The Cytoplasmic Domains of Phospholamban and Phospholemman Associate with Phospholipid Membrane Surfaces†

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ABSTRACT: Phospholamban (PLB) and phospholemman (PLM, also called FXYD1) are small transmembrane proteins that interact with P-type ATPases and regulate ion transport in cardiac cells and other tissues. This work has investigated the hypothesis that the cytoplasmic domains of PLB and PLM, when not interacting with their regulatory targets, are stabilized through associations with the surface of the phospholipid membrane. Peptides representing the 35 C-terminal cytoplasmic residues of PLM (PLM^{37-72}), the 23 N-terminal cytoplasmic residues of PLB (PLB^{1-23}), and the same sequence phosphorylated at Ser-16 ($P-PLB^{1-23}$) were synthesized to examine their interactions with model membranes composed of zwitterionic phosphatidylcholine (PC) lipids alone or in admixture with anionic phosphatidylglycerol (PG) lipids. Wide-line ^2H NMR spectra of PC/PG membranes, with PC deuterated in the choline moiety, indicated that all three peptides interacted with the membrane surface and perturbed the orientation of the choline headgroups. Fluorescence and ^{31}P magic-angle spinning (MAS) NMR measurements indicated that PLB^{1-23} and $P-PLB^{1-23}$ had a higher affinity for PC/PG membranes, which carry an overall negative surface charge, than for PC membranes, which have no net surface charge. The ^{31}P MAS NMR spectra of the PC/PG membranes in the presence of PLM^{37-72}, PLB^{1-23}, and $P-PLB^{1-23}$ indicated that all three peptides induced clustering of the lipids into PC-enriched and PG-enriched regions. These findings support the theory that the cytoplasmic domains of PLB and PLM are stabilized by interacting with lipid headgroups at the membrane surface, and it is speculated that such interactions may modulate the functional properties of biological membranes.

The movement of ions across cellular membranes is facilitated in part by the ion-motive P-type ATPases, a family of ion pumps that actively couple the hydrolysis of ATP to the translocation of cations via a phosphorylated intermediate (*1*). The function of these proteins, which are present in virtually all eukaryotic cells, is to maintain the normal ionic homeostasis that is critical for cellular viability. Research over the past decade has led to the identification of several small transmembrane proteins that regulate the rate of ion translocation by the P-type ATPases. These regulatory proteins, which include phospholamban (PLB^1) and phospholemman (PLM), are typically under 100 amino acids in length, contain a single transmembrane helix, and, in some cases, are substrates for phosphorylation by protein kinases (*2*, *3*). The mechanisms with which these proteins regulate their physiological targets have implications for disease and have been subject to intense investigation (*4*).

Phospholamban (PLB) is a 52 amino acid protein that is expressed predominantly in the sarcoplasmic reticulum (SR) of cardiac myocytes (*5*). The primary physiological function of PLB is to regulate the active transport of calcium ions into the SR lumen by exerting a reversible inhibitory effect upon the cardiac isoform of the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA2a). At physiologically relevant calcium concentrations PLB associates with SERCA and reduces the affinity of the enzyme for calcium (*6*), but elevated calcium levels appear to abolish the physical interaction between the two proteins (*7*). In response to β-adrenergic stimulation PLB is phosphorylated at Ser-16 by cAMP-dependent protein kinase A (PKA) and at Thr-17 by Ca^{2+}-calmodulin dependent protein kinase, which relieves SERCA2a inhibition and increases the rate of calcium uptake into the SR leading to muscle relaxation (*8*). Structural

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[1] Abbreviations: PLB, phospholamban; PLM, phospholemman; AAA-PLB, a full-length, null-cysteine variant (C36A, C41A, C46A) of PLB; $AAA-PLB^{29-52}$, a truncated peptide corresponding to the C-terminal transmembrane residues 29−52 of AAA-PLB; PLB^{1-23}, the N-terminal cytoplasmic residues 1−23 of phospholamban; $P-PLB^{1-23}$, PLB^{1-23} phosphorylated at Ser-16; PLM^{37-72}, the C-terminal cytoplasmic residues 37−72 of phospholemman; PC, phosphatidylcholine; PG, phosphatidylglycerol; ATP, adenosine triphosphate; SR, sarcoplasmic reticulum; SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase; SERCA2a, cardiac isoform of SERCA; PKA, cAMP-dependent protein kinase A; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; ^2H NMR, deuterium NMR; ^{31}P NMR, phosphorus NMR; MAS, magic angle spinning; DMPC-d_4, [1,1,2,2-$^2H_4$]-L-α-dimyristoylphosphatidylcholine; DMPC, L-α-dimyristoylphosphatidylcholine; DOPC, L-α-dioleoylphosphatidylcholine; DOPG, L-α-dioleoylphosphatidylglycerol; LMVs, large multilamellar vesicles; SUVs, small unilamellar vesicles; CD, circular dichroism; TFE, 2,2,2-trifluoroethanol; $\Delta\nu_{1/2}$, peak width at half-height; σ_i, isotropic chemical shift; $\Delta\nu_Q$, quadrupolar splitting.

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analysis of PLB in organic solvents has revealed that the N-terminally situated cytoplasmic domain is predominantly helical and is linked to the transmembrane helix via a short disordered region (*9*). Analysis of PLB in lipid bilayers using solid-state nuclear magnetic resonance (NMR) methods showed that the sequence Ala24−Gln26, which connects the cytoplasmic and transmembrane domains, is helical when the protein is alone in the membrane but undergoes a transition to a more extended structure in the presence of SERCA (*10*). It was argued that the conformational adjustment might reflect changes in the orientations of the transmembrane and cytoplasmic domains as PLB encounters, and moves into contact with, SERCA (*10*). Previous NMR studies from Thomas, Veglia, and co-workers have shown that, in lipid-like detergent micelles, monomeric PLB adopts an "L-shape" structure with the cytoplasmic domain lying parallel to the membrane surface (*11*, *12*). More recent NMR and electron paramagnetic resonance (EPR) experiments by Thomas and colleagues (*13*, *14*) support this hypothesis by suggesting that, in the absence of SERCA, the cytoplasmic domain of PLB interconverts between a motionally restrained state and a disordered state in dynamic equilibrium. The two states were attributed to the amphipathic cytoplasmic helix associating with the membrane surface or being directed toward the aqueous environment. These observations led to the suggestion that the cytoplasmic domain of PLB acts as a conformational switch, which is drawn away from the membrane surface when in close proximity to SERCA (*14*).

Phospholemman (PLM) is a small regulatory protein of 72 amino acids (plus a posttranslationally cleaved 20-residue signal sequence) which has a number of features in common with PLB. PLM belongs to a group of small single-span transmembrane proteins known as the FXYD family, named in recognition of the invariant N-terminal amino acids that are their signature motif (*15*). The protein, also called FXYD1, associates specifically with the $\alpha 1/\beta$ and $\alpha 2/\beta$ isoforms of Na^+/K^+-ATPase in the sarcolemma of cardiac and skeletal muscle cells. The interaction results in a small decrease in the external K^+ affinity for the enzyme and a nearly 2-fold decrease in the internal Na^+ affinity for the enzyme (*16*). A physical association between PLM and Na^+/K^+-ATPase has also been observed in brain tissue, where it reduces enzyme activity without altering Na^+ affinity (*17*). The association of PLM and Na^+/K^+-ATPase is believed to occur via hydrophobic contacts between the transmembrane domains of the two proteins, while interactions between the C-terminal cytoplasmic domains may modulate Na^+/K^+-ATPase activity (*18*, *19*). It has therefore been suggested that the regulation of Na^+/K^+-ATPase by PLM is achieved through a similar mechanism to the regulation of SERCA by PLB, by affecting enzyme activity at one or more of the rate determining steps (*19*). PLM contains several sites in the cytoplasmic domain for phosphorylation by PKA and protein kinase C (*20*, *21*), which may play a role in the regulation of Na^+/K^+-ATPase in the same way that PLB phosphorylation reverses the inhibition of SERCA enzymes.

