Sorting of Lipids and Transmembrane Peptides Between Detergent-Soluble Bilayers and Detergent-Resistant Rafts

Thomas J. McIntosh,* Adriana Vidal,* and Sidney A. Simon[†‡]
*Department of Cell Biology, [†]Department of Neurobiology, and [‡]Center for Neuroengineering, Duke University Medical Center, Durham, North Carolina

ABSTRACT Specific proteins and lipids sequester to regions of cell membranes called rafts. Due to their high content of sphingomyelin (SM) and cholesterol, raft bilayers are thicker than nonraft bilayers and, at least at 4°C, are resistant to Triton X-100 extraction. It has been postulated that rafts concentrate proteins with long transbilayer domains because of "hydrophobic matching" between the transbilayer domain and the thick bilayer hydrocarbon region. However, because the area compressibility and bending moduli of SM:cholesterol bilayers are larger than that of nonraft bilayers, there should be an energy cost to partition proteins or peptides into rafts. To determine the effects on peptide sorting of raft thickness and mechanical properties, we incorporated two transbilayer peptides (P-23, P-29) into bilayers composed of SM, dioleoylphosphatidylcholine, and cholesterol, separated detergent-soluble membranes (DSMs) from detergent-resistant membranes (DRMs), and measured their peptide and lipid compositions. P-23 and P-29 were designed to have transbilayer domains that matched the hydrocarbon thicknesses of DSMs and DRMs, respectively. At both 4°C and 37°C DSMs were enriched in dioleoylphosphatidylcholine and DRMs were enriched in SM and cholesterol. At both temperatures both P-23 and P-29 preferentially localized to DSMs, demonstrating the importance of bilayer mechanical properties relative to hydrophobic mismatch. However, at 37°C significantly more P-29 than P-23 was located in DRMs, implying that hydrophobic matching played a role in peptide sorting at physiological temperature. These experiments demonstrate that the sorting of peptides as measured by detergent extraction is temperature-dependent and both bilayer mechanical properties and hydrophobic matching impact peptide distribution between DSMs and DRMs.

INTRODUCTION

Strong evidence has been obtained for the presence of microdomains or ''rafts'' in both biological membranes (Simons and van Meer, 1988; Simons and Ikonen, 1997; Brown and London, 1998; Brown, 1998; Kabouridis et al., 2000; Simons and Ikonen, 2000; Galbiati et al., 2001; Gkantiragas et al., 2001; Zacharias et al., 2002) and in bilayers with lipid compositions approximating those found in cell plasma membranes (Ahmed et al., 1997; Dietrich et al., 2001; Rinia et al., 2001; Samsonov et al., 2001). Although the lateral size of rafts in membranes is controversial (Edidin, 1998; Kenworthy and Edidin, 1998), in both natural and bilayer membranes rafts have been characterized by their insolubility at low temperatures in detergents such as Triton X-100 (Hanada et al., 1995; Ahmed et al., 1997; London and Brown, 2000; Simons and Toomre, 2000; Heerklotz, 2002). Previous studies have found that detergent-resistant membranes (DRMs) are enriched in specific lipids, including sphingomyelin (SM) and cholesterol (Simons and Ikonen, 1997; Brown, 1998; Brown and London, 2000; Simons and Ikonen, 2000), as well as specific classes of proteins, including dually acylated proteins, GPI-linked proteins, and certain transmembrane receptors (Rodgers et al., 1994; Arreaza and Brown, 1995; Field et al., 1997; Brown and London, 1998; Baird et al., 1999; Melkonian et al., 1999; Brown and London, 2000; Moffett et al., 2000; Prinetti et al., 2000). Due to their ability to sequester and concentrate such lipids and proteins in regions of the plasma membrane, rafts are thought to play important roles in many physiological processes, such as signal transduction (Field et al., 1997; Baird et al., 1999; Kawabuchi et al., 2000; Moffett et al., 2000; Simons and Toomre, 2000), membrane fusion (Chamberlain et al., 2001; Lang et al., 2001), neuronal maturation (Ledesma et al., 1999), lipid sorting (Simons and van Meer, 1988; Brown, 1998; Simons and Ikonen, 2000), and protein trafficking (Bretscher and Munro, 1993; Simons and Ikonen, 1997, 2000; Lafont et al., 1999; Ikonen, 2001).

In terms of lipid and protein sorting, two potentially important features of rafts are their structure and mechanical properties. That is, due to their high content of sphingomyelin and cholesterol (Simons and Ikonen, 1997, 2000; Brown, 1998; Brown and London, 2000), raft bilayers should be thicker and have larger area compressibility and bending moduli than nonraft bilayers.

Relative to the bilayer structure, since a typical SM molecule contains a sphingosine moiety and a long, saturated fatty acid chain, SM:cholesterol bilayers are thought to have thicker bilayers than the surrounding lipid matrix that contains more unsaturated phospholipids (Bretscher and Munro, 1993). In this regard Gandhavadi et al. (2002) used x-ray diffraction analysis to show that detergent-resistant membranes (DRMs) are ~9 Å thicker than detergent-soluble membranes (DSMs) for a bilayer system containing an equimolar mixture of SM, cholesterol, and an unsaturated phospholipid (dioleoylphosphatidylcholine or DOPC). This

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Address reprint requests to Thomas J. McIntosh, 443 Sands Bldg., Duke University Medical Ctr., Durham, NC 27710. Tel.: 919-684-8950; Fax: 919-681-9929; E-mail: t.mcintosh@cellbio.duke.edu.

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commonly studied lipid system (Ahmed et al., 1997; Dietrich et al., 2001; Rinia et al., 2001; Samsonov et al., 2001; Gandhavadi et al., 2002) contains ''liquid-ordered'' DRMs enriched in SM and cholesterol and ''liquid-disordered'' DSMs enriched in DOPC (Gandhavadi et al., 2002). The difference in thickness of raft and nonraft membranes has been proposed to be a factor in trafficking of membrane proteins to the plasma membrane through the Golgi apparatus, where rafts are first formed (Simons and Ikonen, 1997; Gkantiragas et al., 2001; Holthuis et al., 2001). It has been hypothesized that in the Golgi apparatus resident Golgi proteins with relatively short transmembrane domains (TMDs) tend to be localized in thin nonraft membranes, whereas certain proteins with longer TMDs are segregated to thicker microdomains enriched in SM and cholesterol that form transport vesicles destined for the plasma membrane (Munro, 1991; Bretscher and Munro, 1993; Munro, 1995; Rayner and Pelham, 1997; Webb et al., 1998). Although other factors, such as the specific amino acid composition of the TMDs, also appear to be involved in protein sorting (Swift and Machamer, 1991; Nilsson et al., 1991; Banfield et al., 1994; Scheiffele et al., 1997), the importance of TMD length in protein trafficking is indicated by experiments showing that the cell localization of specific membrane proteins depends on their TMD length. That is, shortening the TMDs of proteins normally transported to the cell plasma membrane promotes a significant accumulation of those proteins in the Golgi membranes (Munro, 1995; Cole et al., 1998), whereas increasing the length of the TMD of a specific galactosyltransferase releases that protein from the Golgi for transport to the plasma membrane (Masibay et al., 1993). Consistent with a role for hydrophobic matching of TMD length and bilayer thickness are in vitro experiments showing that the amount of peptide incorporated into lipid bilayers and the orientation of the peptides in bilayers depend on the extent of matching of the hydrophobic length of the TMD and the hydrophobic core of the bilayer (Ren et al., 1997, 1996b; Webb et al., 1998; dePlanque et al., 1999, 2001; Morein et al., 2002; Ridder et al., 2002).

