Hydrophobic Mismatch and the Incorporation of Peptides into Lipid Bilayers: A Possible Mechanism for Retention in the Golgi[†]

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ABSTRACT: Preferential interaction of trans-membrane α-helices whose hydrophobic length matches the hydrophobic thickness of the lipid bilayer could be a mechanism of retention in the Golgi apparatus. We have used fluorescence methods to study the interaction of peptides Ac-K_2-G-L_m-W-L_n-K_2-A-amide (P_{m+n}) with bilayers of phosphatidylcholines with chain lengths between C14 and C24. The peptide P_{22} ($m = 10$, $n = 12$) incorporates into all bilayers, but P_{16} ($m = 7$, $n = 9$) does not incorporate into bilayers when the fatty acyl chain length is C24 and only partly incorporates into bilayers where the chain length is C22. The strongest binding is seen when the hydrophobic length of the peptide matches the calculated hydrophobic thickness of the bilayer. It is suggested that a too-thin bilayer can match to a too-long peptide both by stretching of the lipid and by tilting of the peptide. However, a too-thick bilayer can only match a too-thin peptide by compression of the lipid, which becomes energetically unfavorable when the difference between the bilayer thickness and the peptide length exceeds about 10 Å. The presence of cholesterol in the bilayer leads to a marked reduction in the incorporation of P_{16} into bilayers where the chain length is C18. Hydrophobic mismatch could explain retention of proteins with short trans-membrane α-helical domains in the Golgi, the effect following largely from the low concentration of cholesterol in the Golgi membrane compared to that in the plasma membrane.

In the bulk flow model, the majority of proteins synthesized in the endoplasmic reticulum (ER) are believed to leave the ER by default and flow along the exocytic pathway until they reach the plasma membrane (*1*). Some proteins, however, have to be retained at particular points along the exocytic pathway. Compartmental localization is achieved either with a retention signal in the protein, which, at the appropriate point in the exocytic pathway, prevents forward movement of the protein by denying it access to budding transport vesicles of the onward pathway, or with a retrieval signal, leading to recapture of the protein after it has left the compartment in which it resides. The classic retrieval signal is the KDEL sequence found in many ER resident proteins; the nature of the retention signal is much less clear (*2*).

A clear example of localization is provided by the Golgi apparatus which, despite an extensive flux of proteins, maintains its own distinctive population of resident proteins. There is also evidence for localization of proteins within specific regions of the Golgi apparatus itself; the distributions of glycosyltransferases and glycosidases for example, although overlapping, are distinct (*3, 4*). Many of the proteins in the Golgi membrane are predicted to be type II membrane proteins, with a single trans-membrane α-helix, oriented with the N- and C-termini on the cytoplasmic and lumenal faces of the membrane, respectively. In some cases the Golgi retention signal has been shown to involve the membrane-spanning domain even though the membrane-spanning domains show no sequence homology and it has not been possible to identify any particular motif leading to retention (*5*−*10*). Thus sialyltransferase remains localized in the Golgi even when its 17 amino acid trans-membrane domain is replaced by 17 Leu residues (*7*). However, a longer stretch of 23 Leu residues did not provide an efficient retention signal (*7*). Similarly, a 4-residue insertion into the trans-membrane domain of galactosyltransferase reduced its retention in the Golgi (*11*). The reverse effect has been shown with the influenza virus neuraminidase which shifted from the plasma membrane to the Golgi and the ER when the number of residues in the trans-membrane domain was reduced (*12*).

The lack of a clear retention motif, together with the inability to saturate the mechanism for Golgi retention by overexpression, suggests that retention is not a receptor-mediated event (*1*). One possible model is retention by preferential interaction with membranes of optimal thickness (*1*). Both Bretscher and Munro (*10*) and Masibay et al. (*11*) have shown that trans-membrane domains of Golgi proteins are shorter (average 15 residues) than trans-membrane domains of plasma membrane proteins (average 20 residues). It has therefore been suggested that, if membrane proteins with short trans-membrane domains interact better with thin lipid bilayers and if the lipid bilayer in the Golgi is thinner than that in the plasma membrane, then this could be the mechanism of retention in the Golgi (*10*, *11*). Lipid composition has, in fact, been shown to vary throughout the Golgi, with the *cis*-cisternae having a composition more like that of the ER and the *trans*-cisternae being more like the

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plasma membrane. In particular, the concentrations of cholesterol and sphingolipids (sphingomyelin and glycolipids) are low in the *cis*-cisternae and high in the *trans*-cisternae (*13*, *14*). Since sphingolipids and cholesterol tend to produce a thicker bilayer (*10*, *15*, *16*), this would imply that the bilayer thickens on moving from the *cis*- to the *trans*-cisternae, consistent with a retention mechanism based on bilayer thickness (*10*, *11*). Retention of membrane proteins in the ER could also depend on the length of the transmembrane domain of the protein. For example, cytochrome b_5 has a trans-membrane domain containing just 17 hydrophobic amino acid residues (*17*). If the length of the hydrophobic domain is increased to 22 residues, the protein is transported out of the ER along the secretory pathway (*17*).

