The myristoylated alanine-rich protein kinase C sub- 
strate (MARCKS) may function to sequester phos- 
pholipids within the plane of the bilayer. To characterize 
this interaction with phosphatidylinositol 4,5-bisphos- 
phate (PI(4,5)P₂), a novel spin-labeled derivative, proxyl- 
PIP₂, was synthesized and characterized. In the presence 
of molecules known to bind PI(4,5)P₂, the EPR spectrum of 
this label exhibits an increase in line width because of a 
decrease in label dynamics, and titration of this probe 
with neomycin yields the expected 1:1 stoichiometry. 
Thus, this probe can be used to quantify the interactions 
made by the PI(4,5)P₂ head group within the bilayer. In the presence of a peptide comprising the effect- 
dor domain of MARCKS the EPR spectrum broadens, but 
the changes in line shape are modulated by both 
changes in label correlation time and spin-spin interac- 
tions. This result indicates that at least some proxyl- 
PIP₂ are in close proximity when bound to MARCKS and 
that MARCKS associates with multiple PI(4,5)P₂ mole- 
cules. Titration of the proxyl-PIP₂ EPR signal by the 
MARCKS-derived peptide also suggests that multiple 
PI(4,5)P₂ molecules interact with MARCKS. Site-di- 
rected spin labeling of this peptide shows that the posi- 
tion and conformation of this protein segment at the 
membrane interface are not altered significantly by 
bounding to PI(4,5)P₂. These data are consistent with the 
hypothesis that MARCKS functions to sequester multi- 
ple PI(4,5)P₂ molecules within the plane of the mem- 
brane as a result of interactions that are driven by elec- 
 trostatic forces.

Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂)₁ is one of the major polyphosphoinositols found in the plasma mem- 
brane, and it plays a key role in cell signaling. PI(4,5)P₂ a 
substrate for phospholipase Cs and the precursor for the second 
 messengers inositol trisphosphate and diacylglycerol (1). In 
addition, PI(4,5)P₂ is itself an important signaling molecule (2). 
PI(4,5)P₂ regulates cell motility and morphogenesis through 
its interaction with PH domains and actin-binding proteins 
(3–5) and plays a critical role in membrane trafficking (6, 7), 
the regulation of ion channel activity (8), and the activation 
of guanine nucleotide exchange (9). Although not as abundant as 
the major phospholipids phosphatidylcholine or phosphatidyl-
serine, PI(4,5)P₂ appears to be present in the plasma mem- 
brane at roughly constant levels on the order of 1 mol% (3). At 
these levels of PI(4,5)P₂ there would be large numbers of these 
lipids available to interact with proteins, and there is consid- 
erable interest in understanding how these interactions are 
regulated within the bilayer.

One concept that has emerged to explain the specific action 
of PI(4,5)P₂ is that local free levels of PI(4,5)P₂ within the 
bilayer are controlled by modulating its lateral heterogeneity. 
There are several ways in which the distribution of PI(4,5)P₂ 
within the cell might be regulated. Clearly, the enzymes that 
make PI(4,5)P₂ may be sequestered in certain regions of the 
bilayer, or PI(4,5)P₂ may be specifically accumulated into spe- 
ialized lipid domains such as cholesterol-rich lipid rafts (10). 
Proteins might also sequester or mask the presence of free 
PI(4,5)P₂, and there is evidence that the myristoylated ala- 
mine-rich protein kinase C substrate (MARCKS) specifically binds 
and regulates the levels of free PI(4,5)P₂ within the bilayer 
(11–14). MARCKS is a Ca²⁺-dependent protein kinase C sub- 
strate that is present in high concentrations in many cell types 
(15), it associates with the membrane in- 
terface though its 
N-terminal myristoylation as well as an electrostatic interac- 
tion of its highly basic effector domain with the membrane 
interface (16). The MARCKS effector domain (residues 151– 
175) is highly basic having the sequence KKKKKRFSFKKS- 
FKLSGFSSFKKKK. MARCKS binds strongly to Ca²⁺- 
calmodulin through this domain (17, 18), and this domain interacts 
with actin (19). Membrane-bound MARCKS may be dissociated 
from the membrane interface by calmodulin and by protein 
kine C, which phosphorylates MARCKS within its effector 
domain (20).