In light of recent EPR and NMR data (*13*, *14*) there is growing speculation that interactions between the cytoplasmic domain of PLB and cell membrane surfaces may be important for stabilizing the protein and could play a role in the proposed molecular switch mechanism for SERCA2a regulation. The similarities in the regulatory functions of PLB and PLM suggest that a switch mechanism involving membrane interactions could be a common feature of both proteins. To date, however, specific interactions of regions of these proteins with lipid molecules have not been probed in detail. The work presented here investigates the hypothesis that the cytoplasmic domains of PLB and PLM associate with membrane surfaces by interacting specifically with the polar headgroups of the phospholipids. ^{2}H NMR experiments on a null-cysteine variant of full length PLB (AAA-PLB) and on a truncated peptide comprising the C-terminal transmembrane residues 29−52 of AAA-PLB (AAA-PLB$^{29-52}$) reported elsewhere (*22*) and described here suggest that the cytoplasmic domain of PLB associates with lipid headgroups. The results from these experiments do not provide conclusive evidence that the PLB cytoplasmic domain interacts with membrane surfaces, however. To address these questions further we have synthesized water-soluble peptides encompassing the first 23 N-terminal residues within the cytoplasmic domain of PLB, in which Ser-16 either was phosphorylated or retained the free hydroxyl group, and the C-terminal 35 residues of PLM (Figure 1). This approach made it possible to screen for any interactions occurring between the cytoplasmic sequences of PLB and PLM and model phospholipid vesicles without encountering any interference from their transmembrane domains. By using a combination of fluorescence, circular dichroism, ^{2}H NMR, and ^{31}P NMR spectroscopy it is shown that the three peptides interact weakly with vesicles containing zwitterionic phosphatidylcholine (PC), but have a higher affinity for the negative surface charge of vesicles containing a mixture of PC and anionic phosphatidylglycerol (PG). Intriguingly, it was found that the association of the peptides with mixed PC/PG vesicles sequestered the anionic PG lipids into peptide-bound clusters leaving peptide-free regions enriched in the PC lipids. These observations provide direct evidence that the cytoplasmic domains of PLB and PLM are capable of binding to membrane surfaces and raise the possibility that PLB, PLM, and other members of the FXYD may play a role in modulating the lipid environment.

AAA-PLB

N-**MEKVQYLTRSAIRRASTIEMPQQARQKLQNLFINFALILIALLLIAIIVMLL**-C

AAA-PLB$^{29-52}$

N-CYT-**QNLFINFALILIALLLIAIIVMLL**-C

PLM$^{37-72}$

N-TM-**SRRCRCKFNQQQRTGEPDEEEGTFRSSIRRLSTRRR**-C

PLB$^{1-23}$

N-**MEKVQYLTRSAIRRASTIEMPQQ**-TM-C

P-PLB$^{1-23}$

N-**MEKVQYLTRSAIRRAS(P)TIEMPQQ**-TM-C

FIGURE 1: The sequences of AAA-PLB, AAA-PLB$^{29-52}$, and the soluble PLM and PLB peptide fragments. The labels "CYT" and "TM" denote respectively the locations of the cytoplasmic and transmembrane domains in the full-length proteins from which the fragments were derived, being situated N-terminally or C-terminally with respect to the sequences shown. The label "P" denotes the site of phosphorylation in P-PLB$^{1-23}$.

MATERIALS AND METHODS

Materials. A full-length, null-cysteine variant (C36A, C41A, C46A) of PLB (AAA-PLB), a truncated peptide

comprising residues 29−52 of AAA-PLB (AAA-PLB$^{29-52}$), and a peptide consisting of 35 residues within the PLM C-terminal cytoplasmic domain (PLM$^{37-72}$) were all purchased in pure (>95%) form from the Department of Biochemistry, University of Southampton. The null-cysteine variants of PLB were examined because the three substitutions reduce the propensity of PLB to oligomerize in lipid bilayers. Synthetic peptides comprising residues 1−23 within the N-terminal cytoplasmic domain of PLB were prepared phosphorylated at Ser-16 (P-PLB$^{1-23}$) and as the nonphosphorylated form (PLB$^{1-23}$). These were obtained in pure form from Peptide Protein Research Ltd (U.K.). The sequences of all the peptides studied and the labeling schemes used are summarized in Figure 1.

[1,1,2,2-^{2}H$_4$]-L-α-Dimyristoylphosphatidylcholine (DMPC-d_4) was purchased from Avanti Polar Lipids. Unlabeled DMPC in solid form, L-α-dioleoylphosphatidylcholine (DOPC) and L-α-dioleoylphosphatidylglycerol (DOPG), both in chloroform solution, and 2,2,2-trifluoroethanol were all purchased from Sigma Chemicals Limited.

Preparation of Vesicles. Small unilamellar vesicles (SUVs) were prepared from DMPC only or from DMPC and DOPG at a molar ratio of 2:1, with DMPC in excess, using a tip sonication method (*23*). Lipids were prepared in chloroform and then dried under argon and high vacuum, before resuspension in 10 mM phosphate, pH 7 such that 10 μL aliquots contain 0.05 mg of lipid for fluorescence spectroscopy and 0.3 mg of lipid for circular dichroism. The suspension was sonicated at a probe energy of 10 μm for 3 cycles (1 min on/1 min off) on ice. The formation of SUVs was assumed to occur when the suspensions became clear.

Large multilamellar vesicles (LMVs) of DMPC-d_4/DOPG, DMPC/DOPG, DMPC/DOPC, and DOPC/DOPG were prepared by first dissolving 2−10 mg of each lipid in chloroform and mixing the required lipid solutions together in the appropriate molar ratio. In mixtures containing DMPC (or DMPC-d_4), this lipid was added to a 2-fold molar excess over the other lipid component. In the DOPC/DOPG mixtures, DOPC was present in a 2-fold molar excess over DOPG. The chloroform solutions were dried to a film under argon and high vacuum overnight, and the lipid film was then resuspended in 50 μL of sample buffer containing 10 mM Tris and 1 mM EDTA at pH 7.4. The suspension was vortexed and subjected to five freeze−thaw cycles. Where appropriate, the peptide was added in 5 μL of sample buffer to attain a lipid:peptide molar ratio of 50:1 or 20:1, and the vortex, freeze−thaw process repeated.

Membranes of DMPC-d$_4$/DOPG in a 2:1 molar ratio were prepared containing either AAA-PLB or AAA-PLB$^{29-52}$ to a lipid:peptide molar ratio of 20:1 using a method described elsewhere (*22*). Briefly, the peptides were dissolved in 1:1 chloroform/methanol and added to the mixed lipids dissolved in chloroform. The mixture of peptide and lipid was then dried under argon and high vacuum. The peptide-containing membranes were then rehydrated in aqueous buffer. For NMR experiments, the lipid samples were transferred to a 4 mm diameter zirconium magic-angle sample spinner.

Intrinsic Fluorescence Assay. SUVs of DMPC/DOPG at a 2:1 molar ratio and DMPC alone were prepared in 500 μL of 10 mM phosphate, pH 7, as described above. The SUV solutions were titrated into an initial peptide (PLM$^{37-72}$, PLB$^{1-23}$, or P-PLB$^{1-23}$) solution of 0.1 mg/mL in 10 mM phosphate, pH 7, and the fluorescence emission spectrum from 290 to 340 nm was measured using a Varian Cary Eclipse fluorescence spectrophotometer, with excitation at 280 nm. Titrations of SUVs were carried out until a final lipid to peptide molar ratio of 20:1 was reached. Emission spectra were corrected for the presence of lipid by subtracting control spectra containing only buffer and the appropriate concentration of SUVs.