The idea that, for optimal interaction, the length of a hydrophobic trans-membrane α-helix has to match the thickness of the hydrophobic core of a lipid bilayer is an attractive one, since the energetic cost of exposing either fatty acyl chains or hydrophobic amino acids to water is high (*18*). Theoretical calculations suggest the essential correctness of the idea, but all treat the peptide as a rigid structure, fixed within the bilayer (*19*−*21*). Here we introduce an experimental approach to study the effects of a mismatch between the peptide and the lipid bilayers. We use peptides of the type Ac-K_2-G-L_m-W-L_n-K_2-A-amide (P_{m+n}), consisting of a long sequence of hydrophobic Leu residues capped at both the N- and C-terminal ends with two polar lysine residues with a centrally located tryptophan residue to act as a fluorescence reporter group. It has been shown that peptides of this type (without the tryptophan residue), when mixed with phospholipids, form stable α-helices spanning the phospholipid bilayer, the ends of the helix being anchored in the phospholipid headgroup region by the charged Lys caps (*16*, *22*). A fluorescence quenching method is used to measure the strength of the interaction between the peptides and phosphatidylcholines as a function of fatty acyl chain length, in bilayers in the fluid, liquid crystalline phase (*23*). The peptide is incorporated into bilayers containing the brominated phospholipid dibromostearoylphosphatidylcholine (di(Br_2C18:0)PC);[1] di(Br_2C18:0)PC behaves much like a conventional phospholipid with unsaturated fatty acyl chains because the bulky bromine atoms have effects on lipid packing that are similar to those of a *cis* double bond (*23*). In mixtures of brominated and nonbrominated phospholipids, the degree of quenching of the fluorescence of the tryptophan residue in the peptide is related to the fraction of the surrounding phospholipids which are brominated, and thus to the strength of binding of the nonbrominated lipid to the peptide.

MATERIALS AND METHODS

Materials and General Procedures. Dimyristoleoylphosphatidylcholine (di(C14:1)PC), dipalmitoleoylphosphatidylcholine (di(C16:1)PC), dioleoylphosphatidylcholine (di(C18:1)PC), dieicosenoylphosphatidylcholine (di(C20:1)PC), dierucoylphosphatidylcholine (di(C22:1)PC), and dinervonylphosphatidylcholine (di(C24:1)PC) were obtained from Avanti Polar Lipids, and [^{3}H]dipalmitoylphosphatidylcholine was obtained from New England Nuclear. Di(C18:1)PC was brominated to give di(Br_2C18:0)PC as described in East and Lee (*23*). Peptides $KKGL_7WL_9KKA$ (P_{16}), $KKGL_{10}WL_{12}KKA$ (P_{22}), and $KKGL_6WL_{18}KKA$ ($P_{6,18}$) were synthesized using *t*-Boc chemistry (*24*), and purity was confirmed using electrospray and MALDI-TOF (MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight) mass spectroscopy.

Peptides (20 nmol) were incorporated into phospholipid bilayers by mixing peptide, lipid, and cholesterol in the desired molar ratios in chloroform/methanol (2:1, v/v). Solvent was removed under vacuum, and the mixture was resuspended in a 400-μL buffer (10 mM Hepes, 15% Sucrose, pH 8.0) by sonication in a bath sonicator for 5−10 min. Aliquots (100 μL) were diluted into buffer (2.5 mL; 20 mM Hepes, pH 7.2, 5 mM $MgSO_4$, 100 mM KCl), and fluorescence intensities were recorded at 25 °C using an SLM-Aminco 8000C fluorimeter, with excitation and emission wavelengths of 280 and 340 nm, respectively.

Analysis of Fluorescence Quenching. Fluorescence quenching in mixtures of brominated and nonbrominated phospholipids was fitted to the equation

$$F/F_0 = F_{min} + (F_0 - F_{min})(1 - f_{Br})^n \qquad (1)$$

where F_0 and F_{min} are the fluorescence intensities for the peptide in di(C18:1)PC and in brominated phospholipid, respectively, F is the fluorescence intensity in the phospholipid mixture when the mole fraction of brominated lipid is x_{Br} and the fraction of sites at the lipid−peptide interface occupied by brominated lipid is f_{Br}, and n represents the number of lipid sites making contact with the tryptophan residue (*23*). The fraction of sites occupied by brominated lipid is related to x_{Br} by,

$$f_{Br} = x_{Br}/(x_{Br} + K[1 - x_{Br}]) \qquad (2)$$

where K is the relative binding constant of the brominated phospholipid with respect to the nonbrominated phospholipid (*25*).

Analysis of Peptide Incorporation. Gel filtration and gradient centrifugation were used to determine whether or not peptide was incorporated into liposomes. Peptide (80 nmol) was mixed with phospholipid (8 μmol) containing 0.001% [^{3}H]dipalmitoylphosphatidylcholine and reconstituted as described above. Samples were resolved by passing down a Sephadex G-50 column (5 mL) equilibrated with buffer (10 mM Hepes, 15% sucrose, pH 8.5). Fractions (0.5 mL) were collected and analyzed for lipids by liquid scintillation counting, and for peptides by addition of 0.1 mM *o*-phthaldialdehyde in the presence of 20 mM β-mercaptoethanol; after incubation for 10 min, fluorescence was measured using an SLT Fluostar plate-reading fluorimeter, with excitation and emission wavelengths of 355 and 405 nm, respectively.

