# A Synergistic Effect between Cholesterol and Tryptophan-Flanked Transmembrane Helices Modulates Membrane Curvature†

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ABSTRACT: The aim of this study was to gain insight into the structural consequences of hydrophobic mismatch for membrane proteins in lipid bilayers that contain cholesterol. For this purpose, tryptophan-flanked peptides, designed to mimic transmembrane segments of membrane proteins, were incorporated in model membranes of unsaturated phosphatidylcholine bilayers of varying thickness and containing varying amounts of cholesterol. Analysis of the lipid organization by $^{31}$P NMR and cryo-TEM demonstrated the formation of an isotropic phase, most likely representing a cubic phase, which occurred exclusively in mixtures containing lipids with relatively long acyl chains. Formation of this phase was inhibited by incorporation of lysophosphatidylcholine. These results indicate that the isotropic phase is formed as a consequence of negative hydrophobic mismatch and that its formation is related to a negative membrane curvature. When either peptide or cholesterol was omitted from the mixture, isotropic-phase formation did not occur, not even when the concentrations of these compounds were significantly increased. This suggests that formation of the isotropic phase is the result of a synergistic effect between the peptides and cholesterol. Interestingly, isotropic-phase formation was not observed when the tryptophans in the peptide were replaced by either lysines or histidines. We propose a model for the mechanism of this synergistic effect, in which its dependence on the flanking residues is explained by preferential interactions between cholesterol and tryptophan residues.

There is a growing understanding of the importance of interactions between membrane proteins and their lipid environment for membrane function. A major aspect of such protein−lipid interactions is the hydrophobic-matching principle (*1*). For example, hydrophobic mismatch between the length of the transmembrane segments of integral proteins and the bilayer thickness of the membrane has been shown to affect the activity of a number of different membrane proteins (for reviews, see refs *2*−*4*). On a molecular level, hydrophobic mismatch would lead to the exposure of hydrophobic surfaces to the polar aqueous phase, which is energetically unfavorable. The mechanisms by which such an unfavorable mismatch situation might be alleviated have been studied using model membranes of various bilayer thickness, in which protein transmembrane segments were mimicked by hydrophobic transmembrane peptides of various lengths (*5*−*9*). Insights have been obtained in situations of both positive and negative mismatch, meaning that the peptides were either too long or too short, respectively, to fit in the bilayer. Using mostly phosphatidylcholine (PC)[1] as host lipids, it was found that hydrophobic mismatch can lead to subtle but systematic changes in the membrane organization, such as a modification of the tilt of the transmembrane helices (*6*, *10*) and a partial adjustment of the bilayer thickness (*11*). At high peptide concentrations, negative mismatch can even lead to the formation of nonbilayer phases in PC (*12*, *13*). These studies have improved our understanding of the possible roles of protein−lipid interactions in the transient and local formation of nonbilayer structures, such as those that may occur in vesicular trafficking and in particular in membrane fusion (*14*, *15*).

In model membrane studies, the bilayer thickness is usually varied by using lipids with different acyl chain lengths. However, in mammalian membranes, the bilayer thickness may also be influenced by their cholesterol content (*16*, *17*).

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[1] Abbreviations: PC, phosphatidylcholine; LPC, lysophosphatidylcholine; 20:1$_c$, eicosenoyl; 18:1$_c$, oleoyl; 16:1$_c$, palmitoleoyl; 14:1$_c$, myristoleoyl; WALP, tryptophan-alanine-leucine peptide; KALP, lysine-alanine-leucine peptide; HALP, histidine-alanine-leucine peptide; L/P, lipid/peptide molar ratio; cryo-TEM, cryogenic transmission electron microscopy; NMR, nuclear magnetic resonance.

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The flat rigid ringsystem of cholesterol orders the acyl chains in its vicinity (*18*, *19*), which causes an increase in the hydrophobic thickness of the bilayer (*20*). Besides this, cholesterol affects the properties of a bilayer also in other ways. The stretched conformation of the acyl chains leads to a tight acyl chain packing, which increases the bending rigidity of the membrane (*19*, *21*−*23*). Additionally, because the polar hydroxyl group of cholesterol is much smaller than its hydrophobic part and less hydrated than for instance a phospholipid headgroup, cholesterol has a negative spontaneous curvature (*24*) and its presence can facilitate a negative curvature of the membrane (*25*−*29*). Because cholesterol can influence a membrane in different ways, the effects of cholesterol-induced mismatch may be quite different from the response to mismatch in the absence of cholesterol.