In addition to their thickness, another relevant feature of SM:cholesterol (raft) bilayers is that they have a much larger area compressibility modulus than do unsaturated phosphatidylcholine bilayers (Needham and Nunn, 1990; McIntosh et al., 1992). Since the bilayer bending modulus is proportional to the compressibility modulus times the square of the bilayer thickness (Evans and Rawicz, 1990), the bilayer bending modulus of SM:cholesterol should also be much larger than that of unsaturated phosphatidylcholine bilayers. Therefore, to accommodate proteins or peptides of a given hydrophobic length, it should take more energy to separate or deform adjacent lipid molecules in raft bilayers compared to nonraft bilayers with lower compressibility and bending moduli. This implies that, for a given extent of hydrophobic mismatch, the liquid-ordered SM:cholesterol (raft) bilayer would provide a more energetically unfavorable environment for a protein or peptide than would a liquid-disordered phospholipid bilayer. In this regard, Gandhavadi et al. (2002) showed that a larger percentage of a peptide equivalent to the third transmembrane helix in bacteriorhodopsin (Hunt et al., 1997) partitioned into DOPC bilayers than into SM:cholesterol bilayers.

Lundbaek et al. (2003) have theoretically analyzed the energetics of sorting of transmembrane proteins between cholesterol-containing raft and nonraft membranes. They derived an expression for the energy of deformation to insert a peptide or protein into a bilayer as $\Delta G_{def} = H_B (2u_o)^2$, where u_o is the depth of deformation of each monolayer and H_B is a ''spring constant'' determined by the membrane thickness, the radius of the peptide, and the bilayer's compressibility and bending moduli. They found that whereas changes in bilayer thickness by themselves have only a modest effect on protein sorting, cholesterol-induced increases in bilayer compressibility and bending moduli increase the energetic penalty for incorporating proteins with short transmembrane domains into rafts. Lundbaek et al. (2003) concluded that cholesterol-induced changes in both bilayer structure and mechanical properties permit effective protein sorting between membrane domains.

Recently van Duyl et al. (2002) have addressed this sorting issue experimentally by measuring at 4°C the distribution of transmembrane peptides with different hydrophobic lengths in detergent-treated bilayers composed of equimolar DOPC, SM, and cholesterol. Van Duyl et al. (2002) found that peptides of different transbilayer lengths all preferred the DSMs and concluded that hydrophobic matching of peptide length with lipid thickness is insufficient to cause the partitioning of long peptides into rafts. They also argued that the tight packing of the lipids in the liquid-ordered phase of DRMs constitutes an unfavorable environment for transmembrane domains of proteins. Thus, some of the theoretical predictions of protein sorting between membrane domains (Lundbaek et al., 2003) are not observed experimentally with simple transmembrane peptides (van Duyl et al., 2002).

In this article we extend and attempt to rationalize the seemingly inconsistent results of these theoretical (Lundbaek et al., 2003) and experimental (van Duyl, 2002) studies by using quantitative assays to compare the distribution of both lipids and simple transmembrane peptides in DSMs and DRMs for the 1:1:1 DOPC:SM:cholesterol system. In particular, we compare the distribution of transmembrane peptides of different lengths at temperatures where most detergent solubility studies are performed (4°C) and at physiological temperature (37°C). The influence of temperature change on peptide distribution is of interest since both bilayer thickness (Evans and Needham, 1987; Simon et al., 1995) and bilayer mechanical properties (Evans and Needham, 1987) are temperature-dependent. Moreover, structural analyses of similar bilayer systems have shown that large rafts (several micrometers in diameter) are clearly visible with the light

microscope at low temperatures but become much smaller or undetectable at higher temperatures (Dietrich et al., 2001; Samsonov et al., 2001). In our study we primarily use two transmembrane peptides, P-23 and P-29 (Fig. 1), whose hydrophobic transbilayer domains are designed to match the hydrocarbon thicknesses of DSMs and DRMs, respectively, from the 1:1:1 DOPC:SM:cholesterol system (Gandhavadi et al., 2002). We also use our data to calculate apparent free energies of transfer (ΔG_a) of lipids and transmembrane peptides from DSMs to DRMs. These values of ΔG_a, which provide estimates of the work required to transfer peptides and lipids between DSMs and DRMs, give insights as to the effectiveness of sorting mechanisms based on differences in mechanical and structural properties of raft and nonraft bilayers.

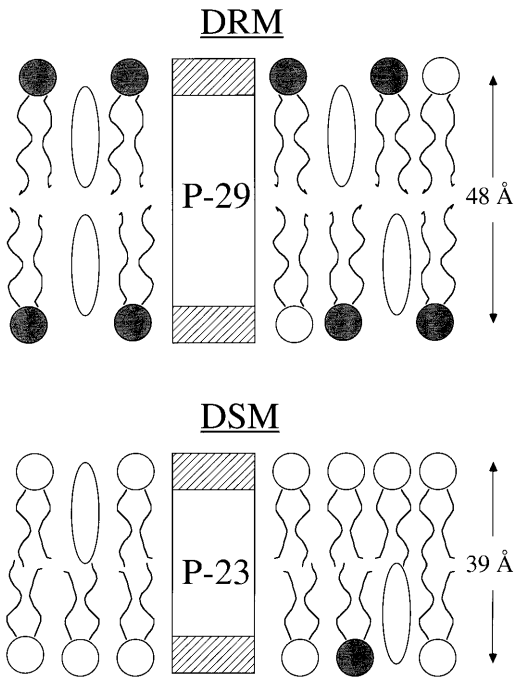

FIGURE 1 Schematic drawing showing peptides P-29 and P-23 in bilayers with thicknesses corresponding to DRM and DSMs, respectively, isolated from 1:1:1 DOPC:SM:cholesterol bilayers. Phospholipids are depicted with circular headgroups (shaded headgroups represent SM and open headgroups represent DOPC) with wavy hydrocarbon chains, cholesterol is depicted as an open oval, and the peptides are depicted as rectangles. For each peptide the white central box corresponds to the transbilayer α-helical core region and the hatched boxes correspond to the hydrophilic regions containing two lysine residues that anchor each end of the peptide to the interfacial region. DRMs are enriched in both SM and cholesterol (Table 1). The headgroup peak separations for DSMs (39 Å) and DRMs (48 Å) obtained from x-ray analysis by Gandhavadi et al. (2002) are shown on each diagram. The length of the 17 amino-acid hydrophobic α-helix for P-23 is estimated to be 25.5 Å (1.5 Å per amino acid residue in an α-helix), which closely matches the hydrocarbon thickness of the DSMs, whereas the length of the 23-amino acid hydrophobic α-helix region for P-29 is estimated to be 34.5 Å, which closely matches the hydrocarbon thickness of the DRMs. For some experiments, we also used the peptide P-31, which contained 25 amino acids in its hydrophobic α-helix region. See text for details.