Other samples were resolved by centrifugation on a linear 5−40% (w/v) sucrose density gradient containing 20%

[1] Abbreviations: di(C14:1)PC, dimyristoleoylphosphatidylcholine; di(C16:1)PC, dipalmitoleoylphosphatidylcholine; di(C18:1)PC, dioleoylphosphatidylcholine; di(C20:1)PC, dieicosenoylphosphatidylcholine; di(C22:1)PC, dierucoylphosphatidylcholine; di(C24:1)PC, dinervonylphosphatidylcholine; di(Br_2C18:0)PC, dibromostearoylphosphatidylcholine; P_{16}, $KKGL_7WL_9KKA$; P_{22}, $KKGL_{10}WL_{12}KKA$; $P_{6,18}$, $KKGL_6WL_{18}KKA$.

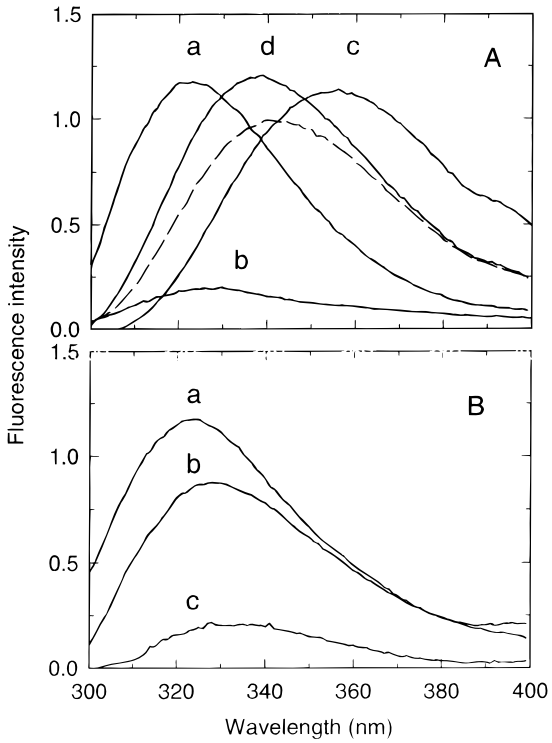

FIGURE 1: Fluorescence emission spectra. (A) The solid lines show fluorescence emission spectra for (a) P_{16} in di(C18:1)PC at a molar ratio of peptide:lipid of 1:100; (b) P_{16} in buffer (20 mM Hepes, pH 7.2, 5 mM Mg, 100 mM KCl); (c) tryptophan in buffer; (d) $P_{6,18}$ in di(C18:1)PC at a molar ratio of peptide:lipid of 1:100. The dotted line shows the emission spectrum of P_{16} in methanol. (B) Fluorescence emission spectra for P_{22} in di(C24:1)PC (a) and P_{16} in di(C22:1)PC (b) or di(C24:1)PC (c). For all spectra the concentration of peptide or tryptophan was 0.02 μM.

glycerol, 40 mM Hepes, pH 7.0, 5 mM $MgSO_4$, 100 mM KCl, and 0.1 mM EGTA at 4 °C for 18 h at 100000*g*. One-milliliter fractions were collected and analyzed for lipids and peptides by liquid scintillation counting and bicinchoninic acid assay, respectively.

Circular Dichroism (CD). For CD spectra, samples containing 22 μM peptide and 2.2 mM lipid were sonicated in a bath sonicator for 15 min until the sample appeared optically clear. CD measurements were carried out using a JASCO J-600 spectropolarimeter, using a 0.1 mm path length cell, a 1-nm bandwidth, a 500-nm slit width, a 0.05-nm resolution, a 4-s response time, and a 50 nm/min scan speed.

RESULTS

Fluorescence Properties of the Peptides. We synthesized two peptides, $KKGL_7WL_9KKA$ (P_{16}) and $KKGL_{10}WL_{12}KKA$ (P_{22}), containing a tryptophan residue in the center of the hydrophobic domain and one peptide, $KKGL_6WL_{18}KKA$ ($P_{6,18}$), in which the tryptophan residue was asymmetrically located. Peptide was incorporated into lipid bilayers by mixing peptide and lipid in organic solvent, removing the solvent, and hydrating the mixture. The fluorescence emission spectrum of the tryptophan residue is environmentally sensitive, the emission maximum moving to a shorter wavelength with decreasing environmental polarity (*26*). The emission spectrum of P_{16} incorporated into bilayers of di(C18:1)PC is centered at 323 nm (Figure 1), indicating a very hydrophobic environment for the tryptophan, consistent with the expected localization in the middle of the bilayer. In contrast, for the peptide $P_{6,18}$ with the asymmetrically located tryptophan residue, the spectrum indicates a less hydrophobic environment, suggesting a location for the tryptophan nearer to the lipid−water interface (Figure 1). The spectra for all the peptides in methanol solution were similar to the spectrum shown for P_{16} in Figure 1.