In this study, we have investigated the consequences of hydrophobic mismatch in model membranes of PC and cholesterol, in which both the acyl chain length of the phospholipid and the cholesterol content were varied. As in previous studies, hydrophobic transmembrane peptides were used, consisting of a sequence of alternating leucine and alanine with varying length, flanked on both sides by two tryptophans. These peptides, called WALP peptides, were designed to mimic the transmembrane segments of integral membrane proteins that contain tryptophans as anchoring residues near the interfacial region of the membrane (*12*). They form stable transmembrane α helices, and their interaction with phospholipids has been characterized extensively (*9*). Because cholesterol was suggested to have a preferential interaction with aromatic amino acid residues, such as tryptophans (*30*, *31*), we also compared the effects of the WALP peptides with those of peptides that contain lysines or histidines as flanking residues. Here, we show, using $^{31}$P nuclear magnetic resonance (NMR) and cryogenic transmission electron microscopy (cryo-TEM), that low concentrations of WALP peptides can induce the formation of cubic-phase-like structures in PC in synergism with cholesterol. However, this did not occur if the tryptophan-flanking residues of the peptides were replaced by lysines or histidines. A model is proposed, which explains these results in terms of preferential interactions between tryptophan and cholesterol.

## EXPERIMENTAL PROCEDURES

*Materials.* 1,2-dieicosenoyl-*sn*-glycero-3-phosphocholine (di-20:1$_c$-PC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (di-18:1$_c$-PC), 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphocholine (di-16:1$_c$-PC), 1,2-dimyristoleoyl-*sn*-glycero-3-phosphocholine (di-14:1$_c$-PC), 1-oleoyl-*sn*-glycero-3-phosphocholine (lysoPC), and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Peptides were synthesized, purified, and analyzed by analytical high-performance liquid chromatography and electrospray mass spectrometry as described before (*12*). The amino acid sequences of the peptides used were Ac-GWW(LA)$_8$LWWA-amide (WALP23), Ac-GWW(LA)$_{10}$LWWA-Etn (WALP27), Ac-GKK(LA)$_8$LKKA-amide (KALP23), and GHH(LA)$_8$LHHA-amide (HALP23).

*Sample Preparation.* Lipids were dissolved in chloroform/methanol (1:1, v/v) as 10−20 mM stock solutions. The exact concentrations of the phospholipid stocks were determined by a phosphorus assay according to Rouser et al. (*32*). Peptides were dissolved in trifluoroethanol (TFE, 1−2 mM). The appropriate amount of indicated lipids and peptides were mixed in solution, after which the organic solvents were evaporated under a flow of nitrogen. The resulting films were further dried by overnight storage under high vacuum. The mixed dry lipid films were hydrated with milliQ water, typically 250 μL. For samples containing HALP23, the dry lipid films were hydrated with buffer (25 mM MES and 25 mM HEPES), of which the pH was adjusted to 4.5 using citric acid or 7.4 using NaOH. The samples were vortexed repeatedly at 45 °C. Some samples were very viscous at this temperature and were more easily vortexed at low temperatures. Therefore, all samples were frozen, partially thawed, and vortexed at 0 °C. After the film was dispersed, samples were pelleted in glass tubes (3 cm long, ∅ ∼ 0.5 cm) and freeze-thawed 10 times.

*$^{31}$P NMR Measurements.* $^{31}$P NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer (Bruker Biospin, Karlsruhe, Germany) at 30 or 60 °C where indicated. $^{31}$P NMR measurements were performed at 202.5 MHz, using a single-pulse experiment with a 12.0 μs pulse, 1.2 s relaxation delay time, and broadband proton decoupling. Typically, between 500 and 2000 scans were acquired for samples containing 10 μmol of phospholipids. An exponential multiplication, corresponding to a line broadening of 100 Hz, was applied to the FID prior to Fourier transformation.