MATERIALS AND METHODS

Materials

Bovine brain sphingomyelin (SM) and dioleoylphosphatidylcholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, AL). The CBQCA Protein Quantification Kit was obtained from Molecular Probes (Eugene, OR). Cholesterol, cholesterol infinity reagent, Triton X-100, Sephadex G-50, silica gel thin layer chromatography plates, and HEPES (*n*-(2-hydroxy-ethyl)-piperazine-*n*′-2-ethanesulfonic acid) were purchased from Sigma Chemical Company (St. Louis, MO). Our standard buffer was 5 mM HEPES, 25 mM KCl adjusted to pH 7.4 with KOH.

The two principle peptides used in our studies were P-23 ($KKG(LA)_4W(LA)_4KKA$), which contained 23 amino acids with a central hydrophobic stretch of 17 amino acids, and P-29 ($KKG(LA)_5LW(LA)_5LKKA$), which contained 29 amino acids with a central hydrophobic region of 23 amino acids. Some experiments were also performed with P-31 ($KKG(LA)_6WA(LA)_5LKKA$), which contained 31 amino acids with a central hydrophobic region of 25 residues. These peptides are similar to the KALP peptides that have been shown to form transbilayer α-helices in bilayers (dePlanque et al., 1999). All peptides were synthesized by the Micro Protein Chemistry Facility at the University of North Carolina (Chapel Hill, NC) using FMOC chemistry in a Symphony (Rainin) peptide synthesizer. The peptides were purified by HPLC and analyzed by time-of-flight MALDI III (Shimadzu/Kratos) mass spectrometry.

Methods

Multilamellar vesicles containing the P-23, P-29, or P-31 peptides were made by the following procedure. Peptide:lipid mixtures (1:10 wt:wt) were co-dissolved in chloroform:methanol (2:1 v:v) and then rotary-evaporated to dryness. The dry films were hydrated by adding buffer and then vortexing and incubating the suspensions for 1 h at 60°C. To break up large particles the suspensions were briefly probe-sonicated.

Detergent extraction procedures were similar to those of Ahmed et al. (1997), with each of the following procedures performed at either 4°C or 37°C. After incubation of the suspensions at the appropriate temperature for 30 min, unincorporated peptide was removed by passing the suspensions through a Sephadex G-50 column that had been equilibrated at the appropriate temperature. The multilamellar vesicles were treated with 1% Triton X-100 for 30 min at either 4°C or 37°C and then centrifuged 30 min with an Eppendorf bench centrifuge at the appropriate temperature. The supernatant was removed and the pellet was resuspended in an equal volume of buffer and briefly probe sonicated.

After detergent treatment, the total phospholipid contents of the supernatant (DSMs) and resuspended pellet (DRMs) were determined by phosphate assay (Chen et al., 1956) and the cholesterol content was determined using the Sigma infinity (cholesterol oxidase) assay. Control experiments showed that 1% Triton did not affect the results of either of these assays. The DOPC:SM ratio in DRMs was obtained using thin layer chromatography (TLC) following the methods of Gandhavadi et al. (2002). For TLC analysis, the DRMs were washed three times in water to remove detergent, then lyophilized and resuspended in chloroform:methanol (2:1 v:v). TLC was performed using chloroform:methanol:ammonium hydroxide (65:25:4 v:v:v) as the solvent with iodine vapor used to detect the lipid spots. To estimate the DOPC:SM ratio, the ratios of the densities of the respective spots in the iodine-treated TLC plates were compared to those of control lanes containing 6:4, 5:5, 4:6, 3:7, 2.5:7.5, 2:8, 1.5:8.5, 1:9, and 0.5:9.5 mol ratios of DOPC:SM. Relative densities of the DOPC and SM spots were determined by obtaining 200-dpi color scans with an Epson Scanner, converting these scans to grayscale TIFF format through Adobe Photoshop 5.0, and then using NIH Image Version 1.61 to measure the area under each peak.

The peptide content in DSMs and DRMs was determined with the CBQCA assay that uses 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde)

as a chromatographic derivatization reagent for amines, in this case the lysines in P-23, P-29, or P-31. For the CBQCA assay, fluorescence measurements were made at an excitation wavelength of 465 nm and emission detection at 550 nm with a Fluoromax DM3000 (Spex Industries, Edison, NJ). Fluorescence intensities of liposomal samples containing each peptide were compared to standard fluorescence intensity versus peptide concentration relations obtained with the same peptides dissolved in 5% ethanol in buffer.

Fluorescence emission spectra of the peptide's tryptophan residue were measured with suspensions in quartz cuvettes over the range of 320–400 nm with an excitation wavelength of 280 nm. Spectra were collected with a step size of 0.5 nm with an averaging time of 4 s/nm. Spectra were recorded for peptides incorporated into 1:1:1 DOPC:SM:cholesterol vesicles, DSMs, and DRMs, as well as for peptide dissolved in 5% ethanol in buffer. Measurements were corrected for blank ethanol-buffer or lipid-buffer suspensions.

The mole-fraction partition coefficient for a peptide from DSM to DRM was calculated from

$$K_p = ([P]_R/[P]_S) \times (([L]_S + [P]_S)/([L]_R + [P]_R)), \quad (1)$$

where $[P]_R$ and $[P]_S$ represent the molar concentrations of the peptide in the DRM and DSM phase, respectively, and $[L]_R$ and $[L]_S$ are the molar concentrations of total lipid (DOPC + SM + cholesterol) in the DRM and DSM phases, respectively. The mole-fraction partition coefficient for cholesterol (or another lipid component) from DSM to DRM was calculated from

$$K_p = ([C]_R/[C]_S) \times ([L]_S)/[L]_R), \quad (2)$$

where $[C]_R$ and $[C]_S$ represent the molar concentrations of cholesterol in the DRM and DSM phase, respectively. The apparent free energies of transfer from DSMs to DRMs were calculated using

$$\Delta G_a = -RT \ln(K_p), \quad (3)$$

where R is the molar gas constant and T is temperature in degrees Kelvin. The thermal energies (RT) are 0.55 kcal/mol and 0.62 kcal/mol at 4°C and 37°C, respectively. An implicit extra thermodynamic assumption of these calculations is that the presence of 1% Triton X-100 does not alter the distribution (or K_p) of the lipids or peptides between DSMs and DRMs. We therefore refer to the energies as apparent free energies (ΔG_a).