Table 1: Relative Phospholipid Binding Constants for Peptides P_{16} and P_{22}

lipid	bilayer thickness (Å)[a]	emission max (nm)		rel binding constant[b]	
		P_{16}[c]	P_{22}[d]	P_{16}[c]	P_{22}[d]
di(C14:1)PC	22.8	328	330	0.4	0.9
di(C16:1)PC	26.3	325	328	0.8	0.7
di(C18:1)PC	29.8	323	325	1.0	1.0
di(C20:1)PC	33.3	322	323	0.7	1.8
di(C22:1)PC	36.8		323		2.0
di(C24:1)PC	40.3		323		1.5

[a] Bilayer hydrophobic thickness d calculated from $d = 1.75(n - 1)$, where n is the number of carbon atoms in the fatty acyl chain (*38, 39*). [b] Binding constant relative to that for di(C18:1)PC calculated from eqs 1 and 2 with $n = 2.7$ for P_{16} and $n = 2.3$ for P_{22}. [c] Estimated hydrophobic length 27 Å. [d] Estimated hydrophobic length 36 Å.

The fluorescence intensity recorded for P_{16} in water is very low compared to that for an equivalent concentration of tryptophan in water and is shifted to lower wavelengths (Figure 1). These very hydrophobic peptides would be expected to aggregate in water, and low fluorescence intensities are commonly reported for aggregates, a form of "concentration quenching" of fluorescence (*27*). The increase in fluorescence intensity on incorporation into a lipid bilayer can be used to quantitate binding. It was found that the fluorescence intensity of P_{16} in di(C18:1)PC increased with increasing molar ratio of lipid to peptide up to about 15:1, beyond which no further change was seen; the maximum fluorescence intensity was comparable to that for peptide in methanol (data not shown). It can be concluded that 15 lipid molecules allow the full incorporation of one molecule of P_{16} into the bilayer.

For P_{22}, fluorescence intensities were the same in bilayers of phosphatidylcholines with chain lengths between C14 and C24, with the wavelengths of the emission maximum decreasing slightly with increasing lipid chain length (Figure 1B, Table 1), suggesting that the tryptophan residue experiences a site of increasing hydrophobicity as the thickness of the bilayer increases. A very different result was obtained with P_{16}. For P_{16}, fluorescence intensities are constant for chain lengths between C14 and C20, and over this range the wavelength of the emission maximum again increases slightly with increasing chain length. However, in di(C24:1)PC the fluorescence intensity is very low, comparable to that in water (Figure 1B), suggesting that the peptide has formed low-fluorescence aggregates, either in water or associated with the lipid bilayer. Increasing the molar ratio of di(C24:1)PC to P_{16} from 100:1 to 1000:1 had no significant effect on fluorescence intensity. The fluorescence emission intensity of P_{16} in di(C22:1)PC is about 75% of that in di(C18:1)PC (Figure 1B).

P_{16} Does Not Incorporate into di(C24:1)PC. To test for incorporation of P_{16} into a lipid, peptide/lipid mixtures were passed through Sephadex G-50 columns (Figure 2). Peptide

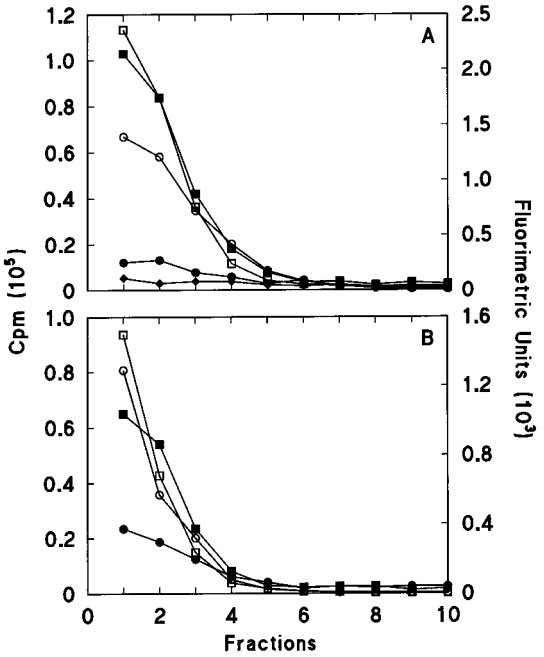

FIGURE 2: Gel filtration gradient profiles for the peptide P_{16}. Fractions were collected from Sephadex G-50 columns and analyzed for peptide (fluorimetric units; ■, ●, ◆) or lipid (cpm; □, ○). (A) Profiles of P_{16} alone (◆), with di(C18:1)PC (■, □), or with di(C24:1)PC (●, ○). (B) Profiles of P_{16} with 1:1 mixtures of di(C14:1)PC and cholesterol (■, □), or with 1:1 mixtures of di(C18:1)PC and cholesterol (●, ○). Fraction 1 represents the top of each gradient and fraction 10 the bottom.