Far UV Circular Dichroism. Secondary structural measurements of PLM$^{37-72}$, PLB$^{1-23}$, and P-PLB$^{1-23}$ were carried out by circular dichroism (CD) spectroscopy on a Jasco J-180 spectropolarimeter at 20 °C. Spectra were recorded from 250 to 180 nm using a 0.1 or 0.02 cm cuvette, a scan rate of 10 nm/min, and an average of 24 scans per sample. Initial measurements were made on solutions of each of the peptides (20 μM PLM$^{37-72}$, 80 μM PLB$^{1-23}$, 50 μM P-PLB$^{1-23}$) in 10 mM phosphate, pH 7 followed by a stepwise titration with trifluoroethanol (TFE) to generate measurements at between 10% and 50% (v/v) TFE. In separate experiments the aqueous peptide solutions (20 μM PLM$^{37-72}$, 50 μM PLB$^{1-23}$, 50 μM P-PLB$^{1-23}$) were measured before and after titrating in SUVs of DMPC and DOPG (in a 2:1 molar ratio) to a final lipid to peptide molar ratio of 100:1. The percentage α-helical content at each lipid or TFE concentration was calculated using the following (*24*):

$$\% \text{ helix} = 100 \times (\theta_{obs} - \theta_C)/(\theta_H - \theta_C)$$

where

$$\theta_H = -40000 \times (1 - x/n) + 100T$$

and

$$\theta_C = 640 - 45T$$

The quantities θ_H and θ_C represent the molar ellipticity values for 100% α-helix and 100% random coil, respectively, both expressed in deg·cm^2/dmol. The quantity θ_{obs} is the observed molar ellipticity measured at 222 nm, T is the temperature in °C, n is the number of amino acids, and x is a constant correcting for non-hydrogen bonded carbonyls (2.5 in this case). This equation can be used when there is a two state transition between random coil and helix, assumed when there is a visible isodichroic point in the CD spectra (*24*).

Solid-State NMR Measurements. Spectra were recorded on a Bruker Avance 400 MHz spectrometer operating at frequencies of 400.13 MHz for ^{1}H, 162.12 MHz for ^{31}P, and 61.00 MHz for ^{2}H. Wide line ^{2}H NMR spectra of membranes containing DMPC-d_4 were obtained using a double-tuned nonspinning probe head with fixed horizontal 5 mm coil. Spectra were recorded as a result of accumulating 10000−20000 transients with a 1 s recycle delay. The quadrupole echo sequence ($90_x-\tau-90_y-\tau-$acquisition) sequence (*25*) was used with a 90° pulse length of 4 μs and delay τ of 22 μs. The experimental temperature was 30 °C. Magic-angle spinning (MAS) ^{31}P NMR spectra were recorded using a double tuned MAS probe with sample rotation rate of 4 kHz. The samples were contained within a 4 mm diameter zirconia rotor, and spectra were recorded after applying a single 4 μs 90° pulse at the frequency ^{31}P followed by 50 kHz proton

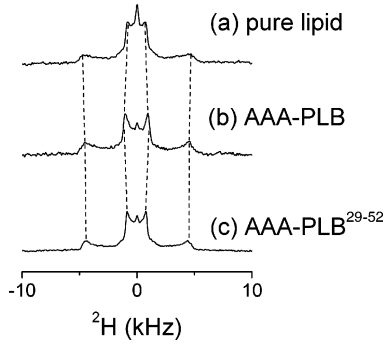

FIGURE 2: Wide line ^{2}H NMR spectra of membranes containing DMPC-d_4 and DOPG in a 2:1 molar ratio (5 mg total lipid) alone (a) and in the presence of AAA-PLB (b) and AAA-PLB$^{29-52}$ (c) in a lipid to peptide molar ratio of 20:1. The quadrupole splittings for the deuterons at the α- and β-choline positions of DMPC-d_4 are denoted by the separation between the pairs of dotted lines. The spectra were obtained at 30 °C and are the result of accumulating 8000 scans.

decoupling during the acquisition period. The experimental temperature was maintained at 4 °C.

RESULTS

Lipid Headgroup Perturbation by Full-Length and Transmembrane PLB Peptide Analogues. Wide-line ^{2}H NMR was used to probe whether the cytoplasmic domain of PLB has an effect upon negatively charged membrane surfaces. For this purpose two membrane samples were prepared, which consisted of DOPG, DMPC containing deuterons at the α and β positions of the choline moiety (i.e., DMPC-d_4) and one of two PLB variants. One variant was the full length, null-cysteine protein AAA-PLB, and the other was the truncated peptide AAA-PLB$^{29-52}$ from which the cytoplasmic domain was absent. The line shape of the ^{2}H NMR spectrum from DMPC-d_4 is highly sensitive to the changes in the orientation of lipid headgroups resulting from interactions with peptides and proteins (*26*). By comparing the ^{2}H NMR spectra of the membranes containing the two PLB analogues it is possible, in principle, to assess whether the cytoplasmic domain perturbs the membrane surface.

In Figure 2 are shown ^{2}H NMR spectra at 30 °C from membranes of DMPC-d_4/DOPG alone and in the presence of AAA-PLB or AAA-PLB$^{29-52}$ at a lipid to peptide molar ratio of 20:1. The spectrum of the pure lipid sample exhibits two sets of Pake doublets with quadrupole splittings ($\Delta\nu_Q$) of ∼9.3 kHz for the α-deuterons and ∼1.5 kHz for the β-deuterons of DMPC-d_4. In the presence of AAA-PLB, the splitting for the α-deuterons decreased ∼9.0 kHz and the β-deuteron splitting increased to ∼2.0 kHz (Table 1), indicating that the protein has an effect upon the lipid headgroups. In the presence of AAA-PLB$^{29-52}$, the splittings for the α-deuterons decreased to 8.8 kHz but the β-deuteron splitting remained essentially the same as for the pure lipid membranes. Hence, the two PLB analogues give rise to different changes in the line shape of the ^{2}H NMR spectra, which suggests that the cytoplasmic domain of PLB plays a role in perturbing the choline lipid headgroups. These observations do not confirm that the cytoplasmic domain interacts directly with the lipid headgroups, however. The dissimilarities in the spectra of the two PLB analogue samples may, for example, reflect differences in headgroup perturbations arising from the transmembrane region in the presence and absence of the cytoplasmic domain. Owing to the inconclusiveness of these results, further experiments were carried out with peptides corresponding to the cytoplasmic domain of PLB (residues 1−23) or PLM (residues 37−72).

Table 1: Summary of Quadrupole Splitting ($\Delta\nu_Q$) Values for the α- and β-Deuterons of DMPC-d_4 Measured from ^{2}H NMR Spectra at 30 °C of LMVs Containing DMPC and DOPC (2:1 molar ratio) in the Presence and Absence of Peptide Fragments at Lipid:Peptide Molar Ratios of 50:1 and 20:1

	$\Delta\nu_Q$ α (Hz)			$\Delta\nu_Q$ β (Hz)		
peptide	lipid only	50:1	20:1	lipid only	50:1	20:1
AAA-PLB	9356		9093	1630		1988
AAA-PLB$^{29-52}$	9356		8830	1630		1637
PLM$^{37-72}$	9354	7505	7400	1521	2323	2480
PLB$^{1-23}$	9363	9107	8808	1550	2112	2309
P-PLB$^{1-23}$	9363	8925	8369	1550	1878	2273

Screening for PLM and PLB Cytoplasmic Domain Peptide Interactions with Lipid Vesicles. The association of peptides with membrane surfaces can be detected by observing the intrinsic fluorescence from aromatic residues after titrating small unilamellar vesicles into the peptide solutions (e.g., ref *23*). Small vesicles are used as the model membrane system to avoid problems of light scattering that are encountered with large unilamellar or multilamellar vesicles. Contacts between the peptide aromatic groups and the polar or charged headgroups of phospholipids can give rise to perturbations of fluorescence emission, thereby providing a primary screen for membrane interactions of a peptide.