RESULTS

Peptide binding and orientation

Phosphate, cholesterol, and CBQCA assays were used to determine the lipid:peptide ratio before and after the peptide-containing liposomes were passed through the G-50 Sephadex column. For P-23 at both 4°C and 37°C the lipid:peptide ratio after passing through the column was the same as the starting material, indicating that, within experimental uncertainty, ~100% of P-23 was bound to the bilayers. A smaller amount of P-29 was bound, ~62% at 4°C and 74% at 37°C.

To determine whether the peptides incorporated into the bilayers were in a transbilayer configuration, we recorded fluorescence spectra from lipid suspensions collected from the column, as well as from the DSMs and DRMs after treatment with 1% Triton X-100. Both P-23 and P-29 contained a tryptophan residue in the center of their hydrophobic region. The environment of the tryptophan influences the maximum of its fluorescence spectrum; typically there is a blue shift when the tryptophan is located in a hydrophobic (low dielectric constant) environment (Lakowicz, 1983). For P-23 and P-29 in 5% ethanol, the maxima of the emission spectrum were at 353 nm and 335 nm, respectively (data not shown). The lower value for P-29 may arise from shielding of its tryptophan residue from water due to peptide aggregation as a consequence of its longer hydrophobic segment. When incorporated into membranes the emission maxima of P-23 and P-29 were similar, with both being blue-shifted compared to spectra from the peptide in 5% ethanol. P-23 had fluorescence emission maxima of 324 nm, 323 nm, and 320 nm in 1:1:1 DOPC:SM:cholesterol, DSMs, and DRMs, respectively, whereas P-29 gave emission maxima of 325 nm, 319 nm, and 319 nm in 1:1:1 DOPC:SM:cholesterol, DSMs, and DRMs, respectively (data not shown).

Formation of DSMs and DRMs

The 1:1:1 DOPC:SM:cholesterol dispersions were fractionated into DSMs and DRMs by 1% Triton X-100 treatment at both 4°C and 37°C. After detergent treatment and centrifugation at 4°C, a pellet (DRMs) and a clear supernatant (DSMs) were obtained. A pellet was also obtained at 37°C, but at this temperature the supernatant exhibited a milky appearance, indicating the presence of larger particles. Compositional analyses of the DSMs and DRMs are presented in Table 1 and Fig. 2. The percentage of the starting lipid found in DSMs is shown in Table 1, the percentage of phospholipid (PL = DOPC + SM), cholesterol, and peptide in DSMs and

TABLE 1 Peptide and lipid distribution between rafts (DRMs) and matrix bilayer (DSMs)

Peptide present	T (°C)	DSM % SL	DSM Chol/L_S	DSM DOPC/L_S	DSM SM/L_S	DSM Peptide/L_S	DRM Chol/L_R	DRM DOPC/ L_R	DRM SM/ L_R	DRM peptide/ L_R
P-23	4	32 ± 9	0.12 ± 0.03	–	–	0.129 ± 0.007	0.38 ± 0.04	–	–	0.012 ± 0.001
P-29	4	32 ± 3	0.12 ± 0.03	–	–	0.108 ± 0.016	0.37 ± 0.09	–	–	0.017 ± 0.004
None	4	23 ± 7	0.17 ± 0.03	0.72	0.08	–	0.40 ± 0.03	0.15	0.46	–
P-23	37	55 ± 6	0.21 ± 0.02	–	–	0.069 ± 0.016	0.30 ± 0.03	–	–	0.018 ± 0.001
P-29	37	50 ± 12	0.22 ± 0.01	–	–	0.055 ± 0.020	0.31 ± 0.01	–	–	0.032 ± 0.005
None	37	46 ± 14	0.23 ± 0.02	0.68 ± 0.14	0.05 ± 0.05	–	0.36 ± 0.05	0.11 ± 0.02	0.54 ± 0.04	–

All ratios represent molar ratios and are mean ± SD for three or four separate experiments, except for SM and DOPC values at 4°C, which were calculated from data of Gandhavadi et al. (2002). Abbreviations: *SL*, Starting Lipid (DOPC + SM + cholesterol) before detergent treatment; and L_S and L_R, total lipid after detergent treatment in DSMs or DRMs, respectively.