P_{16}, when suspended alone in buffer, remained on the column and was not eluted. For mixtures of P_{16} and di(C18:1)PC, both the peptide and the lipid were recovered in the first 4 fractions, with 80−100% recovery. However, for mixtures of P_{16} and di(C24:1)PC, while lipid was again recovered in the first 4 fractions, the peptide did not elute from the column. For the mixture of P_{16} and di(C24:1)PC, recovery of lipid from the column was less than that for mixtures of P_{16} and di(C18:1)PC (Figure 2). If P_{16} alone was first passed through the column followed by di(C18:1)PC, reduced recovery of di(C18:1)PC was again observed (data not shown), showing that the reduced recovery was due to the presence of free P_{16} retained in the column. For mixtures of P_{22} in di(C14:1)PC or di(C24:1)PC, both the lipid and the peptide were recovered in the first four fractions (data not shown). These results therefore show that, whereas P_{22} incorporates fully into all bilayers tested and P_{16} incorporates fully into di(C18:1)PC, P_{16} does not incorporate into di(C24:1)PC.

The results with P_{16} were also confirmed by resolution of lipid/protein mixtures on continuous sucrose gradients (5−40%). For mixtures of P_{16} and di(C18:1)PC, the peptide and the lipid were recovered in the same fractions; but for mixtures of P_{16} and di(C24:1)PC, the peptide and the lipid were recovered in separate fractions, lipid at the top of the gradient and peptide at the top and in the middle of the gradient; when P_{16} alone was run on the gradient, it was recovered in the middle of the gradient (data not shown).

Fluorescence Quenching. The fluorescence intensity for the peptide P_{16} incorporated into bilayers of di(Br_2C18:0)-PC at a molar ratio of lipid to peptide of 100:1 is 5% of that in di(C18:1)PC, demonstrating highly efficient quenching of the tryptophan by the bromine-containing fatty acyl chains

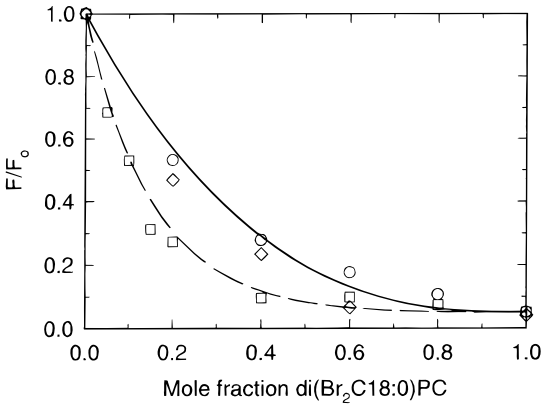

FIGURE 3: Fluorescence intensity for P_{16} in mixtures containing di(Br_2C18:0)PC. P_{16} was incorporated into mixtures of di(Br_2C18:0)PC with di(C18:1)PC (○), di(C14:1)PC (□), or a mixture of di(C14:1)PC and cholesterol at a 1:1 molar ratio (◇), at a molar ratio of peptide:phospholipid of 1:100. Fluorescence intensities are expressed as a fraction of that recorded for P_{16} in di(C18:1)PC. The solid lines show the best fits to the data in di(C18:1)PC and di(C14:1)PC with values for the relative binding constant K given in the text.

(Figure 3). The fluorescence intensity in mixtures of di(Br_2C18:0)PC and di(C18:1)PC decreases with increasing content of di(Br_2C18:0)PC and fits to eq 1 with a value of n, the number of "sites" around the peptide where binding can result in quenching, of 2.7 (Figure 3). Comparison of the fluorescence quenching curve for mixtures of di(Br_2C18:0)PC and di(C18:1)PC with that for mixtures of di(Br_2C18:0)PC and lipid X gives the binding constant for lipid X relative to that for di(C18:1)PC, according to eqs 1 and 2 (*23*, *28*). For example, the quenching curve in mixtures of di(Br_2C18:0)PC and di(C14:1)PC is consistent with a relative binding constant of 0.4 for di(C14:1)PC (Figure 3), showing that di(C14:1)PC binds to the peptide more weakly than di(C18:1)PC, by a factor of 2.5. P_{22} shows levels of quenching in di(Br_2C18:0)PC equal to the level seen with P_{16} and that fit to eq 1 with a value for n of 2.3 (Table 1). Table 1 lists the relative binding constants determined from fluorescence quenching plots for the peptides P_{16} and P_{22}.

The quenching curve for P_{16} in mixtures of di(Br_2C18:0)PC and di(C24:1)PC is more complex (Figure 4). Fluorescence intensity increases with increasing content of di(C24:1)PC up to a mole fraction of di(C24:1)PC of about 0.6, beyond which the intensity stays almost constant. In mixtures of di(C18:1)PC and di(C24:1)PC, the fluorescence intensity of P_{16} starts to decrease at this same point, suggesting that this is the maximum level of di(C24:1)PC for which incorporation of the peptide into the bilayer is normal (Figure 4). The average fatty acyl chain length in a mixture of 60% di(C24:1)PC and 40% di(C18:1)PC is about C22, and as shown in Figure 1B, the fluorescence intensity of P_{16} in di(C22:1)PC is less than that in di(C18:1)PC. The fluorescence intensity in mixtures of di(C24:1)PC and di(C18:1)PC drops very sharply beyond a mole fraction of 0.8 of di(C24:1)PC, corresponding to an average chain length of about C23. Fluorescence quenching in mixtures of di(Br_2C18:0)PC and di(C24:1)PC containing less than 60% di(C24:1)PC is consistent with a relative binding constant for di(C24:1)PC close to 1 (Figure 4).