*Cryo-TEM.* An aliquot of a 3 μL sample, containing 100−200 nmol of lipid, was pipetted onto a glow discharged Quantifoil 2/2 grid at room temperature in the environmental chamber of a Vitrobot with a relative humidity of 100%. The sample was blotted with filter paper during 0.5 s and rapidly plunged into liquid ethane. The grid was kept at cryogenic temperatures and was transferred to a Gatan cryoholder Model 626. The transmission electron microscope used was a Philips Tecnai12 equipped with a Biotwin-lens. Images were captured with a Megaview *II* CCD-camera in Low Dose microscope mode and processed with AnalySIS software.

## RESULTS

*Synergism between Cholesterol and WALP Peptides.* Aqueous dispersions of di-18:1$_c$-PC spontaneously form multilamellar vesicles. The $^{31}$P NMR spectrum of such a dispersion (Figure 1A) displays an anisotropic pattern with a low-field shoulder and a high-field peak, which is typical for a planar bilayer organization (*33*, *34*). The incorporation of the transmembrane peptide WALP23, which fits well in bilayers of di-18:1$_c$-PC (*35*), does not affect this phospholipid organization at a phospholipid/peptide (L/P) molar ratio of 50:1 (Figure 1B) and neither does mixing di-18:1$_c$-PC with cholesterol at a 2:1 molar ratio (Figure 1C). However, a dramatic spectral change occurs when both cholesterol and WALP23 are present in these amounts. The $^{31}$P NMR spectrum of the di-18:1$_c$-PC/cholesterol/WALP23 mixture with a molar ratio of 50:25:1 is dominated by an isotropic peak, and the intensity of the bilayer pattern is greatly reduced (Figure 1D). This cannot simply be due to the additive effects of both compounds on the membrane organization, because the formation of the isotropic signal does not occur in binary mixtures when the amount of WALP23 in di-18:1$_c$-PC is doubled or when the content of

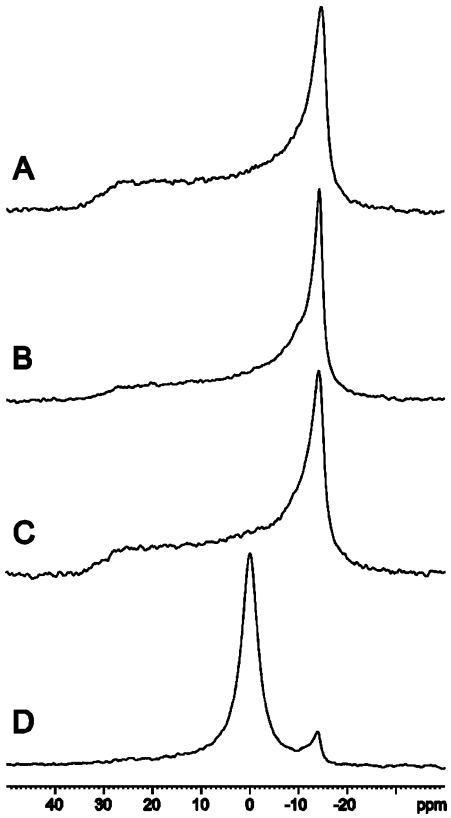


FIGURE 1: $^{31}$P NMR spectra of aqueous dispersions of di-18:1$_c$-PC, with or without WALP23 and/or cholesterol, measured at 30 °C. (A) di-18:1$_c$-PC, (B) di-18:1$_c$-PC/WALP23 (molar ratio of 50:1), (C) di-18:1$_c$-PC/cholesterol (molar ratio of 2:1), (D) di-18:1$_c$-PC/cholesterol/WALP23 (molar ratio of 50:25:1).

cholesterol is increased up to a di-18:1$_c$-PC/cholesterol molar ratio of 11:9 (data not shown). Therefore, we conclude that the formation of the isotropic phase in the di-18:1$_c$-PC/cholesterol/WALP23 mixture with a molar ratio of 50:25:1 must be the result of a synergistic effect of WALP23 and cholesterol.