Although it has been implicated in a number of cellular 
processes, the exact role of MARCKS has been unclear. 
Because phosphorylation prevents MARCKS from binding to 
calmodulin, it was suggested that MARCKS might allow for an 
interaction between protein kinase C and calmodulin-dependent 
signaling pathways (17); however, recent work suggests 
that MARCKS actually functions to sequester PI(4,5)P₂ within 
the plane of the bilayer. The binding of MARCKS (151–175) to 
the membrane interface has been shown to inhibit the hydroli-
ysis of PI(4,5)P₂ by either phospholipase C (PLC)-δ or PLC-β (11, 14), presumably because this peptide competes successfully with the active site of PLC for PI(4,5)P₂. MARCKS has been shown to accumulate at lipid rafts and to codistribute with PI(4,5)P₂ (13), and a peptide derived from the effector domain of MARCKS (MARCKS (151–175)) has been shown to bind strongly to membranes containing PI(4,5)P₂ and PI(3,4)P₂ (12, 14). As a result of this interaction, MARCKS may bind a significant fraction of PI(4,5)P₂ within the plasma membrane. Free levels of PI(4,5)P₂ in the membrane could then be controlled either through protein kinase C, by controlling the phosphorylation state of the MARCKS effector domain, or through the cytoplasmic levels of Ca²⁺-calmodulin.

In the present study, we examined the interaction between MARCKS (151–175) and PI(4,5)P₂ using two approaches. First, we synthesized a novel spin-labeled derivative of PI(4,5)P₂ in which a proxyl nitroxide spin label was incorporated into the sn-1 acyl chain of the phospholipid (21). EPR spectroscopy then was used to examine the interactions between this spin label and known PI(4,5)P₂-binding macromolecules and the interactions between this label and MARCKS (151–175). Second, we derivatized a series of cysteine-substituted peptides based on MARCKS (151–175) with a sulfhydryl reactive spin label (Fig. 1) and used EPR spectroscopy to examine the conformation and structure of MARCKS (151–175) in the presence and absence of PI(4,5)P₂. The data demonstrate that the proxyl-PIP₂ is a useful probe for the interactions between PI(4,5)P₂ and molecules that bind the PI(4,5)P₂ head group within the plane of the bilayer. The data also indicate that MARCKS (151–175) associates with multiple PI(4,5)P₂ molecules within the plane of the membrane by a process that is driven largely by electrostatic interactions.

**EXPERIMENTAL PROCEDURES**

**Materials**

Palmitoyloleoylphosphatidylserine (PS), palmitoyloleoylphosphatidylcholine (PC), 5-doxyl phosphatidylcholine (5-doxyl PC), and the ammonium salt of phosphatidylinositol 4,5-bisphosphate (PIP₂) were obtained from Avanti Polar Lipids (Alabaster, AL). Neomycin was supplied from Avanti Polar Lipids (Alabaster, AL). N-Hydroxysuccinimydyl-3-carboxylate Proxyl, Free Radical (1)—A suspension of 3-carboxy proxyl, free radical (99.9 mg, 0.54 mmol) and dimethylaminopyridine (24.0 mg, 0.20 mmol) were added, and the reaction mixture was stirred overnight at room temperature. The precipitate was filtered off, and the filtrate was evaporated to dryness. The residue was suspended in EtOAc, filtered, and the filtrate evaporated to dryness. The product was purified through a plug of silica, eluted with 3:2 hexanes:EtOAc. The yield was 91.7 mg (60%) as an orange oil. This product yielded a single spot by TLC, Rₚ: 0.44 (3:7 hexanes:EtOAc); ES-MS: m/z = 285.1 [M+H⁺].

**Methods**

**Synthesis of a Spin-labeled Derivative of PI(4,5)P₂ (Fig. 2)**

N-Hydroxysuccinimimidyl-3-carboxylate Proxyl, Free Radical (1)—A suspension of 3-carboxy proxyl, free radical (99.9 mg, 0.54 mmol) and N-hydroxysuccinimide (67.6 mg, 0.59 mmol) was prepared in 10 ml of CH₂Cl₂ (distilled from CaH₂) and stirred for 20 min. Dicyclohexylcarbodiimide (116.1 mg, 0.56 mmol) and dimethylaminopyridine (24.0 mg, 0.20 mmol) were added, and the reaction mixture was stirred overnight at room temperature. The precipitate was filtered off, and the filtrate was evaporated to dryness. The residue was suspended in EtOAc, filtered, and the filtrate evaporated to dryness. The product was purified through a plug of silica, eluted with 3:2 hexanes:EtOAc. The yield was 91.7 mg (60%) as an orange oil. This product yielded a single spot by TLC, Rₚ: 0.44 (3:7 hexanes:EtOAc); ES-MS: m/z = 285.1 [M+H⁺].