The membrane affinities of the water-soluble peptides representing the cytoplasmic domains of PLM (PLM$^{37-72}$) and PLB (PLB$^{1-23}$ and P-PLB$^{1-23}$) were investigated by measuring the intensity of intrinsic fluorescence emission before and after titrating phospholipid SUVs into aqueous solutions of each peptide. The SUVs contained either pure DMPC phospholipids, which carry a zero net surface charge, or a mixture of DMPC with DOPG at a 2:1 molar ratio, which possess a negative surface charge. Titration of the pure DMPC or mixed DMPC/DOPG vesicles into the peptide solutions was, in all cases, accompanied by a progressive decrease in fluorescence intensity as the lipid concentration was increased to a final lipid-to-peptide ratio of 20:1 (Figure 3, a−c). Similar fluorescence quenching effects have been observed for α-synuclein interactions with SUVs (*23*) and may reflect direct interactions of the aromatic group with the phosphate headgroups or structural rearrangements within the peptides upon association with the membrane. Hence, the preliminary screen indicates that the peptides interact with vesicles either of negative surface charge or with no net surface charge.

The extent of fluorescence quenching for each peptide was dependent upon the lipid composition of the vesicles. A greater reduction of PLM$^{37-72}$ fluorescence was observed at the end of the DMPC/DOPG titration experiment (Figure 3a) than after titration of DMPC alone, whereas the converse was observed for the PLB peptides (Figure 3, b−c). It is not possible to draw firm conclusions about the relative affinities of the peptides for the two vesicle samples from the extent of fluorescence perturbation, however, because quenching may be influenced by differences in how the

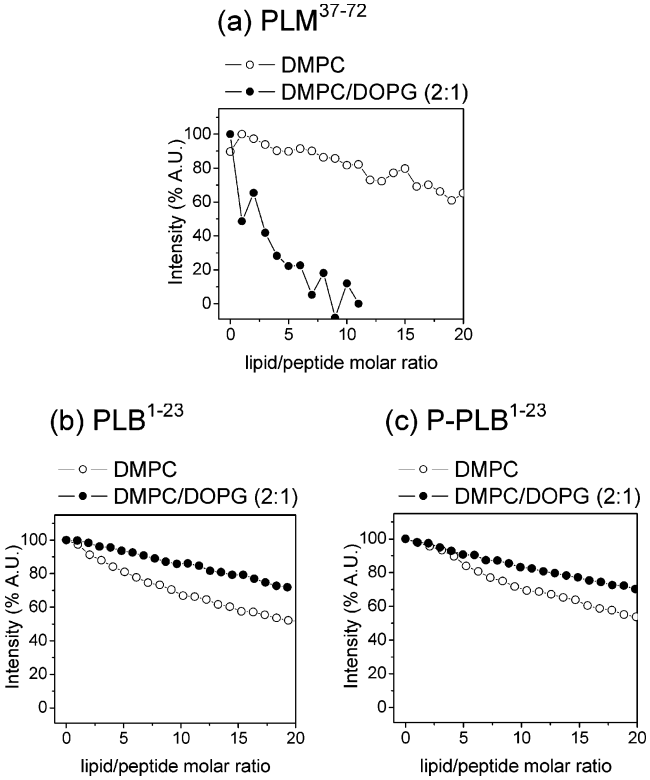

FIGURE 3: Interactions of PLM and PLB peptide fragments with phospholipid SUVs from measurements of intrinsic peptide tyrosine fluorescence. Fluorescence emission at 305 nm of 0.1 mg/mL aqueous solutions of PLM^{37-72} (a), PLB^{1-23} (b), and P-PLB^{1-23} (c) was measured following excitation at 280 nm. SUVs of DMPC/DOPG in a 2:1 molar ratio (filled circles) or DMPC (open circles) were titrated into the peptide solutions to the lipid:peptide molar ratios shown. The fluorescence intensity values were corrected for the presence of lipid by subtracting measurements from lipid only samples at the appropriate concentrations. The experimental temperature was 25 °C.

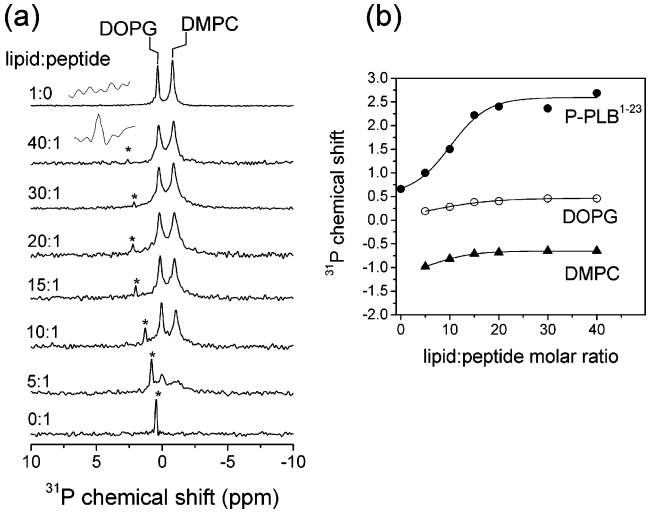

FIGURE 4: The association of P-PLB^{1-23} with DMPC/DOPG membranes observed from the perspective of the lipids and peptide using ^{31}P MAS NMR spectroscopy. Spectra were obtained at 4 °C from a suspension of LMVs containing 5 mg of lipid (with a 2-fold molar excess of DMPC over DOPG) before and after the titration of P-PLB^{1-23} into the vesicles to the lipid:peptide ratios stated (a). The three peaks in the spectrum were assigned to DMPC and DOPG as stated and to the phosphoserine group of P-PLB^{1-23} as denoted by the asterisk. Chemical shifts for the three components are plotted as a function of lipid:peptide molar ratio (b).

peptides associate with membranes of different lipid compositions. It is noted, however, that the curves for the two PLB peptides (PLB^{1-23} and P-PLB^{1-23}) in the DMPC/DOPG titration experiments (Figures 3b, 3c; black circles) are virtually identical to each other, as they are in the DMPC titration (Figures 3b, 3c; open circles). This observation suggests that the two PLB peptide fragments have equal affinity for the DMPC/DOPG vesicles and also for the DMPC vesicles, which implies that phosphorylation of PLB^{1-23} at Ser-16 does not affect the interactions of the peptide with these model membranes.

Affinity of PLM and PLB Peptides for Membrane Vesicles. The preliminary fluorescence experiments above indicate that the PLB and PLM peptide fragments all associate with phospholipid membranes, but the data do not provide a measure of their binding affinities. Further experiments were conducted using ^{31}P MAS NMR spectroscopy to assess the affinity of one peptide, P-PLB^{1-23}, for lipid membranes possessing either a neutral or a negative net surface charge. Two major modifications were made to the experimental conditions used in the fluorescence experiments. First, LMVs were used rather than SUVs as the model membrane system. This was because the LMVs do not interfere with the NMR experiment and present a greater surface area to the peptide, minimizing surface curvature and crowding issues that could influence peptide binding. Second, the experimental temperature was reduced from 25 °C to 4 °C. The lower temperature was adopted for reasons of consistency with data obtained in subsequent experiments which are presented below. Spectra were obtained from solutions of P-PLB^{1-23} alone and during titration of LMVs of DMPC/DOPG or DMPC/DOPC into the peptide solution. Both membranes contain lipids with unsaturated acyl chains, and the membranes will therefore remain in the fluid phase at 4 °C provided the lipids are mixed homogeneously. The high-resolution MAS experiment was conducted with the phosphorylated peptide because it was then possible to resolve individual peaks in the NMR spectrum from the serine phosphate group of the peptide, as well as from the lipid phosphate moieties. In so doing, the experiment provided the opportunity to observe the nature and strength of the peptide−membrane interactions from the perspective of all the peptide and lipid species present, by measuring changes in peak widths at half-height ($\Delta\nu_{1/2}$) and isotropic chemical shift values (σ_i).