When the concentration of either WALP23 or cholesterol in this ternary mixture is reduced, resulting in di-18:1$_c$-PC/cholesterol/WALP23 molar ratios of 50:25:0.5 or 50:17:1, respectively, the spectrum completely changes back to the anisotropic bilayer pattern (data not shown). Apparently, the di-18:1$_c$-PC/cholesterol/WALP23 mixture with a molar ratio of 50:25:1 is near the boundary composition(s) at which the synergistic effect of cholesterol and WALP23 takes place. Increasing the concentration of either WALP23 or cholesterol in the ternary mixture up to di-18:1$_c$-PC/cholesterol/WALP23 molar ratios of 50:25:2 or 50:41:1, respectively, does not lead to any significant changes in the $^{31}$P NMR spectrum (data not shown), suggesting that the formation of the isotropic phase because of the synergistic effect of WALP23 and cholesterol occurs over a broad range of compositions.

*Identity of the Isotropic Phase.* The presence of an isotropic signal in the $^{31}$P NMR spectrum implies a motional averaging of the chemical-shift anisotropy, which usually indicates a change in the morphology of the lipid membrane. To identify the morphology of the isotropic phase, cryo-TEM was performed on the di-18:1$_c$-PC/cholesterol/WALP23 50:25:1 mixture. Various cryo-TEM images showed that this mixture contains lamellar structures, as well as patches with a highly regular structure. A particularly large patch displays

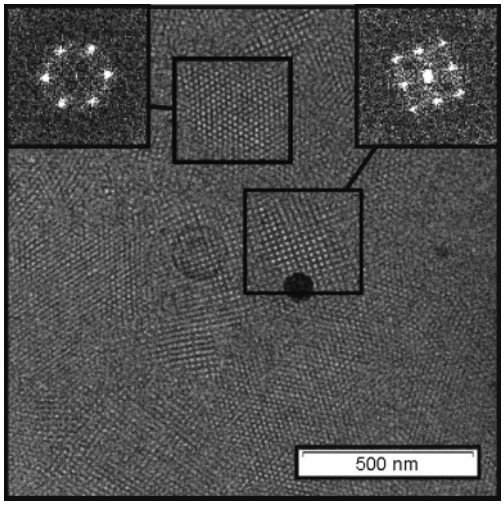


FIGURE 2: Cryo-TEM image of di-18:1$_c$-PC/cholesterol/WALP23 (molar ratio of 50:25:1), showing a large patch of the cubic phase and two sections to which Fourier transformation was applied (insets).

both cubic and hexagonal patterns with a periodicity of 18.2 ± 0.5 and 17.9 ± 0.5 nm, respectively (Figure 2). Such regular patterns were proposed to correspond to cubic-phase-like structures, such as aggregated interlamellar attachment sites (ILAs) (*36*, *37*), and were found persistently in only those samples that displayed an isotropic signal in their $^{31}$P NMR spectrum (data not shown). Singular ILAs and ILAs in less densily packed arrays were also observed in these samples (data not shown). A common feature of such structures is a high membrane curvature. The formation of these structures is consistent with the formation of an isotropic $^{31}$P NMR signal, because lateral diffusion of the lipids in highly curved membranes allows for a rapid reorientation of the lipids in the magnetic field, which leads to the motional narrowing of the chemical-shift anisotropy of the phosphorus nucleus in the lipid headgroup (*34*).

*Hydrophobic Mismatch.* Although WALP23 fits well in bilayers of di-18:1$_c$-PC, the addition of cholesterol may cause negative hydrophobic mismatch because of its bilayer-thickening effect. To test whether the synergistic effect of WALP23 and cholesterol in di-18:1$_c$-PC is related to hydrophobic mismatch, the acyl chain length of the PC and the hydrophobic length of the peptide were varied. The molar ratio of PC/cholesterol/WALP was kept constant at 50:25:1.

