Influence of hydrophobic mismatch and palmitoylation on the association of transmembrane α-helical peptides with detergent-resistant membranes

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Abstract The aim of this study was to gain insight into the mechanism through which transmembrane proteins are targeted to liquid ordered (L_o) phase domains or rafts. This was investigated by analyzing the Triton X-100 resistance of designed transmembrane peptides in model membranes of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, sphingomyelin and cholesterol (1/1/1, molar ratio), which contain both L_o phase domains and fluid bilayers. By using peptides with one or two palmitate chains covalently linked to their N-terminus or with variable hydrophobic lengths, the roles of protein palmitoylation and of mismatch between the transmembrane segment of the protein and the bilayer thickness, respectively, were investigated. The results show that neither hydrophobic matching nor palmitoylation is sufficient for partitioning of peptides into L_o phase domains. It is concluded that the L_o phase itself, due to the tight packing of the lipids, constitutes an unfavorable environment for accommodation of protein transmembrane segments.

Key words: Raft; Sphingolipid; Cholesterol; Triton X-100; Model membrane

1. Introduction

Much evidence has been found for the existence of sphingolipid and cholesterol rich lipid domains, called rafts, in the eukaryotic cell membrane [1,2]. Lipid rafts are thought to be in the liquid ordered (L_o) phase, which was first described using model membranes [3–5]. The L_o phase arises from the interaction of cholesterol with lipids with long saturated acyl chains, like sphingolipids. Cholesterol stretches and orders these chains, leading to an increase in membrane thickness [6,7] and a tight packing of the acyl chains [8,9]. This tight packing gives an explanation for the characteristic resistance of L_o phase membranes to solubilization by non-ionic detergents like Triton X-100 (TX-100) [10]. Detergent-resistant membranes (DRMs) can indeed be isolated from both eukaryotic cells and model membranes containing the L_o phase [11–13].

Rafts have been proposed to play a role in several biological processes, such as signal transduction [14], viral budding [15] and lipid sorting [16]. Although many proteins have been reported to be localized in rafts and/or to be enriched in DRMs [17,18], little is known of the mechanisms with which transmembrane proteins are targeted to rafts. One possible mechanism is based on hydrophobic matching. It has been shown that the bilayer thickness of a membrane with a raft-like composition is larger than that of a fluid bilayer [11]. Since hydrophobic mismatch between a transmembrane α-helix and the bilayer thickness of the membrane is energetically unfavorable [19–21], it is possible that, in biological membranes, proteins with transmembrane domains (TMDs) that are too long to fit in a fluid bilayer preferentially partition into the thicker rafts. Vice versa, proteins with shorter TMDs that do fit in the fluid bilayer might be excluded from the rafts due to mismatch. Such a mismatch-based mechanism has been suggested to play a role in protein sorting in the Golgi. Plasma membranes are expected to be thicker than Golgi membranes, due to their higher content of cholesterol and long chained sphingolipids. In accordance with this, it was found that TMDs of plasma membrane proteins are on average five residues longer than those of Golgi proteins [22]. Moreover, it was shown that lengthening the TMD of a Golgi protein can result in cell surface expression [23,24], while shortening the TMD of a plasma membrane protein can lead to retention in the Golgi [24]. These results support the notion that partitioning of proteins into membrane domains may be modulated by the extent of hydrophobic matching.

Another possible mechanism for targeting both transmembrane and peripheral proteins into rafts may be protein acylation. Many of the proteins that are enriched in DRMs from cells are covalently modified with saturated fatty acyl chains, which are expected to pack well in the L_o phase and to increase the affinity of the protein for L_o phase domains. These modifications are, for instance, GPI anchors, closely spaced myristate and palmitate or dual palmitate chains [25–30]. Disruption of palmitoylation sites can result in the loss of association of a protein with DRMs [27,29]. It has therefore been proposed that acylation and especially palmitoylation can act as a targeting signal for partitioning into rafts [26,27,29].