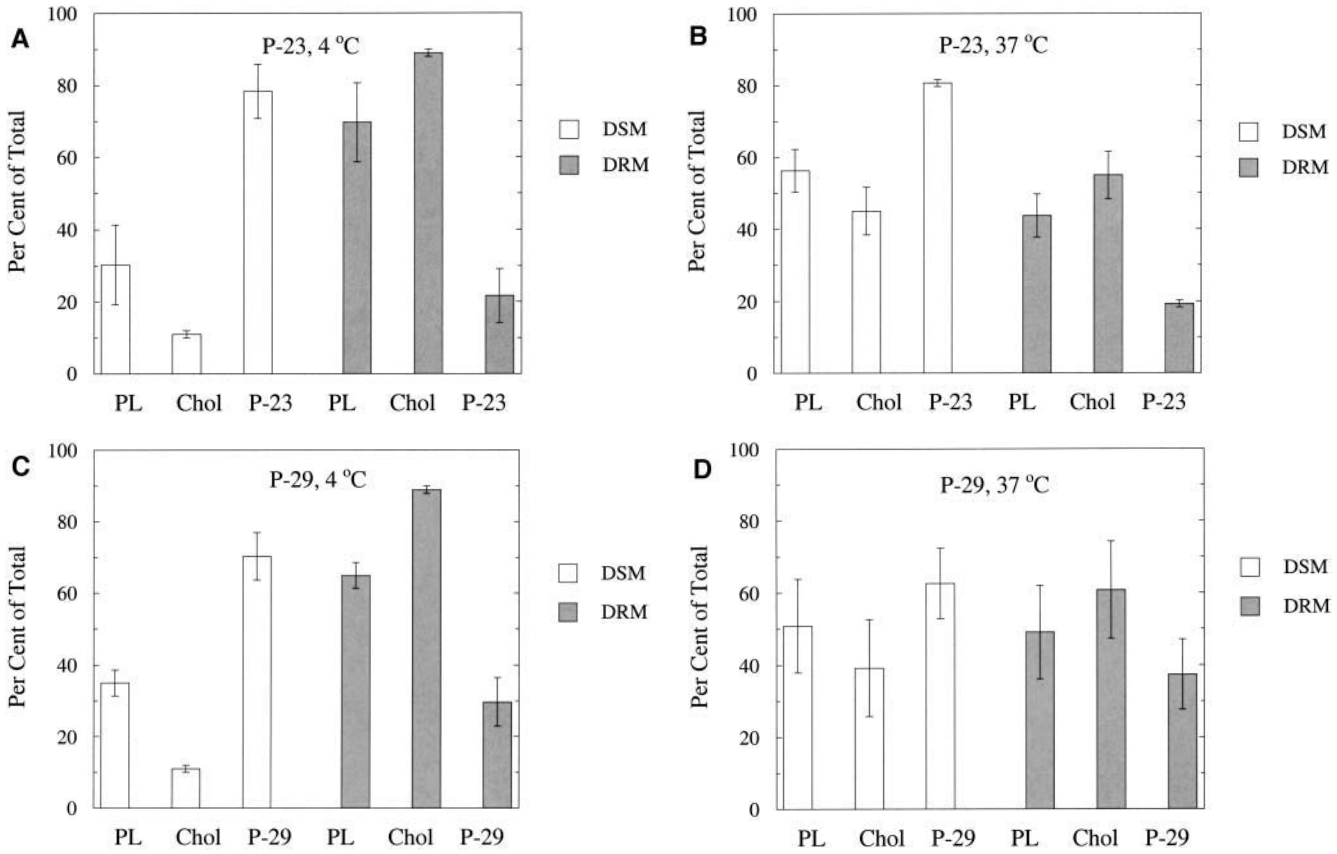

FIGURE 2 Percentage distribution of total phospholipid (DOPC plus SM), cholesterol, and peptides between DSMs and DRMs. *A* shows the distribution of P-23 at 4°C, *B* shows the distribution of P-23 at 37°C, *C* shows the distribution of P-29 at 4°C, and *D* shows the distribution of P-29 at 37°C. The values are mean ± SD for three or four separate experiments.

DRMs at 4° and 37°C are shown in Fig. 2, and the ratios of peptide to total lipid in each fraction (TL = PL + cholesterol) are shown in Fig. 2 and Table 1. We now summarize the key findings from these studies.

Lipid distribution between DSMs and DRMs

At 4°C, in the presence of either peptide, ~70% of the starting lipid (SL = DOPC + SM + cholesterol) was found in DRMs. The DRMs were enriched in cholesterol. In particular, in the presence of either P-23 or P-29 the cholesterol to total lipid ratio was ~3× higher in DRMs than in DSMs (Table 1). Previous work (Gandhavadi et al., 2002) has shown that the DRMs were also enriched in SM, whereas the DSMs were enriched in DOPC and depleted in cholesterol (Table 1).

At 37°C in the presence of either peptide less of the starting lipid (~50% at 37°C compared to 70% at 4°C) was found in DRMs. However, the lipid compositions of DSMs and DRMs were similar at 4°C and 37°C (Table 1). That is, in the presence or absence of peptide, the cholesterol assays showed that at 37°C the DRMs were enriched in cholesterol and TLC analysis (Fig. 3) showed that DRMs contained ~5× more SM than DOPC at 37°C (Table 1).

Peptide distribution between DSMs and DRMs

At 4°C most of the peptide, either P-23 or P-29, was located in DSMs (Fig. 2, *A* and *C*, and Fig. 4 *A*). Specifically, even though a majority of the lipid (both phospholipid and cholesterol) was in DRMs, only ~20% of P-23 and 30% of P-29 was in DRMs (Fig. 2, *A* and *C*). Thus, the P-23:lipid ratio was ~10× larger for DSMs than for DRMs (Fig. 4, Table 1), whereas the P-29:lipid ratio was ~6× larger for DSMs than for DRMs (Fig. 4, Table 1).

At 37°C the peptide:lipid ratio increased in DRMs and decreased in DSMs. At this temperature the peptide:lipid ratio for DSMs compared to DRMs was ~4 for P-23 and <2 for P-29. In other words, at 4°C and 37°C both peptides were preferentially localized in DSMs. However, at 37°C P-29 was found at a significantly higher concentration (P-29:lipid ratio of 0.032) in DRMs than it was at 4°C (P-29:lipid ratio of 0.017). Thus, at 37°C DRMs incorporated a significantly higher ($P < 0.01$) concentration of P-29 than P-23 (Fig. 4).

Apparent free energies of transfer

With the assumption that the Triton X-100 does not change the composition of bilayer DRMs, these peptide and lipid

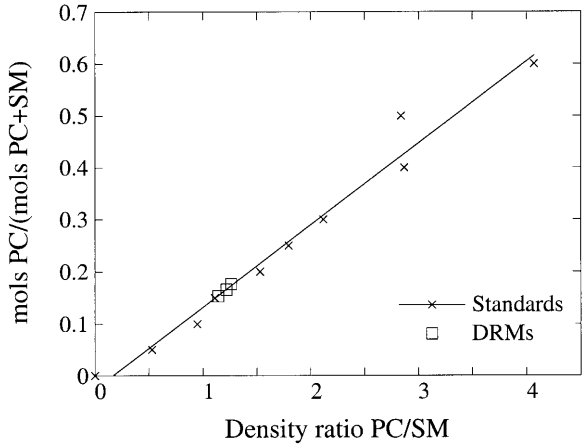

FIGURE 3 Results from thin layer chromatography of DRMs at 37°C. The mol ratio of DOPC/(DOPC + SM) is plotted versus the density ratio of DOPC and SM spots on TLC plates. The × symbols represent results from standards with mol ratios of DOPC/(DOPC + SM) varying from 0.0 to 0.6, and the solid line is a least squares-fit to the standards data ($R^2 = 0.98$). The open squares represent results from three separate DRM samples.

distributions can be put on a thermodynamic basis by calculations of the molar partition coefficients (K_p) and apparent free energies of transfer (ΔG_a) from DSMs to DRMs (Eqs. 1–3 in Methods). Table 2 gives K_p and ΔG_a values for peptides (P-23, P-29, and P-31) and lipids (cholesterol, DOPC, and SM). At both 4°C and 37°C, ΔG_a was negative for SM and cholesterol but positive for DOPC, P-23, P-29, and P-31. This means that to partition from DSMs into DRMs it was energetically favorable for SM and cholesterol, but energetically unfavorable for DOPC, P-23, P-29, and P-31. Interestingly, at 4°C ΔG_a was similar in magnitude (~1 kcal/mol) for the phospholipids (DOPC and SM) and for the peptides (P-23, P-29, P-31). This implies that a similar amount of work was required to move DOPC or any of the peptides into DRMs, and that that was approximately the same as the work required to move SM out of DRMs. Also,

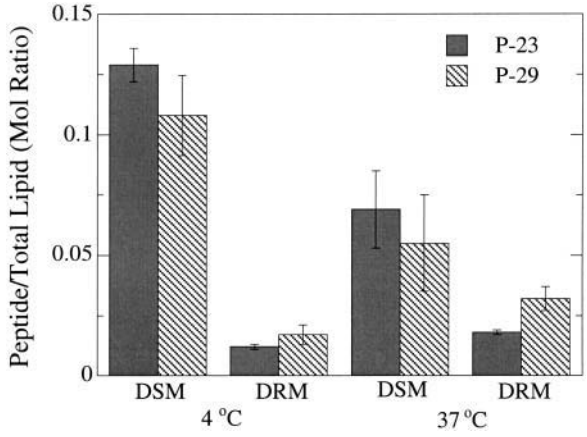

FIGURE 4 Peptide/lipid ratios in DSMs and DRMs for both P-23 and P-29 at 4°C and 37°C. The values are means ± SD for three or four separate experiments.