In Figure 4 are shown the results of a titration experiment to monitor the interaction between P-PLB^{1-23} and DMPC/DOPG membranes, which carry a net negative surface charge. The spectrum of P-PLB^{1-23} alone in aqueous solution exhibited a single peak at 0.38 ppm (Figure 4a, bottom). As the membranes were titrated into the peptide solution, peaks from DMPC at −1.00 ppm and DOPG at 0.05 ppm began to emerge. The two peaks are clearly separated owing to the different chemical properties of the phosphatidylcholine and phosphatidylglycerol headgroups. Several prominent changes in the features of the spectra were also observed as the lipid concentration increased (Figure 4a). The peak from the peptide became increasingly broadened as the lipid concentration was raised, which is suggestive of increasing motional restraint of P-PLB^{1-23} and signifies a shift in the equilibrium of the peptide toward a predominantly membrane-associated state. In parallel, the peaks for the serine phosphate

group and the two lipid components moved progressively downfield. The change in chemical shift for P-PLB$^{1-23}$, which is particularly sensitive to the addition of membranes, suggests that the peptide−membrane interactions expose the serine phosphate group to the electrostatic effects of the lipid polar headgroups. In turn, the lipid phosphate groups respond to changes in the electrostatics at the membrane surface. The chemical shift values for P-PLB$^{1-23}$, DMPC, and DOPG all had a sigmoidal dependence on the peptide to lipid molar ratio (Figure 4b), which suggests that peptide binding to the membrane may be cooperative. Moreover, only one peak from the peptide was observed at each of the lipid concentrations, which indicates that exchange of the peptide on and off the membrane surface is fast on the NMR (i.e., millisecond) time scale and therefore the interaction is probably rather weak. If the exchange rate were slow with respect to the difference in the resonance frequencies for the free and bound peptide species, individual peaks from the two species would be observed at their characteristic frequencies. No further changes in chemical shift values occurred above a lipid to peptide molar ratio of 20:1, at which point it was assumed that the peptide had saturated all the binding sites at the membrane surface. One further feature of the ^{31}P NMR spectra (Figure 4a) is that, as the lipid-to-peptide ratio increases, the peak for DMPC at −1.0 ppm broadens to a greater extent than does the peak for DOPG. This phenomenon was investigated further in subsequent experiments, which are described below.

The titration experiment was repeated with P-PLB$^{1-23}$ and membranes composed of DMPC and DOPC. This lipid combination was chosen because the hydrocarbon chain composition of the lipids is identical to that of the DMPC/DOPG membranes used in the previous experiment, but the headgroups are zwitterionic and therefore do not present a net surface charge to the peptide. Hence, the relative affinity of P-PLB$^{1-23}$ for the DMPC/DOPC membranes and for the DMPC/DOPG membranes will be influenced only by the surface charge density of the membranes. The spectra obtained in the titration experiment exhibit one peak from the identical headgroups of the mixed phosphatidylcholine lipids and a peak from the peptide (Figure 5a). Much smaller changes in $\Delta\nu_{1/2}$ and σ_i occurred over the lipid titration range than were observed in the DMPC/DOPG experiment. The chemical shift value for the peptide, which again suggested a sigmoidal dependence on lipid concentration, continued to increase at lipid to peptide molar ratios above 20:1, and saturation of binding was not attained even at the highest molar ratio (80:1) that could be examined reliably (Figure 5b). Hence, P-PLB$^{1-23}$ appears to bind much more weakly to membranes of neutral surface charge (e.g., DMPC) than to membranes with a net negatively charged surface (e.g., DMPC/DOPG).

The membrane binding affinities of PLM$^{37-72}$ and PLB$^{1-23}$ were not investigated by the ^{31}P NMR method because the peptides did not contain the highly sensitive phosphate reporter group and the chemical shift changes for the lipid peaks were too small to provide a reliable binding curve. In the case of PLB$^{1-23}$, however, the fluorescence data (Figure 3) indicate that the peptide has virtually the same binding affinity for lipid membranes as does P-PLB$^{1-23}$. It is reasonable to assume, therefore, that PLB$^{1-23}$, like its phosphorylated analogue, has a higher affinity for membranes with a negative surface charge than for membranes with zero net surface charge.

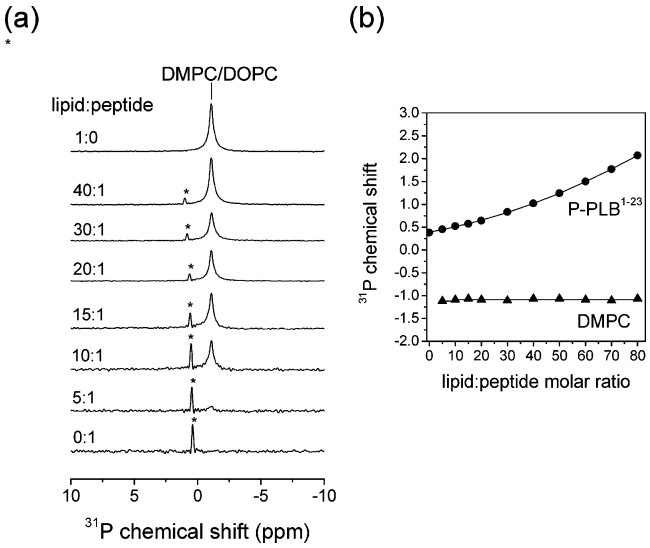

FIGURE 5: The association of P-PLB$^{1-23}$ with DMPC/DOPC membranes observed from the perspective of the lipids and peptide using ^{31}P MAS NMR spectroscopy. Spectra were obtained at 4 °C from a suspension of LMVs containing 5 mg of lipid (with a 2-fold molar excess of DMPC over DOPC) before and after the titration of P-PLB$^{1-23}$ into the vesicles to the lipid:peptide ratios stated (a). The two peaks in the spectrum were assigned to DMPC and DOPC as stated and to the phosphoserine group of P-PLB$^{1-23}$ as denoted by the asterisk. Chemical shifts for the three components are plotted as a function of lipid:peptide molar ratio (b).

The Association of PLM and PLB Peptides with Phospholipid Headgroups. The fluorescence and ^{31}P NMR experiments above suggest, but do not confirm, that the PLM and PLB peptide fragments associate with membranes through contacts with the lipid polar headgroups. Wide-line ^{2}H NMR was used to probe in more detail whether PLM$^{37-72}$, PLB$^{1-23}$, and P-PLB$^{1-23}$ interact directly with the negatively charged surface of membranes composed of DMPC/DOPG (in a 2:1 molar ratio). For this purpose DMPC was deuterated at the α and β positions of the choline moiety (i.e., DMPC-d_4). The ^{2}H NMR spectrum of DMPC-d_4 is highly sensitive to the orientation of the headgroup and any perturbations therein that occur when peptides and other solutes interact with the membrane surface (*26*). LMVs were again used as model membranes in these experiments because the slow tumbling of the vesicles does not average out the quadrupolar anisotropy of the deuterium nuclei and therefore permits site-specific details about the lipid molecules to be observed. Any changes in the quadrupole splittings (the separation between the most intense parts of the spectrum) for DMPC-d_4 in the presence of the PLM and PLB peptides would be diagnostic of their interactions with the membrane surface.