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Abbreviations: DRM, detergent-resistant membrane; TMD, transmembrane domain; TX-100, Triton X-100; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; SM, sphingomyelin; MLV, multilamellar vesicle; WALP, tryptophan-alanine-leucine peptide; TLC, thin layer chromatography; TFE, trifluoroethanol

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In the present study, we investigate the influence of hydrophobic length and palmitoylation on the partitioning of TMDs into L_o phase domains. The properties of such domains, as well as effects of peptide/lipid hydrophobic mismatch, have been well characterized in model membrane systems [3,11,31–34]. Therefore, we have chosen to use model systems in which TMDs are mimicked by synthetic polypeptides. The peptides used consist of a sequence of alternating leucine and alanine, flanked on both sides by two tryptophans. These peptides, called WALP peptides, form stable transmembrane α-helices that incorporate well into model membranes [35]. By varying the number of leucine and alanine pairs, the influence of the hydrophobic length of these peptides on their partitioning into L_o phase domains is studied. The influence of palmitoylation is investigated by using peptides to which one or two palmitate chains are covalently linked to the N-terminus of the peptide.

2. Materials and methods

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), cholesterol and egg sphingomyelin were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Egg sphingomyelin consists of a mixture of sphingomyelins with exclusively saturated acyl chains, mainly $C_{16:0}$ acyl chains. The unpalmitoylated WALP peptides (Table 1) were synthesized and analyzed by analytical high-performance liquid chromatography (HPLC) and electrospray-mass spectrometry (ES-MS) as described before [36].

2.1. Synthesis of palmitoylated peptides

The palmitoylated WALP peptides were prepared from a WALP23 derivative with a C-terminal amide group, which was synthesized and analyzed by analytical HPLC and ES-MS as described for several other WALP analogs [36]. After coupling of the final amino acid and removal of the Fmoc functionality, the resin was split into three portions. One portion was treated with acetic anhydride/*N*,*N*-diisopropylethylamine (DIPEA)/*N*-hydroxybenzotriazole (HOBt) in *N*-methylpyrrolinone (NMP) for 30 min to yield WALP23 with an acetyl group attached to the N-terminus. The second portion was reacted with palmitic acid in the presence of benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP)/HOBt and DIPEA in 1,2-dichloroethane (DCE)/NMP 1/1 v/v for 16 h to yield WALP23 with one palmitate chain linked at the N-terminus via an amide bond (1PW23). The third portion was treated with succinimidyl-$Glu^{inv}(OC_{16}H_{33})$-$OC_{16}H_{33}$/BOP/HOBt and DIPEA in DCE/NMP 1/1 v/v for 16 h to yield the WALP23 with two hexadecanyl chains linked to the N-terminus (2PW23). In this construct, the two hexadecanyl chains are esterified to the acid moieties of a glutamic acid, which is connected to the peptide via succinimidyl as a flexible linker. The succinimidyl-$Glu^{inv}(OC_{16}H_{33})$-$OC_{16}H_{33}$ was synthesized according to [37]. When necessary (as judged by high performance thin layer chromatography (TLC)), the peptides were further purified by preparative TLC on Merck Silicagel 60 F_{254} plates running with $CHCl_3$/MeOH/H_2O (65/25/4, by volume) as liquid phase.

2.2. Preparation of multilamellar vesicles (MLVs)

DOPC and sphingomyelin (SM) were dissolved in $CHCl_3$/MeOH (1/1, by volume) as 10–20 mM stock solutions. The exact concentrations of these stocks were determined by a phosphorus assay according to Rouser [38]. Cholesterol was dissolved in $CHCl_3$ (20 mM). The concentration of WALP peptides, dissolved in trifluoroethanol (TFE; ca. 2 mM), was determined by the absorbance of tryptophan at 280 nm ($\varepsilon = 22\,400\ M^{-1}\ cm^{-1}$). DOPC, SM and cholesterol were mixed in solution in a 1/1/1, 1/2/1 or a 0/2/1 molar ratio. Where present, WALP in TFE was added at 2 mol% with respect to the total amount of phospholipids. This amount of peptide was chosen, because it can be adequately detected in the presence of lipids without exceeding the concentration at which the bilayer structure of a membrane would be disturbed [39]. Next, organic solvents were evaporated using a rotary evaporator. The resulting films were further dried by overnight storage under high vacuum. Multilamellar vesicles were made by hydrating the mixed dry lipid or lipid/peptide films with buffer (20 mM Tris, 150 mM NaCl, pH 7.4) at 60°C under repeated vortexing until the film was dispersed. The total lipid concentration was 4 mM. The peptides have been shown to adopt a stable transmembrane conformation when incorporated in MLVs using this method [35,36].