TABLE 2 Partition coefficients and apparent free energies of transfer for peptides and lipids from DSMs to DRMs

Molecule	Peptide present	Temp.	K_p	ΔG_a (kcal/mol)
P-23	P-23	4°C	0.10 ± 0.01	+1.29 ± 0.01
P-29	P-29	4°C	0.17 ± 0.04	+0.97 ± 0.15
P-31	P-31	4°C	0.10 ± 0.04	+1.29 ± 0.21
Cholesterol	P-23	4°C	3.40 ± 1.05	−0.65 ± 0.19
Cholesterol	P-29	4°C	2.74 ± 0.39	−0.55 ± 0.08
Cholesterol	None	4°C	2.30 ± 0.41	−0.46 ± 0.10
DOPC	None	4°C	0.20	+0.88
SM	None	4°C	5.75	−0.96
P-23	P-23	37°C	0.28 ± 0.06	+0.82 ± 0.13
P-29	P-29	37°C	0.63 ± 0.14	+0.30 ± 0.16
P-31	P-31	37°C	1.15 ± 0.76	+0.04 ± 0.49
Cholesterol	P-23	37°C	1.40 ± 0.02	−0.20 ± 0.01
Cholesterol	P-29	37°C	1.42 ± 0.09	−0.22 ± 0.04
Cholesterol	None	37°C	1.58 ± 0.39	−0.27 ± 0.15
DOPC	None	37°C	0.15 ± 0.08	+1.22 ± 0.39
SM	None	37°C	4.26 ± 1.22	−0.87 ± 0.20

Values represent mean ± SD for three or four separate experiments, except for SM and DOPC at 4°C, which were calculated from data of Gandhavadi et al. (2002). For comparison, thermal energies are 0.55 kcal/mol and 0.62 kcal/mol at 4°C and 37°C, respectively.

the apparent free energy to transfer cholesterol from DSMs to DRMs (between −0.46 and −0.65 kcal/mol) was independent (within experimental uncertainty) of the presence or absence of peptide in the bilayer or on the length of the peptide present in the bilayer. For cholesterol the magnitude of ΔG_a at 37°C was smaller (less negative) than at 4°C, meaning that there was less of an energy gain to transfer cholesterol from DSMs to DRMs at the higher temperature.

Although at both 4°C and 37°C ΔG_a was positive for P-23, P-29, and P-31, its magnitude depended on both temperature and peptide length. Specifically the magnitudes of ΔG_a for all peptides (P-23, P-29, and P-31) were smaller at 37°C than at 4°C, meaning that less energy was required to transfer the peptides from DSMs to DRMs at the higher temperature. Moreover, the magnitude of ΔG_a at 37°C was significantly smaller for P-29 (0.30 kcal/mol) than for P-23 (0.82 kcal/mol).

DISCUSSION

The data presented in this article show that the peptide distribution and the work required to transfer simple transbilayer peptides from detergent-soluble bilayers to detergent-resistant bilayers depend both on the structure of the peptide (length of its transbilayer α-helix) and on the temperature at which the bilayers are fractionated into DSMs and DRMs.

Peptide design

There is relatively limited information on the factors that make a ''good'' transmembrane raft protein. To test the possible role of hydrophobic length, in this study we use peptides

similar to the KALP peptides (dePlanque et al., 2001; Morein et al., 2002; Strandberg et al., 2002) that form transbilayer α-helices in bilayers (dePlanque et al., 1999). These peptides have a central core of hydrophobic residues (leucines and arginines) flanked by charged lysines that anchor each end of the peptide to the hydrocarbon-water interface. As shown in Fig. 1, P-23 and P-29 were designed to have hydrophobic lengths similar to the hydrocarbon thicknesses of DSMs and DRMs, respectively, measured by x-ray diffraction for the 1:1:1 DOPC:SM:cholesterol system (Gandhavadi et al., 2002). Two factors that must be considered in the design of these peptides are that 1), the position of the hydrocarbon/water interface is not precisely determined from medium-resolution electron density profiles (Gandhavadi et al., 2002); and 2), the bilayer hydrocarbon thickness decreases with increasing temperature (Evans and Needham, 1987; Simon et al., 1995). The following analysis indicates that at 37°C the lengths of the helices of P-23 and P-29 should closely match the hydrocarbon thicknesses of DSM and DRM bilayers, respectively. At 20°C the headgroup peak separations in electron density profiles of DSM and DRM bilayers were found to be 39 Å and 48 Å, respectively (Gandhavadi et al., 2002). Since the distance between the headgroup peak and the deeper carbonyl of the phospholipid molecule is ~5 Å (McIntosh and Simon, 1986), at 20°C the hydrocarbon thicknesses of DSMs and DRMs are ~29 Å and 38 Å, respectively. Given that the thermal expansion modulus of PC bilayers in the absence and presence of cholesterol are -3.3×10^{-3}/°C and -2.0×10^{-3}/°C, respectively, we estimate that at 37°C the hydrocarbon thickness of DSMs and DRMs are ~27.4 Å and 36.7 Å, respectively. Given that the length of an α-helix is ~1.5 Å per amino acid residue, the lengths of the hydrophobic helices of P-23 and P-29 are 25.5 Å and 34.5 Å, respectively. With the assumption that the peptide helices are oriented perpendicular to the plane of the bilayer, these calculations indicate that, within ~2 Å, there is a ''hydrophobic match'' between P-23 and DSMs and between P-29 and DRMs at 37°C. At 4°C the match would not be quite as close because, using the thermal expansion moduli given above, we estimate that the hydrocarbon regions of both DSM and DRM bilayer would be ~3 Å thicker at 4°C than at 37°C. Therefore, to even more closely match the hydrocarbon thickness of DRMs at 4°C, we performed some experiments with P-31, which is 3 Å longer than P-29. As shown in Table 2, we found at both 4°C and 37°C that the distributions of P-29 and P-31 between DSMs and DRMs were similar.