These results suggest that the critical parameter for incorporation of P_{16} into the bilayer is the average fatty acyl

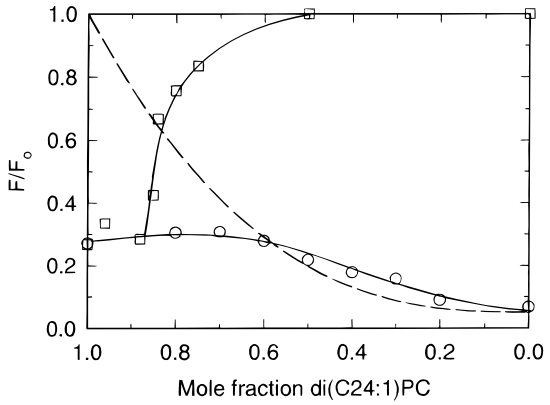

FIGURE 4: Fluorescence intensity for P_{16} in mixtures containing di(C24:1)PC. P_{16} was incorporated into mixtures of di(Br_2C18:0)-PC and di(C24:1)PC (○), or of di(C18:1)PC and di(C24:1)PC (□) at a molar ratio of peptide:phospholipid of 1:100. Fluorescence intensities are expressed as a fraction of that recorded for P_{16} in di(C18:1)PC. The broken line shows the fluorescence quenching curve calculated for the mixture of di(Br_2C18:0)PC and di(C24:1)PC, with a relative binding constant for di(C24:1)PC of 1 (see text).

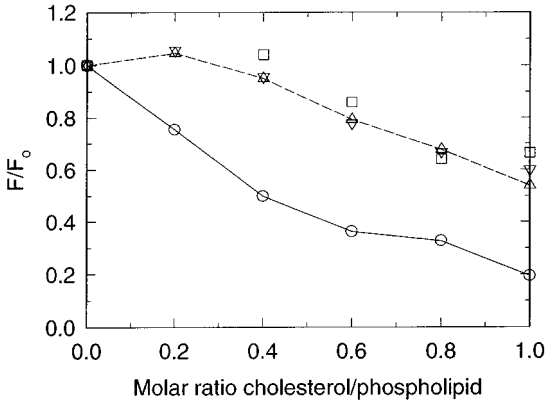

FIGURE 5: Effects of cholesterol. The fluorescence intensity was recorded for P_{16} in di(C18:1)PC (○) or di(C14:1)PC (□) and for P_{22} in di(C18:1)PC (△) or di(C14:1)PC (▽) as a function of the molar ratio of cholesterol:phospholipid.

chain length. As this increases beyond about C22, the partition of P_{16} between the lipid bilayer and the nonincorporated aggregates of P_{16} increasingly favors the aggregates. An alternative explanation for the marked increase in fluorescence intensity for P_{16} in mixtures of di(C18:1)PC and di(C24:1)PC beyond 15% di(C18:1)PC could have been weak binding of di(C24:1)PC to P_{16}, so that P_{16} would be surrounded in the bilayer by di(C18:1)PC, allowing its incorporation into the bilayer. However, this would not be consistent with the relative binding constant close to 1 determined above for di(C24:1)PC and would have resulted in strong quenching of fluorescence in mixtures of di(C24:1)PC containing low mole fractions of di(Br_2C18:0)PC, which was not observed (Figure 4).

Effects of Cholesterol. Incorporation of cholesterol up to a molar ratio of cholesterol:phospholipid of 0.4:1 resulted in no significant change in the fluorescence intensity for P_{22} in either di(C14:1)PC or di(C18:1)PC, but at molar ratios greater than this, addition of cholesterol resulted in a small decrease in fluorescence intensity (Figure 5). Effects of cholesterol on the fluorescence intensity for P_{16} in di(C14:1)PC were comparable to those for P_{22}, but for P_{16} in di-(C18:1)PC, effects were much more marked, the fluorescence intensity decreasing to a value ~20% of that in the absence of cholesterol at a 1:1 molar ratio of cholesterol:phospholipid (Figure 5), consistent with a low level of incorporation of P_{16} into the bilayers.

Effects of cholesterol on incorporation of the peptides into the lipid bilayers were also studied by using gel filtration on Sephadex G-50 columns (Figure 2B). For P_{16} in a 1:1 mixture of di(C14:1)PC and cholesterol, the lipid and the peptide coelute in the first four fractions. However, for P_{16} in a 1:1 mixture of di(C18:1)PC and cholesterol, only about 35% of the peptide was recovered (Figure 2B), suggesting only partial incorporation of the peptide, consistent with the fluorescence results (Figure 5).

For P_{16} in mixtures of di(Br_2C18:0)PC and di(C14:1)PC, addition of cholesterol at a 1:1 molar ratio with respect to total phospholipid resulted in a reduction in the level of fluorescence quenching observed at any given mole fraction of di(Br_2C18:0)PC, consistent with an increase in the relative binding constant K for di(C14:1)PC in the presence of cholesterol to a value close to 1 (Figure 3).