**Expression and Purification of PLC-51 PH Domain**

Recombinant PH domain from human PLC-51 was produced from an Escherichia coli strain that was generously provided by Mario Rebecchi and purified following a procedure described previously (25). The identity of the PH domain was confirmed by gel electrophoresis and mass spectrometry, and the purity as judged by electrophoresis was greater than 95%.

**Lipid Vesicle Preparation**

Lipid bilayers having the desired lipid composition were produced by mixing the appropriate lipids from stock solutions in chloroform, removing the chloroform by vacuum desiccation, and hydrating the resulting lipid film in a buffer containing 100 mM KCl, 10 mM MOPS, pH 7.0. The lipid mixture was freeze-thawed five times, and unilamellar vesicles were produced by extrusion of the mixture through a polycarbonate filter (Porvair, Livermore, CA) using a LiposFast extruder (Avestine, Ottawa, Canada). Proxyl-PIP₂ could be incorporated into both leaflets of the membrane by dissolving the labeled lipid into the lipid chloroform solution; however, in all of the data shown here, the spin label was incorporated into the outer membrane leaflet by adding the vesicle solution to a dried film of proxyl-PIP₂. The addition of unlabeled or spin-labeled MARCKS (151–175) to preformed lipid ves-
The EPR spectrum of proxyl-PIP2 in the presence of neomycin or MARCKS (151–175) was fitted into the loop-gap resonator. The amplification of the central nitroxide resonance, $A(0)$, was then recorded as a function of the concentration of neomycin or MARCKS (151–175) added to the lipid sample. The EPR spectrum of proxyl-PIP2 in the presence of neomycin or MARCKS (151–175) is a linear combination of EPR signals from free lipid and lipid-bound to the macromolecule. If the line shapes for these species is known, the fraction of bound proxyl-PIP2 may then be determined from the value of $A(0)$ (see Equation 6).

**EPR Power Saturation Measurements**

Power saturation measurements were made on proxyl-PIP2 and on spin-labeled MARCKS (151–175) to determine the depth of the nitroxide label from the level of the lipid phosphate. These measurements were carried out in a manner similar to that described previously (27) using gas-permeable TPX capillary tubes (Medical Advances, Milwaukee WI). In each case, the peak-to-peak (or peak-to-trough) amplitude of the central $m_i = 0$ first derivative EPR resonance amplitude, $A(0)$, was then recorded as a function of the concentration of neomycin or MARCKS (151–175) added to the lipid sample. The EPR spectrum of proxyl-PIP2 in the presence of neomycin or MARCKS (151–175) is a linear combination of EPR signals from free lipid and lipid-bound to the macromolecule. If the line shapes for these species is known, the fraction of bound proxyl-PIP2 may then be determined from the value of $A(0)$ (see Equation 6).
the effector domain of MARCKS. In this case we assume the equilibrium in Reaction 2.

\[ M + (\text{PIP}_2)_n \rightleftharpoons M(\text{PIP}_2)_n \]  

**REACTION 2**

In this equilibrium it is assumed that MARCKS (151–175) binds in a single step to \( n \) proxyl-PIP\(_2\), and a quadratic equation that describes this binding may be derived using equations analogous to 3, 4, and 5 shown above. It should be noted that this binding assumes that \( n \) proxyl-PIP\(_2\) are in a preformed complex before binding to MARCKS (151–175). This is almost certainly not the case, and Reaction 2 should be taken only as an approximation of the actual events that take place during peptide binding.

**RESULTS**

Proxyl-PIP\(_2\) Incorporates Spontaneously into Lipid Vesicles—Shown in Fig. 3A is an EPR spectrum of the triethylammonium salt of proxyl-PIP\(_2\) dissolved into chloroform at a concentration of ~200 \( \mu M \). The EPR spectrum exhibits clear evidence for spin exchange, which likely results from the formation of inverted micelles in this nonpolar solvent. In the aqueous phase, PI(4,5)P\(_2\) is known to form micelles (29), and this also appears to be the case for proxyl-PIP\(_2\). Although proxyl-PIP\(_2\) is freely soluble in aqueous solution, no EPR spectrum can be observed at room temperature. This is consistent with the formation of micelles, which would promote strong dipolar interactions and/or spin exchange between labeled nitroxides and result in high relaxation rates. The lack of an observable EPR spectrum also indicates that the critical micelle concentration for proxyl-PIP\(_2\) must be lower than 5 \( \mu M \) because an aqueous concentration of monomeric proxyl-PIP\(_2\) at or above this concentration would have yielded a well resolved signal. When lipid vesicles formed from PC are added to solid proxyl-PIP\(_2\) as described above (see “Methods”), the EPR spectrum shown in Fig. 3B is observed. This spectrum is characteristic of a label that is monomeric in the membrane and undergoing relatively rapid motion, and it is reasonably well simulated assuming an isotropic rotational model where the nitroxide has a correlation time of about 6 ns.