Table 1
Amino acid sequences of the WALP peptides used

Peptide	Sequence
WALP23	Ac-GWWLALALALALALALALALWWA-Etn[a]
WALP27	Ac-GWWLALALALALALALALALALALWWA-Etn
WALP31	Ac-GWWLALALALALALALALALALALALALWWA-Etn

[a]A WALP23 with an amide group linked to the C-terminus instead of an ethanolamine group was used where mentioned.

2.3. Treatment with TX-100

MLV dispersions were divided into two equal portions and cooled on ice. A fourfold molar excess, with respect to the total amount of lipid, of ice-cold 10% (w/w) TX-100 in buffer was added to one portion, resulting in a final concentration of 1% TX-100. As a control, the corresponding volume of buffer was added to the other portion. The samples were incubated on ice for 30 min. Next, the DRMs in the samples treated with TX-100 were separated from the dissolved membrane fraction by ultracentrifugation at $200\,000 \times g$ for 1 h at 4°C. The membranes in the samples treated with buffer were spun down by ultracentrifugation at $200\,000 \times g$ at 4°C for 30 min or 1 h, with similar results. All pellets were washed with MilliQ water and lyophilized.

2.4. TLC analysis

Lyophilized pellets were dissolved in $CHCl_3$/MeOH (3/1, by volume). Samples were analyzed by TLC with $CHCl_3$/MeOH/H_2O (65/25/4, by volume) as liquid phase. Spots were visualized by I_2 staining. DOPC and SM spots were quantified by scraping the spots off the TLC plate, followed by a phosphorus determination according to Rouser [38]. >80% of the original amount of DOPC and SM was recovered from the pellet fraction after TLC when samples had been treated with buffer. Peptide spots were quantified by densitometry after scanning the TLC plate. The detergent resistance of the lipids and peptides is defined as the percentage of the amount of material that was pelleted after treatment with TX-100 as compared to the amount of material that was pelleted after treatment with buffer. The experimental error in the values of the detergent resistance did not exceed 20% of those values.

3. Results

3.1. TX-100 resistance of lipids

First, the resistance to TX-100 was checked of different mixtures of the non-raft lipid DOPC, the raft lipid SM and cholesterol in the absence of peptide. Fig. 1A shows that, after treatment of a 2/1 mixture of SM and cholesterol with either TX-100 (lane 2) or buffer (lane 1), similar amounts of SM are pelleted. This high detergent resistance of SM implies that this mixture consists mainly of DRM. When DOPC is incorporated together with SM and cholesterol in a 1/1/1 molar ratio, again similar amounts of SM are pelleted after treatment of this mixture with TX-100 (lane 4) or buffer (lane 3). In contrast, most of the DOPC in the 1/1/1 mixture is dissolved by TX-100 (lane 4) as compared to treatment with buffer (lane 3). Quantification (Fig. 1B) shows that the detergent resistance of SM is indeed high and comparable in both mixtures, whereas that of DOPC in the 1/1/1 mixture amounts to only 30%, in agreement with results from earlier studies [11,12]. Mixtures lacking either SM or cholesterol were completely dissolved by TX-100 (data not shown).