Circular Dichroism. The CD spectra of P_{16} or P_{22} in methanol solution or incorporated into sonicated lipid vesicles were very similar, with two minima near 222 and 208 nm and a crossover at 202 nm, typical for an α-helical structure (data not shown). Similar spectra were obtained for conditions of maximum or minimum mismatch: P_{22} in di(C14:1)PC and P_{16} in di(C18:1)PC gave mean residual ellipticities at Θ_{222} of 27 025 deg·cm^2/dmol and 23 493 deg·cm^2/dmol, corresponding to fractional helicities of 83% and 72%, respectively (*29*).

DISCUSSION

Effects of Peptide Length and Bilayer Thickness on Peptide Incorporation. Our objective was to test the idea that optimal interaction between a lipid bilayer and a hydrophobic peptide spanning that bilayer required that the hydrophobic length of the peptide should match the hydrophobic thickness of the bilayer. We studied peptides of the type Ac-K_2-G-L_m-W-L_n-K_2-A-amide, consisting of a long sequence of hydrophobic Leu residues capped at both the N- and C-terminal ends with two polar lysine residues. As described earlier, the polyleucine region was designed to form a maximally stable α-helix, particularly in the hydrophobic environment of the lipid bilayer. The charged Lys caps were designed both to anchor the ends of the peptides in the lipid headgroup region and to inhibit the aggregation of the peptides in the membrane. Peptides of this type have been shown to adopt the expected helical structure in lipid bilayers with the long axis of the helix more or less perpendicular to the bilayer surface (*22*). The single Trp residue was introduced into the middle of the peptides to act as a fluorescent reporter group.

The peptides were incorporated into a series of phosphatidylcholines containing monounsaturated fatty acyl chains; at the temperature of the experiments (25 °C) all of the lipids will be in the fluid, liquid crystalline state (*30*). Our first observation is that, whereas P_{22} was fully incorporated into the phospholipid bilayers irrespective of lipid chain length, P_{16} failed to incorporate normally into a bilayer of di(C24:1)PC and was only partly incorporated into a bilayer of di-(C22:1)PC.

The intensity of emission of the tryptophan residue was used to demonstrate incorporation of the peptide into lipid bilayers. The fluorescence intensity of P_{16} suspended in buffer was very low (Figure 1). Under these conditions, the peptide was presumably in a highly aggregated state, and such aggregates often have very low fluorescences possibly due to reabsorption of fluorescence emission prior to its escape from the aggregate (*31*). On incorporation of P_{16} into bilayers of di(C18:1)PC, fluorescence intensity increased to a value comparable to that seen in methanol (Figure 1). Samples, when resolved using gel filtration or on a sucrose gradient, showed that P_{16} had fully incorporated into the lipid bilayer (Figure 2). A further indication that P_{16} incorporates into bilayers of a phospholipid with a C18 fatty acyl chain is the high level of quenching observed in the bilayers of di(Br_2C18:0)PC (Figure 3); quenching of tryptophan fluorescence by bromine occurs by a process of heavy atom quenching which requires contact between the tryptophan and the bromine, indicating that the tryptophan residue is fully accessible to the lipid fatty acyl chains. In contrast to the results with P_{16} in the bilayers of di(C18:1)PC, the fluorescence intensity observed for P_{16} mixed with di(C24:1)PC is very low, comparable to that seen for aggregates in water, suggesting that P_{16} has not incorporated into the bilayer (Figure 1). This is confirmed by the gel filtration results (Figure 2). Thus we conclude that P_{16} does not incorporate into bilayers of di(C24:1)PC but, rather, forms large aggregates in the aqueous medium, which can be separated from the di(C24:1)PC liposomes by gel filtration or centrifugation. For P_{16} and di(C22:1)PC, the intermediate fluorescence intensity level observed (Figure 1) suggests partial incorporation into the bilayer and partial aggregate formation; fluorescence intensity levels in phosphatidylcholines with chain lengths between C20 and C14 are constant (data not shown), suggesting normal incorporation of P_{16} into bilayers with chain lengths less than C22.

If it is assumed that the peptides adopt an ideal α-helical structure, then the hydrophobic length of P_{16} will be about 27 Å, calculated using a helix translation of 1.5 Å per residue and a hydrophobic stretch of 18 residues in total. Thus the thickness of P_{16} will match a bilayer of about di(C16:1)PC (see Table 1). In bilayers of suboptimal thickness, significant changes in the α-helical structures of the peptides are unlikely because of the stability of the α-helix, an expectation confirmed in infrared studies of peptides of this type (*32*, *33*). There are then two mechanisms which would allow matching of a thin bilayer to a long peptide: the fatty acyl chains could stretch, increasing the thickness of the bilayer, or the peptide could tilt away from the direction of the bilayer normal, reducing its effective length across the bilayer. However, when the hydrophobic thickness of the bilayer is greater than that of the peptide, there is only one way to achieve matching and that is by compression of the fatty acyl chains. The results presented here for P_{16} suggest that this becomes energetically unfavorable when the hydrophobic thickness of the bilayer exceeds the hydrophobic length of the peptide by more than about 10 Å.

The peptide P_{22} has been observed to incorporate normally into all bilayers with chain lengths in the range C14−C24 (Table 1). In this case the hydrophobic length of the peptide will be about 36 Å, giving an optimal match to di(C22:1)-PC. For P_{22} in di(C14:1)PC the mismatch is about 13 Å, but since it is the peptide which is too long, the mismatch can be accommodated by tilting the peptide.