When the bilayer thickness is increased by replacing di-18:1$_c$-PC in this mixture by di-20:1$_c$-PC, the $^{31}$P NMR spectrum does not show any significant change (parts A and B of Figure 3). In contrast, shortening the acyl chain length by using di-16:1$_c$-PC reduces the hydrophobic mismatch and results in a clear spectral change: the isotropic signal is broadened and its intensity is reduced (Figure 3C). The isotropic signal disappears completely if the acyl chain is shortened even further by using di-14:1$_c$-PC (Figure 3D). These results suggest that hydrophobic mismatch indeed plays a role in the synergistic effect. This is further demonstrated by the observation that a pure bilayer pattern occurs in the $^{31}$P NMR spectrum of the di-18:1$_c$-PC-containing mixture, when the mismatch is reduced by replacing WALP23 with WALP27 (Figure 3E).

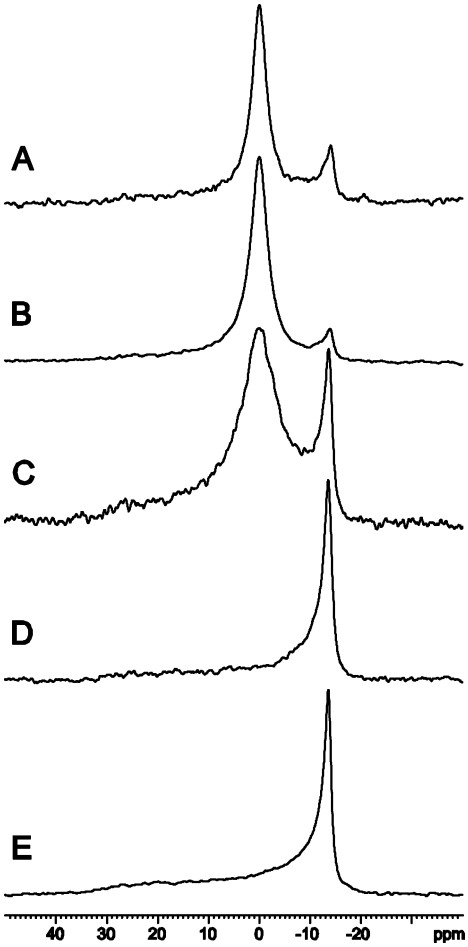


FIGURE 3: $^{31}$P NMR spectra of aqueous dispersions of PC/cholesterol/WALP (molar ratio of 50:25:1) mixtures, containing PC species with unsaturated acyl chains of various length and either WALP23 or the longer peptide WALP27, measured at 30 °C. (A) di-20:1$_c$-PC/cholesterol/WALP23, (B) di-18:1$_c$-PC/cholesterol/WALP23, (C) di-16:1$_c$-PC/cholesterol/WALP23, (D) di-14:1$_c$-PC/cholesterol/WALP23, and (E) di-18:1$_c$-PC/cholesterol/WALP27.

*Negative Curvature.* WALP peptides are known to be able to cause the formation of an isotropic phase in PC in mismatch situations in the absence of cholesterol (*12*, *13*, *38*). However, this requires high peptide concentrations (L/P, 10:1), whereas isotropic-phase formation in the presence of cholesterol is already observed at much lower peptide concentrations (L/P, 50:1).

Therefore, apart from increasing the bilayer thickness, cholesterol must influence the membrane in some additional way. Because the formation of cubic-phase-like structures usually occurs in membranes with a net negative spontaneous curvature, the ability of cholesterol to facilitate a negative curvature of the membrane (*24*−*29*) may play a role. This was tested by observing the effects of introducing lipid molecules with positive spontaneous curvature to the membranes in the di-18:1$_c$-PC/cholesterol/WALP23 50:25:1 mixture (Figure 4A). When 10% of the di-18:1$_c$-PC is replaced with lysoPC (LPC), the $^{31}$P NMR spectrum does not display any isotropic signal (Figure 4B), suggesting that negative curvature indeed is important for the formation of the isotropic phase. The appearance of the additional small peak at 9.7 ppm can be attributed to LPC itself, which has a smaller chemical-shift anisotropy than di-18:1$_c$-PC when present in a lipid bilayer, because of an increased motional freedom of the lipid headgroup (*39*).

