II} phase in $18:1_t$-PE and an isotropic phase in $16:1_c$-PE. Since the acyl chains of these lipids differ only little in length, this result indicates that the effective hydrophobic length of all the charged peptides must be quite similar. This implies that if lysines do not snorkel, neither do the arginine side chains of RALP23. Furthermore, there appears to be no specific interaction between the zwitterionic PE lipid headgroups and the flanking residues of the different peptides, but rather a general tendency of charged groups to be localized in the polar headgroup region.

In $18:1_c$-PE/$18:1_c$-PG lipids, it can be expected that electrostatic interactions between the negatively charged PG lipids and charged flanking residues in the peptides may influence the effects of the peptides on lipid phase behavior.

Role of Electrostatic Interactions. PE/PG mixtures as used in this study have a high charge density, due to the presence of the negatively charged PG. This could be expected to interfere with H_{II} phase formation, since in the H_{II} phase the headgroups are closer together than in a bilayer, resulting in larger repulsive electrostatic forces. Yet these mixtures easily can organize into an H_{II} phase upon incorporation of short mismatching peptides, which can be either neutral, as in the case of WALP, or positively charged, as in the case of KALP or RALP. This is consistent with previous studies on gramicidin, where it was found that the peptide could induce H_{II} phase formation in pure charged lipid systems such as PG or phosphatidylserine and charged gramicidin analogues were found to induce H_{II} phase formation in PC (*31*). Importantly, in the present study, we have shown that incorporation of neutral or positively charged peptides in pure PE has very similar effects as in PE/PG, indicating that

mismatch effects are dominant over electrostatic interactions. However, repulsive electrostatic effects do seem to play a role in the association of the negatively charged DALP23 to bilayers of PE/PG. The low extent of association is likely to be responsible for the induction of an isotropic phase by this peptide instead of an H_{II} phase, which requires much higher peptide concentrations.

The effect of charge was also shown by experiments with HALP peptides at different pH values. At low pH, when the histidine side chain is positively charged, HALP23 induces an H_{II} phase, while at high pH, when the side chain is not charged, it induces an isotropic phase. A similar effect of HALP23 has been observed in PC (*20*), which was ascribed to the side chain in its neutral form tolerating an anchoring position closer to the hydrophobic core of the membrane than the positively charged side chain. This would result in an increase in effective length, comparable to the situation for WALP peptides. We speculate that the same explanation holds for the effects of pH variation on the behavior of HALP in PE/PG.

In this study, all positively charged peptides behave similarly. Preliminary experiments with peptides where the flanking residues are phenylalanine (FALP) or tyrosine (YALP) suggest that also all peptides with aromatic flanking residues behave similarly in the lipid systems used in this study (Strandberg and Killian, unpublished data). In contrast, recent experiments in PC systems on the effects of the same peptides show a much more complex behavior (*20*). This is presumably caused by the fact that it is more difficult for PC lipids to form nonlamellar phases. Therefore, in PC systems but not in the lipid systems investigated here, the phase behavior may be very sensitive to details of the interactions of side chains with the lipids.

Implications for Biological Systems. Two general observations can be made from our results. The first is that mismatching peptides can induce and/or promote the formation of nonlamellar phases. This effect has also been observed for biologically relevant peptides, such as synthetic peptides corresponding to signal sequences (*25*) or transmembrane parts of proteins (*8*). The concentrations at which the peptides exert these effects can be very low, depending on the tendency of the lipids to adopt nonlamellar phases by themselves. This could be important for processes such as membrane fusion and spontaneous insertion and translocation of proteins (*32−35*). Our results suggest that such processes could be modulated by hydrophobic mismatch, and that the efficiency will depend on a variety of factors, including extent of mismatch, nature of the lipids, and amino acid composition of the peptide.

A second observation from our results is that mismatching peptides can cause a macroscopic phase separation in order to surround themselves with lipids in a phase where the best matching is obtained. The mechanism by which this phase separation occurs may be similar to that in processes involved in protein sorting in biological systems, which were suggested to be mismatch-dependent (*36−39*).

As a first step in the process of mismatch-based sorting, it can be expected that transmembrane peptides or proteins become preferentially surrounded by lipids with best matching hydrophobic length. In membrane systems containing different lipid lengths, such microdomain formation could

be an alternative to the macroscopic phase separation as found in the present study [see (*3*) and references cited therein].

In cases of negative mismatch, as studied here, the effective hydrophobic length of the peptides is determined both by the length of the hydrophobic stretch of the peptide and by the "anchoring" positions of the flanking residues in the lipid bilayer. For long, charged side chains, the possibility of snorkeling can also affect the hydrophobic length. Our results show that whether snorkeling indeed occurs depends on the molecular details of the system and on whether energetically more attractive alternatives are available. This sensitivity to the system could explain some findings in the literature. For example, snorkeling was proposed to explain the lipid binding of amphipathic helices from apolipoproteins (*29, 40*), but others did not observe snorkeling in NMR-determined structures of these peptides in SDS micelles (*41, 42*). It should be noted that in these studies peptides lie parallel to the lipid surface, and therefore snorkeling in these peptides is perpendicular to the helix axis, which may result in a different lipid dependence.

In any case, it is clear that the possibility of snorkeling does allow some flexibility to the hydrophobic length of transmembrane segments with lysines as flanking residues. This would allow modulation of the effective hydrophobic length of such segments and hence of their behavior in a biological system.

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