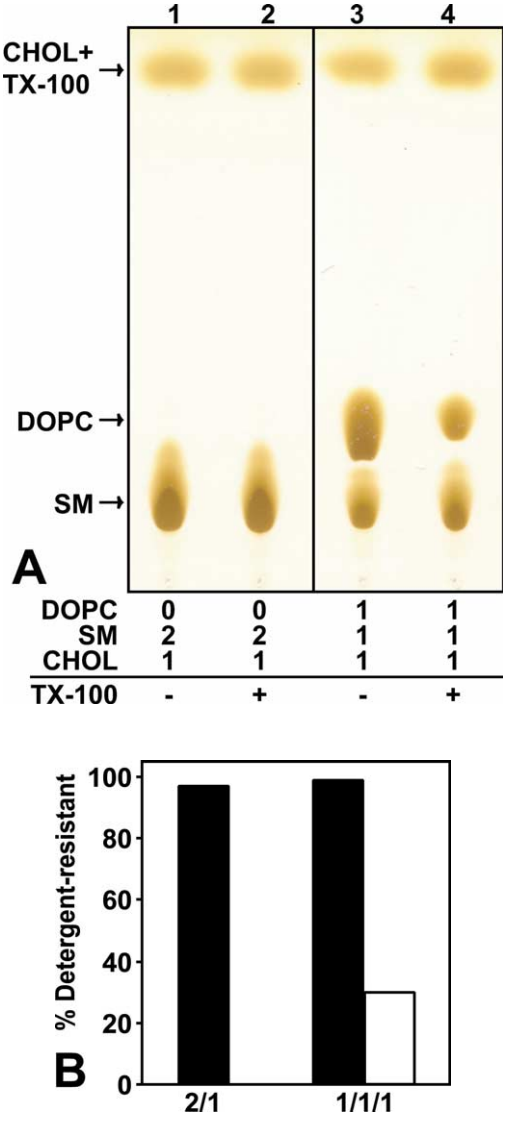

Fig. 1. Detergent resistance of SM and DOPC in different mixtures of DOPC, SM and cholesterol. A: TLC analysis of the pellets after treatment of MLVs with either buffer (−) or 1% TX-100 (+). The MLVs initially contained DOPC, SM and cholesterol (CHOL) at the indicated molar lipid ratios (0/2/1 and 1/1/1). B: Quantification of the detergent resistance of SM (filled bars) and DOPC (open bars) in these MLVs. The detergent resistance is defined as the percentage of the amount of lipid that was pelleted after treatment with TX-100 as compared to the amount of lipid that was pelleted after treatment with buffer.

3.2. Hydrophobic matching of transmembrane peptides

In order to investigate the influence of hydrophobic matching on the partitioning of TMDs of proteins into lipid rafts, WALPs with three different hydrophobic lengths were incorporated into MLVs containing DOPC, SM and cholesterol in a 1/1/1 ratio. The detergent resistances of DOPC and SM appear to be rather similar in the presence of peptides (Fig. 2A) and in the absence of peptides (Fig. 1A, lanes 3,4). The peptides themselves can be easily visualized on TLC (Fig. 2A), well separated from any lipid spots. The shortest peptide, WALP23, has a hydrophobic length that is expected to match well with that of a DOPC bilayer [35]. This peptide (Fig. 2A, lanes 1,2) was mostly dissolved by TX-100 from MLVs containing DOPC, SM and cholesterol in a 1/1/1 ratio (16% remained undissolved, Fig. 2B). This suggests that WALP23 had partitioned into the fluid, DOPC containing bilayer, as expected on the basis of mismatch dependent sorting. However, the longer peptides WALP27 and WALP31 (lanes 3,4 and 5,6, respectively) were also mostly dissolved by TX-100 (14% and 3% remained undissolved, respectively, Fig. 2B). Thus, increasing the hydrophobic length of the WALP peptides and thereby the mismatch with the fluid bilayer does not appear to result in increased DRM association.

The detergent resistance of the different length peptides was also determined in a pure raft-like mixture of SM and cholesterol in a 2/1 molar ratio. For each of the WALP peptides it was found that in this mixture the detergent resistance was much higher than when DOPC was also present (Fig. 2B). A