The fluorescence emission spectra maxima of 319–325 nm for P-23 and P-29 in 1:1:1 DOPC:SM:cholesterol bilayers, DSMs, and DRMs are typical of spectra obtained from peptides whose tryptophan residues are buried deep in the hydrocarbon interior (Ren et al., 1997, 1999a; Webb et al., 1998). Since the tryptophan residue was in the center of each peptide, these spectra provide strong evidence that these peptides were predominantly in a transbilayer orientation in 1:1:1 DOPC:SM:cholesterol bilayers. The observation that a higher percentage of P-23 than P-29 was bound to the 1:1:1 DOPC:SM:cholesterol bilayers at either 4°C or 37°C may be due to the tendency of P-29 to aggregate in the aqueous phase. This hypothesis is supported by the relatively low fluorescence emission maximum (335 nm) observed for P-29 in 5% EtOH (compared to 353 nm for P-23 in 5% EtOH).

Lipid and peptide distribution at 4°C

At 4°C much of the cholesterol was found in DRMs (Table 1, Fig. 2, *A* and *C*), both in the presence and absence of peptide. This result is consistent with previous detergent extraction studies at 4°C of both biological membranes (MacDonald, 1980; Hanada et al., 1995; Fridriksson et al., 1999; London and Brown, 2000) and this 1:1:1 DOPC:SM:cholesterol system (Gandhavadi et al., 2002). These previous studies have shown that the DRMs are also enriched in SM.

At 4°C, P-23, P-29, and P-31 were all preferentially localized to DSMs (Table 1). This means that, independent of peptide length, at this temperature these transmembrane peptides distributed to the liquid-disordered lipids in DSMs, rather than to the liquid-ordered lipids in DRMs where P-29 and P-31 have a better hydrophobic match. Van Duyl et al. (2002) obtained a similar result at 4°C for a series of WALP transmembrane peptides of similar hydrophobic lengths that contain tryptophans (rather than lysines) near the C- and N-termini of the peptides. This implies that similar distributions between DSMs and DRMs are obtained with different residues anchoring the peptides at the interfacial regions of the bilayer (either charged lysines in the KALP peptides or nonpolar tryptophans in the WALP peptides). Moreover, the WALP peptides do not contain a tryptophan residue in the center of the hydrophobic α-helix. This implies that the relatively bulky tryptophan in the center of the helices of P-23, P-29, and P-31 is probably not an important factor in preventing partitioning of these peptides into DRMs.

We argue that the localization of P-23, P-29, and P-31 to DSMs at 4°C is likely due to the much smaller compressibility and bending moduli for bilayers containing primarily DOPC compared to bilayers containing primarily SM and cholesterol (Needham and Nunn, 1990), making it more energetically favorable for the peptides to partition into DSMs than into DRMs. That is, as predicted by Lundbaek et al. (2003), a major role of cholesterol in peptide sorting is to change the bilayer material properties. Or stated another way, the high cohesive energy of SM:cholesterol bilayers tends to ''squeeze out'' molecules that disturb their relatively organized acyl chain packing (van Duyl et al., 2002; Allende et al., 2003). To this point, we found that the magnitude of the negative free energy of transfer of amphipathic peptides, such as melittin, from the aqueous phase to a bilayer is larger for DSM lipids than for DRM lipids (Gandhavadi et al., 2002) and decreases linearly with increasing bilayer compressibility modulus (Allende et al., 2003). Thus, our data

indicate that at 4°C the unfavorable energy cost due to the hydrophobic mismatch of partitioning P-29 or P-31 from DRMs to DSMs is overcome by the energy gain of being in a DSM bilayer with low compressibility and bending moduli. Concerning the partitioning of P-29 and P-31 into DSMs, it is possible that these peptides could tilt in the bilayer to minimize hydrophobic mismatch (Ben-Shaul et al., 1996). However, recent computer simulations indicate that to reduce mismatch it is easier to deform the bilayer than to tilt the transbilayer peptide (Petrache et al., 2002).

Lipid and peptide distribution at 37°C

At physiological temperature less of the starting lipid was localized to DRMs than to DSMs (Table 1). In addition, under the same centrifugation conditions where the supernatant was clear at 4°C due to presence of micelles, the supernatant was cloudy at 37°C, indicating that it may contain bilayers as well as mixed micelles. These observations are consistent with the recent results of Heerklotz (2002), who found that, at a given Triton X-100:PC ratio, as the temperature is increased some of the mixed micelles are converted to liquid-disordered bilayers. In particular, with the Triton X-100:PC ratio used in our work (~1:1), at 4°C suspensions of PC:SM:cholesterol consist of liquid-ordered bilayers and micelles, whereas at ~37°C the suspensions contain liquid-ordered bilayers, micelles, and liquid-disordered bilayers (Heerklotz, 2002).

At 37°C there was a greater peptide/lipid ratio for both P-23 and P-29 in DRMs than at 4°C (Table 1, Fig. 4). We hypothesize that this is due to the compressibility modulus of DRMs decreasing with increasing temperature more than the compressibility modulus of DSMs. Such a decrease in DRM compressibility modulus should decrease the energetic cost of partitioning peptides from DSMs to DRMs (Table 2) due to smaller differences in mechanical properties of the respective bilayers (Lundbaek et al., 2003). Although we are unaware of any compressibility moduli measurements for SM:cholesterol bilayers as a function of temperature, measurements of the temperature dependence of compressibility of dimyristoylphosphatidylcholine (DMPC) bilayers in the presence and absence of cholesterol are consistent with our hypothesis. Evans and Needham (1987) showed that the compressibility modulus of 3:2 DMPC:cholesterol bilayers containing 1 mol % transbilayer peptide similar to P-29 decreased from 945 dyn/cm at 12°C to 193 dyn/cm at 34°C. This value at 34°C was similar to the value for liquid-crystalline phase DMPC at 29°C in the absence of cholesterol. Thus, there appears to be a much closer match of compressibility moduli between DMPC and DMPC:cholesterol:peptide bilayers at elevated temperatures than at low temperatures. A testable prediction of our work is that the compressibility modulus of SM:cholesterol (and DRMs) should decrease more upon going from 4°C to 37°C than the compressibility modulus of DOPC (and DSMs) so that there would be a smaller difference in compressibility moduli between DSMs and DRMs at 37°C than at 4°C. Similar arguments should hold for bending moduli since they are proportional to the compressibility moduli (Evans and Rawicz, 1990).

That P-29 is found at higher concentrations than P-23 in DRMs at 37°C is likely a manifestation of the closer match of length of the hydrophobic α-helix of P-29 with the hydrocarbon thickness of DRMs (see above and Fig. 1). It would cost considerable energy to deform the DRM bilayer to conform to the shorter P-23 peptide (Lundbaek et al., 2003). Thus, at least for these peptides, the effect of hydrophobic mismatch on peptide sorting is larger at physiological temperature than at 4°C.