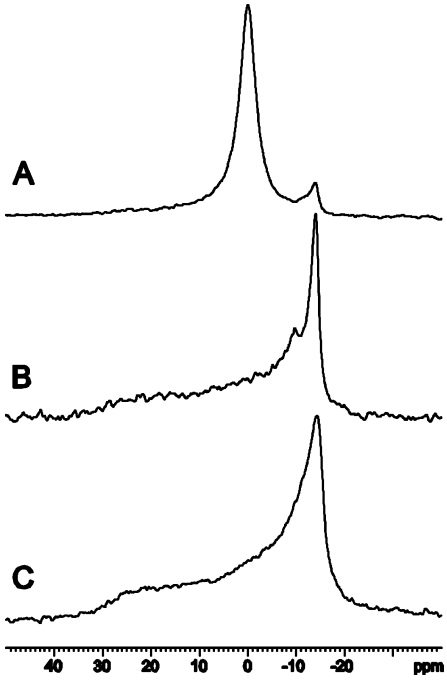


FIGURE 4: $^{31}$P NMR spectra of aqueous dispersions of di-18:1$_c$-PC/cholesterol/WALP23 50:25:1 (A), di-18:1$_c$-PC/18:1$_c$-PC/cholesterol/WALP23 45:5:25:1 (B), and di-18:1$_c$-PC/cholesterol/KALP23 50:25:1 (C).

*Flanking Residues.* Next, the question arose whether the synergistic effect is a general feature of transmembrane helices in cholesterol-containing bilayers. Lysines, like tryptophans, are frequently found as anchoring residues (*12*). Therefore, the effect of the tryptophan-flanked peptide WALP23 was first compared with that of its lysine-flanked counterpart (KALP23). In contrast to the $^{31}$P NMR spectrum of the di-18:1$_c$-PC/cholesterol/WALP23 50:25:1 mixture (Figure 4A), no isotropic signal is present in the spectrum when KALP23 is incorporated instead of WALP23 (Figure 4C). This is unlikely to be due to a difference in the effective hydrophobic length of these peptides (*12*), because no isotropic signal is observed when the mismatch is varied by incorporating KALP23 in mixtures containing cholesterol and either di-16:1$_c$-PC or di-20:1$_c$-PC (data not shown).

In principle, it is possible that KALP23 is less efficient than WALP23 in its synergism with cholesterol and hence that the boundary composition at which the synergistic effect occurs is shifted to a higher peptide concentration for KALP23 as compared to WALP23. However, even when the concentration of KALP23 is doubled, the $^{31}$P NMR spectrum of this mixture still displays only the anisotropic bilayer pattern (data not shown).

Additionally, it is possible that a higher temperature is required for the formation of the isotropic phase in the presence of KALP23 as compared to that with WALP23. However, at 60 °C, the $^{31}$P NMR spectra of KALP23-containing samples still display the anisotropic bilayer pattern, even when the mismatch is increased by incorporating KALP23 in cholesterol-containing di-20:1$_c$-PC and when a double concentration of KALP23 is used (data not shown). Therefore, we conclude that the synergistic effect of cholesterol and peptide is dependent on the flanking residues

of the peptide and is not a general feature of transmembrane helices.

Which of the differences in the properties of tryptophans with respect to lysines may cause the difference in the behavior between WALP23 and KALP23? Tryptophan residues are bulkier and more hydrophobic than lysine residues, which are more flexible and positively charged. The influence of such properties on the synergistic effect was studied by using a peptide, flanked by histidine residues (HALP23), which are also bulkier and more hydrophobic than lysine residues but are positively charged at lower pH. When HALP23 is incorporated in cholesterol-containing di-18:1$_c$-PC, the $^{31}$P NMR spectrum of this mixture displays only the anisotropic bilayer pattern, either at pH 7.4 or 4.5 (data not shown). Therefore, the synergistic effect between WALP23 and cholesterol is probably not directly caused by the bulkiness and hydrophobicity of the tryptophans nor is the effect abolished because of the mere presence of a positive charge on the lysines in the case of KALP23.