In Figure 6 (a−c) are shown ^{2}H NMR spectra at 30 °C from membranes of DMPC-d_4/DOPG in the absence and presence of the three peptides at lipid-to-peptide ratios of 50:1 and 20:1. In the absence of peptide, the spectra exhibit two sets of Pake doublets with quadrupole splittings ($\Delta\nu_Q$) of ∼9.3 kHz for the α-deuterons and ∼1.5 kHz for the β-deuterons of DMPC-d_4. Upon addition of each of the three peptides to the membranes, the splitting for the α-deuterons decreased and the β-deuteron splitting increased with increasing peptide concentration. The changes in the spectrum

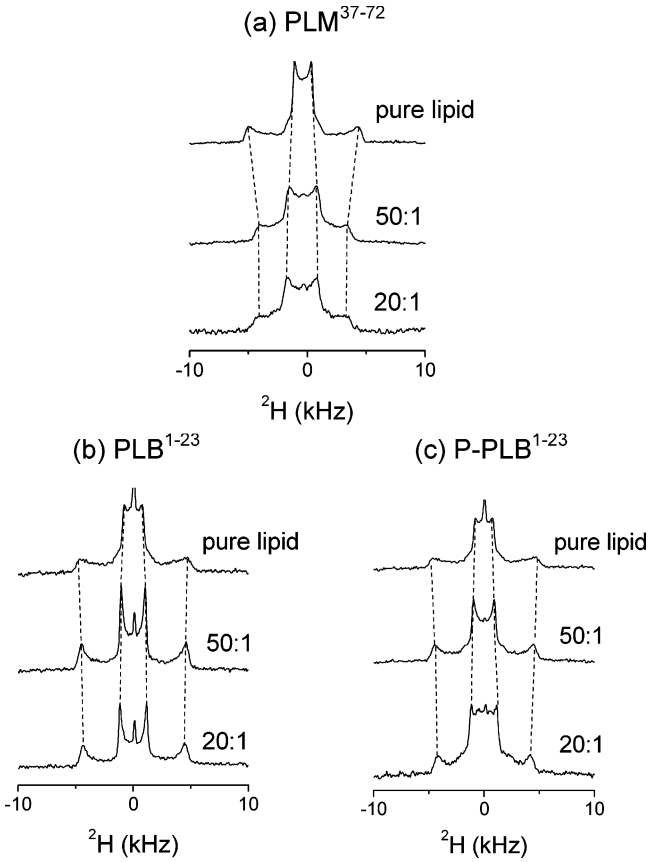

FIGURE 6: Detection of interactions between phospholipid headgroups and peptide fragments of PLM and PLB monitored from ^{2}H NMR spectra of LMVs containing DMPC-d_4 and DOPG. Spectra of vesicles containing DMPC-d_4 and DOPG in a 2:1 molar ratio (5 mg of total lipid) were obtained before (pure lipid) and after the addition of PLM$^{37-72}$ (a), PLB$^{1-23}$ (b), and P-PLB$^{1-23}$ (c) to lipid:peptide molar ratios of 50:1 and 20:1. The quadrupole splittings for the deuterons at the α- and β-choline positions of DMPC-d_4 are denoted by the separation between the pairs of dotted lines. The spectra were obtained at 30 °C and are the result of accumulating 10 000 scans.

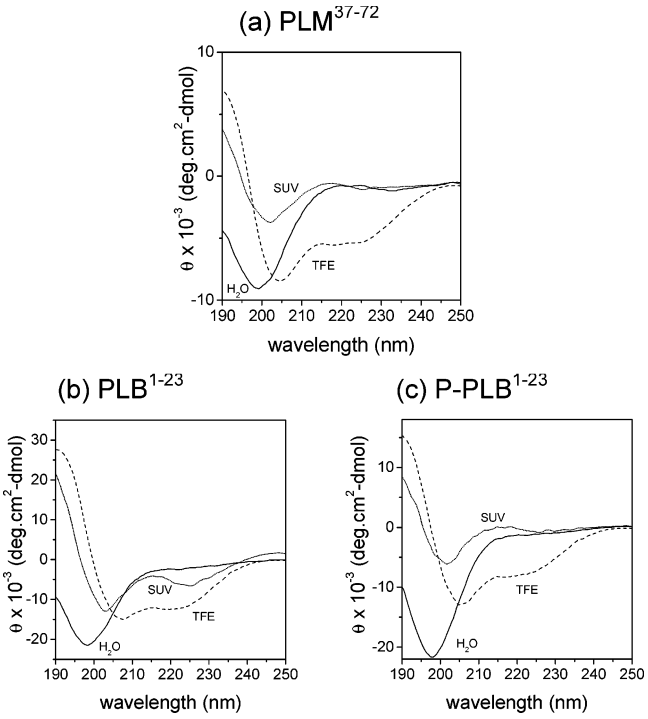

FIGURE 7: Analysis of the secondary structures of PLM and PLB peptides in aqueous/TFE solutions and in the presence of SUVs of DMPC/DOPG using CD spectroscopy. Spectra are shown for PLM$^{37-72}$ (a), PLB$^{1-23}$, (b) and P-PLB$^{1-23}$ (c) in aqueous solution (solid lines), after the addition of 41% (v/v) TFE (dashed lines) and after the addition of SUVs to a lipid:peptide molar ratio of 100:1 (dotted lines). The molar ellipticities were calculated after correcting for dilution by TFE or after correcting for interference from the SUVs by subtracting the spectra of peptide-free lipid suspensions at the appropriate concentrations. In the TFE experiment, initial peptide concentrations were 20 μM PLM$^{37-72}$, 80 μM PLB$^{1-23}$, and 50 μM P-PLB$^{1-23}$. In the SUV experiment, initial peptide concentrations were 20 μM PLM$^{37-72}$, 50 μM PLB$^{1-23}$, and 50 μM P-PLB$^{1-23}$.

provide confirmatory evidence that the peptides interact with the membrane surface, where they perturb the orientation of the choline headgroup. The splitting values obtained from the spectra in the presence of the three peptides are summarized in Table 1. The largest changes in splittings were observed when PLM$^{37-72}$ was added to the membrane, suggesting that this peptide had the greatest disruptive effect on the lipid headgroups. Of the two PLB peptides, PLB$^{1-23}$ invoked the greater increase in the splittings from the β-deuterons but the phosphorylated peptide P-PLB$^{1-23}$ caused the greater reduction in the splittings from the α-deuterons. The different changes seen of the splittings after addition of PLM$^{37-72}$ and the two PLB peptides might reflect differences in the affinities of the peptides for the membrane. Alternatively, the spectra may detect variations in the structures or orientations of the three peptides at the membrane surface, which perturb the lipid headgroup orientations in different ways. It is not possible at this stage to distinguish between these possibilities.

Secondary Structures of PLM and PLB Peptides in the Presence of Lipid Vesicles. Circular dichroism (CD) spectroscopy was used to monitor the secondary structures of PLM$^{37-72}$, PLB$^{1-23}$, and P-PLB$^{1-23}$ in aqueous solution and after the addition of TFE or SUVs of DMPC/DOPG (in a 2:1 lipid molar ratio). Spectra of the peptides alone in aqueous solution (Figure 7, a−c; solid lines) were consistent with all three peptides being predominantly random coil in the aqueous environment. TFE was then titrated into the peptide solutions to assess the intrinsic propensity of the peptides to adopt defined secondary structures (*27*). The spectra obtained after titrating TFE into the aqueous solution (not shown) showed a progressive structural change in each of the peptides up to TFE concentrations of 41% (v/v), but no further changes in the spectra were observed above this TFE concentration. The spectra of the peptides in 41% TFE are shown in Figure 7 (a−c, dashed lines). There is a clear isodichroic point within the spectra of each peptide before and after the addition of TFE at about 204 nm, providing strong evidence that this secondary structural change is a two-state transition at the residue level from random coil to helix. The α-helical content of PLM was estimated to be approximately 3.8% in aqueous solution, increasing to about 15% at the end-point of the TFE titration (Figure 7a). Similarly, the helical content of PLB$^{1-23}$ increased from about 3% in aqueous solution to ∼40% in 41% TFE, but the helical content of P-PLB$^{1-23}$ was rather less (∼20%) at the end of the TFE titration (Figure 7 b−c). These observations agree with previous NMR studies of PLB peptides in TFE solution and detergent micelles, which suggest that the cytoplasmic domain is predominantly helical but undergoes

partial unwinding toward the N-terminus after phosphorylation at Ser-16 (*28*, *33*).