There will be an energetic cost associated with stretching or compressing the lipid fatty acyl chains, which will be reflected in values of relative lipid binding constants. As shown in Table 1, the strongest binding of lipid to P_{16} is observed for di(C18:1)PC, and the strongest binding for P_{22} is observed with di(C22:1)PC, as expected for optimal matching. However, it is clear that effects of a too-thin bilayer are relatively small and level out at a factor of 2−2.5 reduction in binding constant at a mismatch of about 6 Å. These observations, together with the observed much greater effect of too-thick than too-thin bilayers, are not predicted by current theoretical models which suggest that the effects of a mismatch will increase steeply in magnitude with increasing extent of mismatch with effects of too-thick and too-thin bilayers being rather similar (*19*−*21*, *34*).

Biological Relevance. The large effect of too-thick bilayers and the small effect of too-thin bilayers makes the thickness matching model for membrane protein retention more attractive. Membrane proteins are incorporated initially into the ER, and if effects of a too-thin bilayer were large, the biogenesis of membrane proteins with relatively long trans-membrane α-helices would be difficult since the ER is a relatively thin membrane. However, the asymmetry in the effect of too-thick and too-thin bilayers means that insertion into the ER membrane would be relatively little affected by the length of the trans-membrane α-helices, whereas flow of a protein with a short trans-membrane α-helix out of the ER or *cis* Golgi would be strongly disfavored. Also important is the observation that, in a bilayer containing a mixture of lipids with different fatty acyl chains, the peptide shows little selectivity in its interaction with the lipid (Figure 4). Thus the important parameter is the average thickness of the bilayer, and the presence of a small amount of a short-chain lipid will not allow a short peptide to insert into a bilayer which is, on average, too thick.

The differences in fatty acyl chain lengths found for lipids in the ER, the Golgi, and the plasma membrane are probably not sufficient to explain the observed retention in the Golgi of a peptide containing a trans-membrane domain of 17 residues. The average chain lengths of the phosphatidylcholine and phosphatidylethanolamine fractions are between C16 and C18 and are very similar for all compartments of the mammalian cell (*35*). The higher sphingomyelin content of the plasma membrane (19% of the total phospholipid) and the Golgi (12%) than of the ER (4%) may be more significant since about 30% of the fatty acyl chains in sphingomyelin are C24:0 (*13*). However the major factor is likely to be the amount of cholesterol, which is largely absent from ER and increases through the Golgi to a final level of a 1:1 cholesterol:phospholipid in the plasma membrane (*14*).

Cholesterol has a major effect on incorporation of the peptides into the phospholipid bilayers (Figure 5). For P_{22} in di(C14:1)PC or di(C18:1)PC, the fluorescence intensity of P_{22} decreases with increasing cholesterol content, suggesting decreased incorporation of the peptide into the bilayer; inclusion of cholesterol into phospholipid bilayers is known to reduce partitioning of a wide variety of hydrophobic molecules into the bilayer (*36*). For P_{16} in di-(C18:1)PC, however, the effect of cholesterol is very much more marked, with a large decrease in fluorescence intensity

being seen at a 1:1 molar ratio of cholesterol to phospholipid. Since the effect of cholesterol on P_{16} in di(C14:1)PC is comparable to that seen for P_{22} in di(C14:1)PC or di(C18:1)PC, the effect of cholesterol on P_{16} in di(C18:1)PC is likely to follow from increasing membrane thickness preventing incorporation, as well as the above general partition effect. This is supported by the gel filtration experiments, in which P_{16} and di(C14:1)PC coeluted, while P_{16} and di(C18:1)PC did not (Figure 2B). Nezil and Bloom (*16*) have shown that incorporation of cholesterol at 33 mol % increases bilayer thickness by about 4 Å. An effective increase in chain length is also suggested by the fluorescence quenching experiment shown in Figure 3 which shows that, in the presence of cholesterol, the relative binding constant of di(C14:1)PC to P_{16} increases from 0.4 to about 1, as expected if the presence of cholesterol increases the effective chain length of the C14 chain so that it more nearly matches the hydrophobic length of the peptide.

These results therefore show that the presence of cholesterol has a major effect on the incorporation of a short peptide into a bilayer whose thickness, in the absence of cholesterol, is only slightly greater than the hydrophobic length of the peptide. Such an effect of cholesterol, if observed for membrane proteins containing single trans-membrane α-helices, would provide an explanation for the retention of proteins with short trans-membrane α-helices in the *cis* Golgi.

For some proteins, sequences flanking the trans-membrane domain have also been shown to play a role in mediating Golgi retention. It has been suggested that such sequences could be involved in the aggregation of the proteins in the membrane, forming aggregates too large to enter vesicles budding off from the cisternal rims, resulting in retention in the Golgi (*2, 4, 7, 37*). Mismatching of the hydrophobic thicknesses of membrane proteins and lipid bilayers has been predicted to lead to aggregation of the membrane proteins as a way of minimizing lipid−protein contact (*19, 20*). Thus, mechanisms of retention based on aggregation may also depend on membrane thickness and the presence of cholesterol, as described above.

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