Our experiments also showed that the distribution of cholesterol and peptides containing a single hydrophobic α-helix were different at 37°C than at 4°C, the temperature at which most detergent solubility experiments are performed on biological membranes and lipid bilayer systems. These results indicate that detergent solubility experiments performed at low temperatures may not give a completely accurate insight into the concentrations of specific proteins and lipids in raft membranes at physiological temperatures.

Thus, the peptide data in Tables 1 and 2 illustrate the importance of both bilayer mechanical properties and hydrophobic matching on peptide sorting between bilayer domains. That both P-23 and P-29 were found in higher concentrations in DRMs at 37°C than at 4°C shows the importance of bilayer mechanical properties on partitioning between bilayer domains. The higher concentration of P-29 compared to P-23 in DRMs at 37°C demonstrates the effect of hydrophobic matching of peptide length and bilayer hydrocarbon thickness.

Our data can also be related to temperature-dependent observations of lipid raft lateral dimensions for similar lipid systems. Both Dietrich et al. (2001) and Samsonov et al. (2001) observed that rafts became smaller as the temperature was increased. Samsonov et al. (2001) noted that rafts can ''dissolve,'' so that they cannot be resolved with the light microscope, at temperatures above the main melting temperature of SM, which is ~39°C (McIntosh et al., 1992). We argue that this could also be a manifestation of the bilayer compressibility and bending moduli of DSMs and DRMs being closer at 37°C than at 4°C.

Energetics of lipid-peptide sorting

Let us initially consider the apparent free energies of transfer of the lipids from DSMs to DRMs. We found at both 4°C and 37°C that ~−1 kcal/mol was required to transfer SM and ~1 kcal/mol to transfer DOPC (Table 2). SM, a lipid with long, mostly saturated hydrocarbon chains, has strong van der Waals associations with cholesterol and forms detergent-insoluble bilayers with cholesterol (MacDonald, 1980; Li et al., 2001). We argue that unsaturated DOPC preferentially

partitions into DSMs because it would be energetically unfavorable for DOPC to disrupt the relatively organized liquid-ordered SM/cholesterol phase. Our values for the apparent free energy of transfer for SM (Table 2) were close to the values obtained using fluorescent amphiphiles to measure the distribution of saturated lipids (diC18:0) between gel and liquid-disordered phases (Mesquita et al., 2000), and therefore should be a good model for the transfer of long chain unsaturated molecules between DSMs and DRMs. We also found that at 37°C cholesterol preferentially partitioned into DRMs with ΔG_a of -0.2 to -0.3 kcal/mol (Table 2). This value is comparable with the recent results of Niu and Litman (2002) who used a lipid vesicle-cyclodextrin system to measure free energies of transfer of cholesterol between vesicles of different lipid compositions. In particular, Niu and Litman (2002) found that, compared to a DOPC vesicle, cholesterol prefers to be in a vesicle composed of stearoyloleoylphosphatidylcholine (SOPC, a lipid containing a saturated C18:0 hydrocarbon chain) by -0.2 kcal/mol.

As noted in the Methods section, an implicit assumption of our apparent free energy calculations is that Triton X-100 does not alter the composition of DSMs and DRMs. For lipid components the consistency of our values for ΔG_a with measurements using different methods (Mesquita et al., 2000; Niu and Litman, 2002) provides evidence for the validity of that assumption.

We found that there was a small energetic cost ($\sim$1 kcal/mol at 4°C) to transfer either P-23 or P-29 from DSMs to DRMs (Table 2). The small value of ΔG_a for P-23 is lower than what would be predicted from the theoretical studies of Lundbaek et al. (2003). For a peptide with a transbilayer α-helical domain of 17 amino acids (the same as P-23) the bilayer deformation energies calculations of Lundbaek et al. (2003) give ΔG_{def} $\sim$7 kcal/mole for 1:1 SOPC:cholesterol bilayers and ΔG_{def} $\sim$1 kcal/mol for SOPC bilayers, meaning that the energy difference to partition from SOPC to 1:1 SOPC:cholesterol would be $\sim$6 kcal/mol. Lundbaek et al. (2003) did not do calculations for a peptide with a transbilayer domain of 23 amino acids (like P-29). However, extrapolation of their calculations to a peptide of this length would result in a very small energy of deformation ($\sim$1 kcal/mol), similar to our observed apparent free energy of transfer for P-29 (Table 2). In their calculations Lundbaek et al. (2003) used bilayers (SOPC and SOPC:cholesterol) that differed in thickness by 3 Å, whereas in our preparation the difference in hydrocarbon thickness between DSMs and DRMs is $\sim$9 Å. This would make the discrepancy between theory and experiment for P-23 even greater. Indeed, our results are more consistent with the deformation energies (ΔG_{def}) calculated solely on the basis of differences in bilayer thickness between SOPC and SOPC:cholesterol and assuming the same compressibility and bending moduli for the two bilayers (Lundbaek et al., 2003).

Thus, our experimental values for ΔG_a are quite close to the theoretical predictions of Lundbaek et al. (2003) for P-29, but significantly lower than their theoretical predictions for P-23. There are several possibilities to rationalize the differences between experimental (Table 2) and theoretical predictions for P-23. As noted above, one possibility is that the detergent altered the peptide distribution between DSMs and DRMs. However, this factor is unlikely to account for such a large difference in free energy for P-23 between theory and experiment ($\sim$5 kcal/mol), especially given the agreement between experiment and theory for P-29 and the consistency of our lipid apparent free energies with free energies determined using other techniques (see above). Another possibility is that the theoretical calculations used macroscopic values for the bending and compressibility moduli and assumed that these values would be unaffected by the presence of a peptide. This assumption may be critically dependent on both the peptide and bilayer composition. In this regard, Evans and Needham (1987) found that the presence of 1 mol % transbilayer peptide lowered the compressibility modulus of 3:2 SOPC:cholesterol from 363 dyn/cm to 169 dyn/cm and lowered the compressibility modulus of 3:2 DMPC:cholesterol from 600 dyn/cm to 193 dyn/cm. The use of lower compressibility moduli in the Lundbaek et al. (2003) analysis should lower their ΔG_{def} predictions for P-23 and bring them into closer agreement with our experimental values. This issue, however, will have to be addressed by experiments in which peptide distributions are measured in the same bilayers with known mechanical and structural properties.

Finally, as shown in Table 2, at physiological temperature the magnitudes of the apparent free energies of transfer (ΔG_a) for cholesterol, P-29, and P-31 are less than the magnitude of thermal energy (0.6 kcal/mol). This implies that raft formation represents only a marginally effective mechanism for membrane sorting of these molecules. However, the magnitude of ΔG_a is considerably larger than the magnitude of thermal energy for DOPC, SM, and P-23, indicating that raft formation may have a significant impact on the sorting of unsaturated PCs, SM, and proteins with relatively short transmembrane domains.

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