CD spectra of the three peptide solutions (in the absence of TFE) obtained after adding DMPC/DOPG SUVs to a lipid to peptide molar ratio of 100:1 are shown in Figure 7 (a−c, dotted lines). The spectra were rather noisy as a result of subtracting the lipid background spectra, but the spectrum of PLB^{1-23} showed a clear isodichroic point consistent with a coil−helix transition after addition of the vesicles (Figure 7b). The spectra of PLM^{37-72} and $P\text{-}PLB^{1-23}$ were suggestive of small structural changes after the addition of vesicles (Figure 7, a,c), but did not necessarily reflect an increase in helical content of these peptides. Hence, it appears that although the three peptides all interact with these model membranes, only PLB^{1-23} undergoes an appreciable conformational transition from random coil to helix at the membrane surface. It is noted, however, that the structure adopted by PLB^{1-23} in the presence of the lipid vesicles is likely to be different from the structure taken by the peptide in TFE.

Effects of Peptide Binding upon Lipid Organization. The ^{31}P NMR spectra of $P\text{-}PLB^{1-23}$ with DMPC/DOPG membranes (Figure 4a) show that the peptide causes the peaks for the two lipids to broaden, but as the peptide-to-lipid ratio increases the peak for DMPC becomes significantly broader than the peak for DOPG. The dependence of the DMPC peak width upon the lipid-to-peptide ratio suggests that $P\text{-}PLB^{1-23}$ associates with the membrane surface and selectively reduces the rotational and translational mobility of the choline lipid phosphate groups (*29*). Such an effect may occur if the peptide binds selectively to the PG headgroups and sequesters the PG lipids into peptide-associated clusters, leaving behind pools of peptide-free lipids enriched in DMPC. The relationship between lipid clustering and the selective broadening of the ^{31}P NMR peak for DMPC is a consequence of the acyl chain composition of the membrane lipids—DMPC being saturated and DOPG being unsaturated—and the experimental temperature employed (4 °C). The spectrum of the DMPC/DOPG membranes before the addition of peptides exhibits narrow peaks for both lipid components because the lipids are mixed homogeneously and the fluid, unsaturated chains of DOPG prevent the DMPC chains from adopting the ordered gel phase configuration at 4 °C. If a peptide binds to the membrane surface and gathers the DOPG molecules together, the remaining pools of predominantly peptide-free DMPC molecules will have similar properties to membranes composed of pure DMPC. At the experimental temperature of 4 °C, well below the chain melting temperature of DMPC, the saturated myristoyl chains in the DMPC pools have a greater propensity to pack together in a gel phase. The ordered gellike lipids in the clusters will be impaired in their motional freedom, and consequently the NMR peak for DMPC is broadened out to a width close to that observed for pure gel-phase DMPC at 4 °C (1.0−1.5 ppm). Hence, the peptide can exert an *indirect* effect upon the spectrum of DMPC by interacting with the DOPG molecules.

Further ^{31}P NMR measurements were carried out to determine whether the interactions of PLM^{37-72}, PLB^{1-23}, and $P\text{-}PLB^{1-23}$ with the surface of mixed PC/PG membranes induced lipid clustering. In Figure 8 (left) are shown the ^{31}P NMR spectra of DMPC/DOPG membranes alone and after the addition of PLM^{37-72}, PLB^{1-23}, and $P\text{-}PLB^{1-23}$ to a lipid

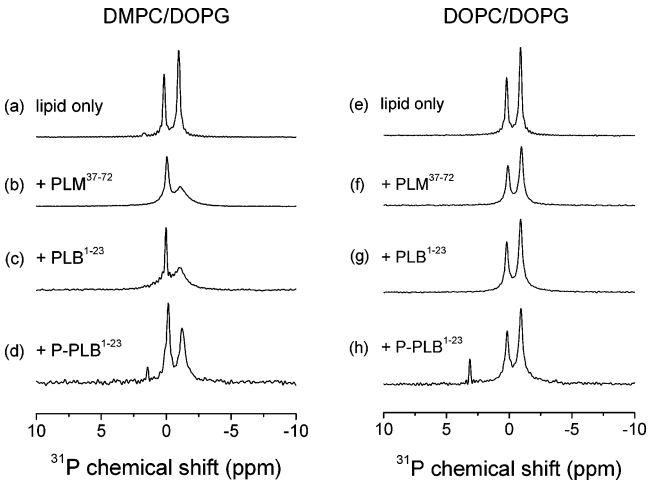

FIGURE 8: Phospholipid segregation in LMVs of PC/PG lipids induced by membrane surface binding of PLM and PLB peptides, as monitored by ^{31}P MAS NMR spectroscopy. Spectra were obtained at 4 °C from vesicles containing 5 mg of DMPC and DOPG (with DMPC in 2-fold molar excess over DOPG) before (a) and after the addition of PLM^{37-72} (b), PLB^{1-23} (c), and $P\text{-}PLB^{1-23}$ (d) at a lipid to peptide molar ratio of 20:1. Spectra were also obtained at 4 °C from vesicles containing 5 mg of DOPC and DOPG (with DOPC in 2-fold molar excess over DOPG) before (e) and after the addition of PLM^{37-72} (f), PLB^{1-23} (g), and $P\text{-}PLB^{1-23}$ (h) to a lipid to peptide molar ratio of 20:1.

Table 2: Summary of ^{31}P NMR Isotropic Chemical Shifts (σ_i) and Line Widths ($\Delta\nu_{1/2}$) at 4 °C for LMVs of PC/PG (with PC in 2-fold Molar Excess over PG) in the Presence and Absence of PLM^{37-72}, PLB^{1-23}, and $P\text{-}PLB^{1-23}$ at a Lipid to Peptide Molar Ratio of 20:1

	$\Delta\nu_{1/2}$ (ppm)			σ_i (ppm)	
sample	PC	PG	ratio	PC	PG
DMPC/DOPG					
lipid only	0.23	0.19	1.21	−1.00	0.05
+PLM^{37-72}	1.53	0.31	4.93	−1.16	−0.12
+PLB^{1-23}	1.29	0.19	6.78	−1.00	−0.03
+$P\text{-}PLB^{1-23}$	0.51	0.2	2.55	−0.99	0.06
DOPC/DOPG					
lipid only	0.25	0.23	1.09	−0.91	0.20
+PLM^{37-72}	0.38	0.37	1.03	−0.98	0.10
+PLB^{1-23}	0.40	0.36	1.11	−0.92	0.19
+$P\text{-}PLB^{1-23}$	0.44	0.43	1.02	−0.94	0.16

to peptide molar ratio of 20:1. In the absence of any peptide the widths of the two peaks from the lipid components are approximately equal and their intensities reflect the molar excess of DMPC over DOPG. After addition of each of the peptides, the peak for DOPG remains relatively narrow but the peak for DMPC broadens markedly, consistent with impaired mobility of this lipid species. The largest increase in line width occurred after the addition of PLM^{37-72}, while $P\text{-}PLB^{1-23}$ had the least effect (Table 2).