## DISCUSSION

This study shows that the structural organization of a membrane can be altered by the combined presence of both cholesterol and transmembrane helices. In particular, it was shown that cholesterol and WALP23 have a synergistic effect on PC bilayers, which results in the formation of cubic-phase-like structures. Because both hydrophobic mismatch and membrane curvature appear to play a role in this synergistic effect, we will first briefly consider these two aspects separately. Next, we will propose a mechanism for the synergism between cholesterol and WALP23, which may also explain why the synergistic effect was not observed with its lysine-flanked counterpart KALP23 or with the histidine-flanked HALP23. Finally, the implications of the synergistic effect for natural membranes will be discussed.

*Hydrophobic Mismatch and Curvature*. A synergistic effect was observed between WALP23 and cholesterol in di-18:1-PC, causing isotropic-phase formation. The formation of this phase is inhibited by either increasing the hydrophobic length of the peptide or by shortening the acyl chain length of the phospholipids, demonstrating that the synergistic effect is related to the negative hydrophobic mismatch. Although WALP23 fits well in bilayers of pure di-18:1$_c$-PC (*35*), cholesterol can increase the bilayer thickness by approximately 4 Å (*40*, *41*) and therefore the addition of cholesterol may cause negative hydrophobic mismatch between the hydrophobic length of the peptide and the thickness of the bilayer. However, the peptide concentrations used in this study (L/P, 50:1) are insufficient to drive the formation of a cubic phase in response to negative mismatch in the absence of cholesterol (data not shown). Because cubic-phase formation is facilitated by negative membrane curvature (*14*, *36*, *42*, *43*), it is likely that the effect of cholesterol on membrane curvature plays an additional role in its synergism with WALP23. Cholesterol has a spontaneous radius of curvature of −23 Å (*24*) and therefore would preferentially reside in lipid structures with a negative curvature (Figure 5A). The fact that negative membrane curvature is related to the synergistic effect is supported by the observation that isotropic-phase formation is inhibited by the addition of LPC, which has a positive spontaneous radius of curvature of +38 Å (*44*) (Figure 5B).

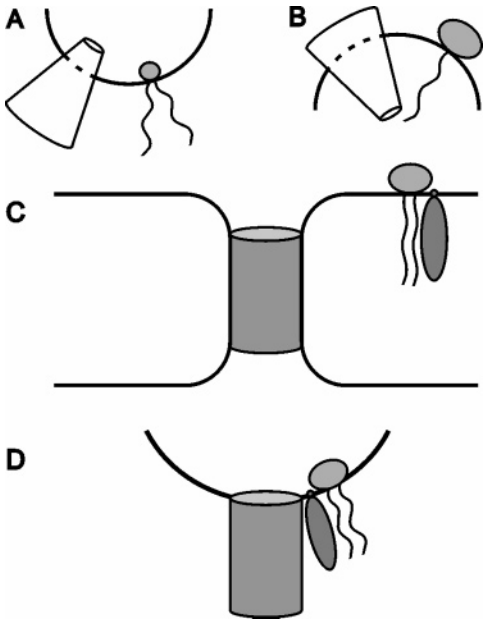


FIGURE 5: Schematic representation of negative membrane curvature (A), positive membrane curvature (B), local positive membrane curvature because of negative hydrophobic mismatch between the hydrophobic length of a transmembrane helix and the hydrophobic thickness of a bilayer (C), and membrane curvature as proposed to be caused by the synergistic effect of cholesterol and WALP23 (D). Phospholipid shapes suited for incorporation in these membrane organizations are indicated. Because of its intrinsic negative curvature, cholesterol fits well in negatively curved membranes (A) but not in positively curved membranes (B) or in the local positive curvature around mismatching peptides (C), which is proposed to cause membrane curvature if cholesterol is attracted close to the mismatching peptides.