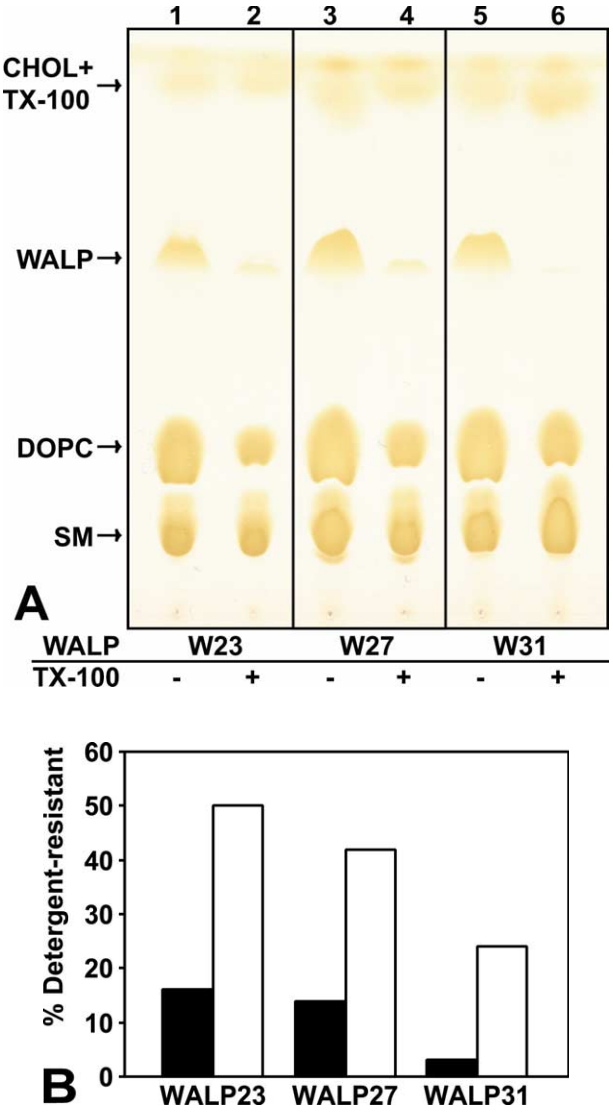

Fig. 2. DRM association of WALP peptides with different hydrophobic lengths. A: TLC analysis of the pellets after treatment of MLVs with either buffer (−) or 1% TX-100 (+). The MLVs initially contained DOPC, SM and cholesterol in 1/1/1 ratio and 2% WALP23 (W23), WALP27 (W27) or WALP31 (W31). The C-terminus of each of these peptides is capped with an ethanolamine group. B: Quantification of the detergent resistance of these peptides in MLVs containing DOPC, SM and CHOL in a 1/1/1 (filled bars) or a 0/2/1 (open bars) ratio.

mixture of SM and cholesterol in a 2/1 molar ratio has been shown to be structurally comparable to DRMs, obtained from a mixture of DOPC, SM and cholesterol in a 1/1/1 ratio by treatment with 1% TX-100 [11]. Therefore, these results suggest that the high degree of solubilization of the peptides from MLVs with DOPC is due to the presence of a fluid, DOPC containing bilayer and hence that all three peptides preferentially partition into the fluid bilayer.

3.3. Palmitoylation of transmembrane peptides

In order to test whether palmitoylation can facilitate the partitioning of TMDs of proteins into lipid rafts, unpalmitoylated WALP23 or WALP23 with one or two palmitate chains, covalently linked to the N-terminus, were incorporated into MLVs with a 1/1/1 ratio of DOPC, SM and cholesterol. Palmitoylation causes an increase in the R_f values of the peptides, reflecting the increase in hydrophobicity (Fig. 3A). The minor peptide spots with smaller R_f values reflect the small amounts of non-palmitoylated peptides generated by the experimental procedure. Fig. 3A (lanes 3,4 and 5,6) shows that the peptides with one or two palmitate chains behave similarly to the unpalmitoylated peptide (lanes 1,2) in that they are mostly dissolved by TX-100. Quantification reveals that increasing the degree of palmitoylation causes a slight, but not significant, decrease in detergent resistance (Fig. 3B). This suggests that palmitoylation of WALP23 is not sufficient to allow the association of this peptide with DRMs.