A control ^{31}P NMR experiment, in which the DMPC/DOPG membranes were replaced with DOPC/DOPG membranes, was carried out to establish whether selective broadening of the DMPC peak arises because the peptide *directly* restrains the DMPC molecules. In the control experiment both lipid components have unsaturated chains but present the same membrane surface environment to the peptide as do the DMPC/DOPG membranes. This change in lipid composition will have little or no effect upon the membrane affinity of surface binding peptides. Now, however, the peak for DOPC will broaden only if the peptide

exerts a direct restraint on the mobility of the lipids. The NMR peaks for the two lipid components will not broaden out if the peptide induces lipid clustering as described above, because the segregated lipid components will remain fully fluid at 4 °C. In Figure 8 (right) are shown the ^{31}P NMR spectra of DOPC/DOPG in a molar ratio of 2:1 before and after the addition of PLM$^{37-72}$, PLB$^{1-23}$, or P-PLB$^{1-23}$. The ratios of the peak widths remain essentially unchanged after the addition of the three peptides (Table 2). Hence, selective broadening of the peak from DOPC did not occur in the control experiment, suggesting that the peptides do not significantly restrain the mobility of PC lipids through a direct interaction with the choline headgroups. The selective broadening observed for the DMPC/DOPG membranes is therefore likely to be related to the chain composition of the lipids and is consistent with each of the peptides inducing clustering of the two lipid components into peptide-free, DMPC-rich domains and peptide-bound, DOPG-rich domains.

DISCUSSION

This paper presents studies of soluble peptide fragments of small regulatory transmembrane proteins, which have provided strong evidence that the cytoplasmic domains of PLB and PLM are capable of binding to the surface of lipid membranes. Previous EPR studies of PLB containing nitroxide spin-labels in the cytoplasmic domain have identified the presence of two protein species with distinct dynamic properties, one which was highly mobile and the other being relatively ordered (*13*). These species were attributed to different PLB conformers which preexist in dynamic equilibrium in the absence of contact with SERCA enzymes. The ordered species was suggested to correspond to a bent form of PLB in which the cytoplasmic domain is stabilized by lying across the membrane surface roughly perpendicular to the transmembrane domain. The more mobile species represents an extended form in which the cytoplasmic domain lies away from the membrane surface. Recent structural studies of PLB pentamers in dodecylphosphocholine micelles (*31*) have suggested that the cytoplasmic domain is, on average, oriented away from the membrane surface, apparently contradicting observations made here and elsewhere (*13*). This recent structural information, which was obtained in a zwitterionic model membrane system, does not preclude the possibility of the PLM cytoplasmic domain interacting with negatively charged membrane surfaces, however. The linker region between the cytoplasmic and transmembrane domains is highly disordered (*12*, *31*) and the motional excursion of the cytoplasmic domain about its average orientation could allow it to interact with negatively charged membrane surfaces.

Here, fluorescence data have shown that peptides corresponding to the cytoplasmic domain of PLB (PLB$^{1-23}$ and P-PLB$^{1-23}$) bind to the surface of lipid vesicles, and NMR data indicate that the peptides have a slightly higher affinity for negatively charged membrane surfaces than for uncharged surfaces. The association appears to be mediated through specific interactions with the lipid polar headgroups, which in mixed membranes of zwitterionic DMPC and anionic DOPG change the orientation of the choline moiety of phosphatidylcholine lipids (Figure 6). Moreover, binding of PLB$^{1-23}$ to membranes with overall negative surface charge gives rise to a marked increase in the helical content of the peptide. PLB$^{1-23}$ can be modeled as an amphipathic helix, with lysine and arginine groups situated on one face, and it is feasible that in full-length PLB these residues are oriented toward the membrane surface (*32*). In this configuration the residues may interact with negatively charged lipid phosphate moieties and stabilize the cytoplasmic domain in a helical conformation. Phosphorylation of PLB$^{1-23}$ at Ser-16 reduces its propensity to adopt a helical structure in TFE, in agreement with earlier NMR experiments which show that the helical region unravels from the C-terminal end after phosphorylation (*28*, *33*). The phosphorylated peptide, P-PLB$^{1-23}$, also has a lower helical content than PLB$^{1-23}$ in the presence of DMPC/DOPG membranes, but phosphorylation does not appear to affect its affinity for the membrane (Figure 3, b and c). It appears, therefore, that the membrane affinity of the PLB cytoplasmic domain may not be entirely dependent upon the N-terminal amino acids being stabilized as a helix at the membrane surface. Interestingly, ^{31}P MAS NMR experiments (Figures 4 and 5) have shown that the ^{31}P chemical shift of the P-PLB$^{1-23}$ serine phosphate is highly sensitive to interactions with the membrane surface. This suggests either that the peptide undergoes a conformational change at or around Ser-16 when it associates with the membrane surface or that the phosphate group is oriented toward the lipid headgroups.

The ^{31}P NMR spectra of P-PLB$^{1-23}$ in the presence of mixed lipid membranes (Figures 4 and 5) further indicate that the association of P-PLB$^{1-23}$ with membrane surfaces is rather weak and that the peptide undergoes rapid exchange (on the sub-millisecond time scale) between free and membrane bound environments. This observation is consistent with the proposal that the cytoplasmic domain of PLB exists in dynamic equilibrium between an ordered membrane-associated state and a disordered state away from the membrane surface (*13*). Clearly, the cytoplasmic domain of the full-length protein is anchored to the lipid bilayer by the transmembrane domain of PLB and has fewer degrees of motional freedom than do the peptides studied here. The weak interactions between P-PLB$^{1-23}$ and membrane surfaces suggest that the cytoplasmic domain of full-length PLB may interact only transiently with the phospholipid headgroups. It should be noted, however, that the equilibrium state of the PLB cytoplasmic domain at the SR membrane surface in vivo will depend upon the density of Ca^{2+}-ATPase and other transmembrane proteins within the SR membrane. Transient interactions between PLB and the SR membrane surface can only occur if the membrane protein density is low enough to expose enough membrane surface area to accommodate the PLB cytoplasmic domain.

The peptide fragment representing the cytoplasmic domain of PLM is, like PLB, capable of interacting with both neutral and negatively charged lipid membrane surfaces, although it is not possible to draw conclusions about relative binding affinities from the data presented. It is tempting to suggest that membrane interactions are a common feature underlying the structure and function of many or all the ATPase-regulating transmembrane proteins including other members of the FXYD family. Some caution should be exercised in drawing parallels between the behavior of the cytoplasmic domains of PLB and PLM, however, since important differences may exist in their modes of membrane binding.

One notable distinction in the behavior of PLM^{37-72} and PLB^{1-23} is that the former peptide remains predominantly unfolded at the membrane surface whereas PLB^{1-23} undergoes a coil−helix transition (Figure 7). The inability of the PLM cytoplasmic domain to adopt a more compact helical structure at the membrane surface could have implications for its ability to interact with membrane surfaces in the biological environment where steric considerations are more important.

An interesting feature of the results presented here is the ability of the PLM and PLB peptides to associate preferentially with anionic lipids within mixed lipid membranes and induce clustering of individual lipid components into peptide-free and peptide-bound domains (Figure 8). Lipid domain formation has been observed in a number of membrane binding peptides and proteins, but the biological role is not well understood. For example, annexins, calcium binding proteins thought to be involved in cellular trafficking, bind rapidly to membrane surfaces and induce lipid clustering (*34*). Annexins are also able to bind to specific regions of cellular membranes, and it has been suggested that they play a role in caveolae organization (*35*). Recently, we have reported that peptide fragments of the presynaptic protein α-synuclein can also organize lipids into clusters, which is consistent with the theory that the protein plays a role in synaptic vesicle formation or in cellular trafficking, by recruiting lipids to sites of endocytosis (*30*). In the case of PLB and PLM, no clear roles for these proteins in membrane ordering, trafficking, or domain formation have been defined, and the observed effects of the peptide fragments on the lipid organization within model membranes may have little or no biological relevance. This phenomenon justifies further investigation, however, since the functions of P-type ATPases including SERCA enzymes and Na^+/K^+-ATPases are highly sensitive to the lipid composition of the membrane, preferring lipids with anionic headgroups (*36*). It is speculated that in the cellular membrane the cytoplasmic domains of PLM and PLB could segregate anionic lipids away from the bulk bilayer environment, reducing the effective concentration of these lipids to support the maximal function of ATPase enzymes. By so doing, PLM and PLB could regulate their physiological targets indirectly, by modulating the membrane environment, as well as through direct contact with the ATPase partner.

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