*Mechanism of the Synergistic Effect.* The synergistic effect between WALP23 and cholesterol is not merely due to an additive effect of curvature and mismatching peptides. This can be deduced from the observation that KALP23 and HALP23, similar to WALP23, are able to induce isotropic-phase formation in response to negative mismatch at high peptide concentrations in the absence of cholesterol (*38*), whereas they are unable to induce the formation of this phase in synergism with cholesterol. To explain the dependence of the synergistic effect on the flanking residues, the effects of negative mismatch and curvature must be considered on a molecular level. At low peptide concentrations, an unfavorable mismatch situation may be alleviated by a deformation of the membrane that would adjust the bilayer thickness in the direct vicinity of the peptides but would leave the rest of the membrane largely unaffected. Such membrane deformations, also called dimples, were proposed to occur under negative mismatch conditions in the presence of gramicidin channels and were found to be related to the stability of these channels (*45*−*48*). In analogy to gramicidin-channel-induced dimple formation, negative mismatch would result in a ring of lipids adopting a positive membrane curvature directly around the peptides (Figure 5C), for WALP23 as well as for KALP23 and HALP23. The formation of such positive curvature would be inhibited in the presence of cholesterol, because of its intrinsic negative curvature (*24*). Hence, the presence of cholesterol may destabilize the phospholipid bilayer under conditions of negative mismatch, while at the same time, the intrinsic curvature of cholesterol can make the formation of cubic-phase-like structures energetically

more favorable (*26*, *27*). The opposite effect would be expected for LPC, consistent with our results. Why then do we observe the isotropic-phase formation for WALP23 but not for KALP23 or HALP23? The most simple explanation for the synergistic effect of cholesterol and WALP23 would be a direct and preferential interaction between these two compounds, which would attract cholesterol to the WALP23 peptide and into the area of positive membrane curvature. The resulting bilayer destabilization, together with the preference of cholesterol to reside in lipid structures with a negative curvature, may then be sufficient as a driving force for isotropic-phase formation in the presence of WALP23 (Figure 5D). In the case of KALP23 and HALP23, isotropic-phase formation may not occur, because cholesterol can remain freely dispersed in the bilayer under negative mismatch conditions, causing less destabilization of the bilayer (Figure 5C). Such a specific interaction between cholesterol and WALP23 could be due to the preferential position of the rigid ring system of tryptophan near the lipid carbonyl region, which may be close to the position of the cholesterol hydroxyl group and the most proximal ring in its ringsystem (*19*, *49*). Indeed, a preferential interaction has been suggested to occur between cholesterol and the tryptophan-anchored gramicidin (*30*), and a special role has been suggested for tryptophans in the interaction of proteins with cholesterol (*31*).

*Natural Membranes.* The question arises what the consequences of the synergistic effect of cholesterol and tryptophan-flanked transmembrane helices would be in natural membranes. Tryptophans are often found in natural transmembrane proteins as membrane-anchoring residues. The results in this study would suggest that the presence of such proteins in cholesterol-containing membranes is particularly unfavorable under negative mismatch conditions and may lead to curved membrane structures. We speculate that such a mechanism for the formation of curved membranes may be involved in various fusion and fission processes (*50*) and even in the formation of cubic phases, which has been suggested to occur in various cell organelles (*51*).

We also propose that the interplay between the intrinsic curvature of cholesterol and mismatching transmembrane helices may play a role in the protein secretion pathway. The cholesterol content of the membranes and the hydrophobic length of the transmembrane segments of integral membrane proteins increase along the secretory pathway (*16*, *17*). Newly synthesized proteins are all initially incorporated in the thin membrane of the endoplasmic reticulum. In the case of integral proteins that are destined for thicker membranes and thus have relatively long transmembrane segments, this would result in positive mismatch, which may lead to a local negative curvature around the proteins, in analogy to the local positive curvature because of negative mismatch. The presence of cholesterol around long transmembrane segments may stabilize such local negative curvature because of its intrinsic negative curvature, as well as partially alleviate the mismatch because of its bilayer thickening effect. Thus, positive mismatch, in particular for proteins containing tryptophans as anchoring residues, may promote the sequestering of the small amounts of cholesterol present in the endoplasmic reticulum. Such a mechanism would not only facilitate the insertion of long transmembrane helices in the endoplasmic reticulum membrane but would also lead to the cosorting of cholesterol and long transmembrane helices, enabling their collective transport along the secretory pathway.

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