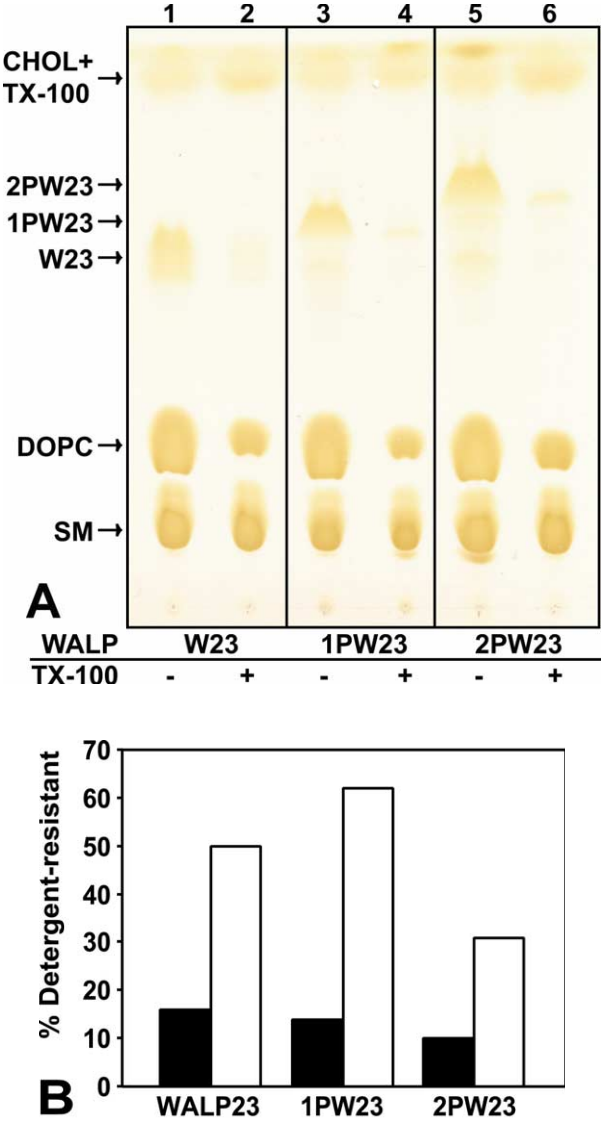

Fig. 3. DRM association of unpalmitoylated WALP23 and WALP23 with one or two palmitate chains. A: TLC analysis of the pellets after treatment of MLVs with either buffer (−) or 1% TX-100 (+). The MLVs initially contained DOPC, SM and cholesterol in 1/1/1 ratio and 2% unpalmitoylated WALP23 (W23) or WALP23 with one (1PW23) or two (2PW23) palmitate chains covalently linked to the N-terminus. The C-terminus of each of these peptides is capped with an amide group. B: Quantification of the detergent resistance of these peptides in MLVs containing DOPC, SM and CHOL in a 1/1/1 (filled bars) or a 0/2/1 (open bars) ratio.

The detergent resistance of the single and double palmitoylated WALP23 peptides was much higher in a pure raft-like mixture of SM and cholesterol in a 2/1 molar ratio (Fig. 3B), as was also observed for the different length analogs, and did not show a clear dependency on degree of palmitoylation. The results indicate that it is the presence of a fluid, DOPC containing bilayer that leads to the solubilization of both the unpalmitoylated and the palmitoylated WALP23 from MLVs containing DOPC, SM and cholesterol.

4. Discussion

4.1. Influence of mismatch on DRM association

Before discussing the effect of mismatch, we must estimate the extent of the mismatch between the hydrophobic length of the peptides and the bilayer thicknesses of the L_o phase domains and the fluid bilayer. An X-ray study has shown that the bilayer thickness of DRMs, isolated from a 1/1/1 mixture of DOPC, SM and cholesterol by treatment with 1% TX-100, amounts to approximately 47 Å between the lipid headgroups. The thickness of the fluid bilayer in this mixture was shown to be 38 Å [11]. From these headgroup–headgroup distances, it can be estimated [40] that the hydrophobic thicknesses of the DRMs and the fluid bilayer amount to approximately 36 Å and 27 Å, respectively.

The optimal bilayer thicknesses for matching the different hydrophobic lengths of the peptides can be estimated from their incorporation efficiencies in PC bilayers with varying thickness [35]. Based on these studies, the hydrophobic length of WALP23 (approximately 25.5 Å) would fit well in the fluid bilayer. The longer WALP27 (approximately 31.5 Å) could be accommodated by both types of bilayer, while the longest peptide, WALP31 (approximately 37.5 Å), is expected to fit better into the DRMs than the fluid bilayer. Therefore, in a 1/1/1 mixture of DOPC, SM and cholesterol, the DRM association of these peptides after treatment with TX-100 could be expected to increase with their length, if hydrophobic matching is indeed an important parameter. However, we found that all three peptides were mostly dissolved by TX-100 from MLVs containing DOPC, SM and cholesterol in a 1/1/1 ratio. In fact, WALP31 was even more dissolved than WALP23 and WALP27. Thus, increasing the hydrophobic length of the WALP peptides does not result in an increase in their DRM association in MLVs containing a fluid bilayer and L_o phase domains.

The most logical explanation for these observations is related to the tight packing of the lipid acyl chains in the L_o phase. This may constitute an energetically unfavorable envi-

ronment for the WALP peptides, impairing the partitioning of the peptides into the L_o phase domains. Consequently, the peptides will partition in the fluid, DOPC containing bilayer and thereby become detergent solubilized together with the DOPC. It is possible that the peptides somewhat facilitate the solubilization of DOPC by TX-100 by inducing local disturbances in the bilayer. The extent of these disturbances could increase with the mismatch between the peptides and the fluid bilayer, thereby increasing the accessibility of the longer peptides to TX-100. This would explain the observed increased solubilization of WALP31 as compared to the other peptides.

In the absence of DOPC, the peptides can to some extent be forced to incorporate into the L_o phase, since the DRM association of the peptides was much higher in MLVs containing only SM and cholesterol in a 2/1 molar ratio. This indicates that the low detergent resistance of the WALPs in the 1/1/1 mixture of DOPC, SM and cholesterol is due to the presence of the fluid, DOPC containing bilayer. However, the peptides were not fully detergent-resistant in the 2/1 mixture of SM and cholesterol, probably because the tight packing of the L_o phase in this mixture still constitutes an unfavorable environment for the peptides and may impair the homogeneous mixing of the peptides with the lipids. This might result in clustering of peptides or the sequestering of peptides to sites in the bilayer where they disturb lipid packing, both increasing their accessibility to TX-100. The extent of clustering or perturbation of packing in peptide rich sites could well be dependent on the type of peptide. This would then explain the dependence of the detergent resistance of the peptides on the type of peptide in a 2/1 mixture of SM and cholesterol.

4.2. Influence of palmitoylation on DRM association

Palmitate chains are saturated and are expected to pack well into L_o phase domains. Therefore, covalently linking palmitate chains to the N-terminus of a WALP peptide can be expected to improve the affinity of the peptides for the L_o phase domains and thereby their DRM association. However, the detergent resistance of the palmitoylated peptides was very similar to that of the unpalmitoylated peptides, both in the presence and the absence of DOPC in the MLVs. It can be concluded that the single or double palmitoylation of the WALP peptide does not result in a significant increase in its DRM association in MLVs containing a fluid, DOPC containing bilayer and L_o phase domains. The most likely explanation for this is that the palmitate chains are unable to sufficiently improve the affinity of the peptides for the L_o phase to overcome the preference of the peptides for partitioning into the fluid bilayer. Alternatively, it is possible that the peptides are located at the interface of the L_o phase domains and the fluid bilayer, possibly with the palmitate chains inserted in the L_o phase, while remaining accessible to TX-100.

4.3. Relevance to biological membranes

The finding that increasing the hydrophobic length of a transmembrane peptide does not increase its association with the DRMs strongly suggests that hydrophobic matching is insufficient to cause integration of membrane proteins with long TMDs into rafts in biological membranes. Palmitoylation of transmembrane peptides also did not lead to enhanced DRM association, which suggests that palmitoylation of transmembrane proteins cannot be a general determinant for association of such proteins with DRMs obtained from biological membranes. This is consistent with the finding that not all transmembrane proteins that are associated with DRMs are palmitoylated. However, palmitoylation appears to play an essential role in the targeting of some proteins into rafts [26,29]. We propose that mechanisms such as hydrophobic mismatch and palmitoylation can only play a role in targeting transmembrane proteins into rafts if certain requirements are met for the insertion of TMDs into the tightly packed lipid environment of rafts. These requirements could be related to both the amino acid composition of the TMD and the exact lipid composition of the raft. Indeed, the amino acid composition of the TMD has been shown to play a role in the DRM association of some proteins [41–43], although a consensus sequence has yet to be found. Furthermore, the composition of rafts in biological membranes will be much more complex than in the model systems studied here and, consequently, packing properties may be different. Moreover, lipids are asymmetrically distributed over both leaflets of the plasma membrane. The significance of this asymmetry is shown by the observation that raft association of influenza virus hemagglutinin is more dependent on the amino acid composition of the TMD at the exoplasmic side than on that at the cytoplasmic side [41]. Although sphingolipids are primarily located in the exoplasmic leaflet [44], rafts probably also exist in the cytoplasmic leaflet. It is possible that such cytoplasmic rafts have packing properties that are less stringent than those of exoplasmic rafts, as is suggested by the sequence dependence of hemagglutinin raft association [41]. Considering that the palmitate chains of palmitoylated proteins usually reside in the cytoplasmic leaflet, palmitoylation might then only increase the affinity of proteins for rafts with moderately tight packing properties.

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