To ensure that the spectrum in Fig. 3B arises from a nitroxide with a membrane location, the EPR spectrum was power saturated (see “Methods”) in the presence of \( O_2 \), NiAA, or NiEDDA. The values of \( \Delta P_{1/2} \) for \( O_2 \) and the metal complexes are consistent with a membrane location for the label. For proxyl-PIP\(_2\) in the presence of PC, we obtain a depth parameter, \( \Phi \), of ~0.77 using 20 \( \mu M \) NiAA. Using a calibration curve determined previously for PC bilayers (28), a location of 3 ± 2 Å below the level of the lipid phosphate is obtained. For proxyl-PIP\(_2\) incorporated into PC:PS membranes, a value of \( \Phi \) = 0.46 is obtained using 20 \( \mu M \) NiEDDA. This value yields a position of 6 ± 2 Å below the level of the lipid phosphate based on a calibration determined recently for PC:PS (30). Thus, the power saturation data are consistent with a membrane location for the proxyl-PIP\(_2\), where the label takes up a position a few Å within the bilayer below the level of the head group phosphate. This position is ~10 Å shallower than that expected if the glycerol backbone of the proxyl-PIP\(_2\) were located at the same position as the membrane lipid, and the acyl chain attached to the proxyl spin label were in a fully extended conformation.

Double integration of the spectrum in Fig. 3B yielded a spin concentration of ~90 \( \mu M \), which is close to the concentration of nitroxide label added to the vesicle suspension. Thus, this label fully incorporates into the lipid bilayer when absorbed to vesicles from the external aqueous solution. Because PI(4,5)P\(_2\) has ~3 negative charges at neutral pH it is not expected to undergo transmembrane migration; as a result, proxyl-PIP\(_2\) that is incorporated in this manner should reside on the external surface of these preformed lipid vesicles. In subsequent experiments, detailed below, proxyl-PIP\(_2\) has been incorporated in this manner into the external vesicle surface.

Proxyl-PIP\(_2\) Is Sensitive to the Interaction with Neomycin and the PH Domain from PLC-51—To determine whether the EPR spectrum of proxyl-PIP\(_2\) is sensitive to interactions of its head group, we compared the EPR spectra of proxyl-PIP\(_2\) in the presence and absence of neomycin and the PH domain from PLC-51, two well characterized PI(4,5)P\(_2\)-binding molecules. Shown in Fig. 4, A and B, are EPR spectra for proxyl-PIP\(_2\) in the presence and absence of neomycin and the PLC-51 PH domain. Both of these molecules are known to exhibit strong 1:1 binding to PIP\(_2\), and in both cases, the EPR spectra exhibit a similar broadening in the presence of these reagents. This
level of broadening corresponds to an increase in the rotational correlation time for the proxyl label of about 25%.

There are several mechanisms that might give rise to this change in rotational correlation time. The proxyl spin label is quite mobile when attached to PI(4,5)P₂, which is not unreasonable given the alkyl chain and rotatable bonds that link the label to the glycerol backbone. However, bond rotations and librations of this chain are expected to be of limited amplitude, and some averaging of the nitroxide magnetic interactions should result from lipid rotational motion that takes place on the ns time scale (31). As a result, any interaction that slows the rotational rate of the labeled lipid, such as attachment to a large macromolecule, should broaden its EPR spectrum. Conceivably, interactions with the PI(4,5)P₂ head group might sterically interfere with the spin label and reduce the amplitude of motion of the proxyl label attached to the sn-1 chain. Finally, the if the interaction with proxyl-PIP₂ altered the membrane position of the label, changes in label motion might result because of the altered environment around the label.

To determine whether more quantitative information can be extracted from the interaction of proxyl-PIP₂ with a PI(4,5)P₂-binding species, we titrated the first derivative EPR line amplitude (see “Methods”) as a function of the neomycin concentration. Shown in Fig. 5 are the first derivative EPR amplitudes obtained from this titration. Addition of neomycin results in the formation of a PI(4,5)P₂-neomycin complex and a change in label dynamics. These data points were then fit using Equations 3–6 assuming a 1:1 binding of neomycin to PIP₂. In this fit, both $K_a$ and $A_m$ were allowed to be adjustable parameters. The agreement between these data and this fit is quite reasonable, and the accuracy of this fit is very sensitive to the stoichiometry of the interaction between PIP₂ and neomycin. The value of the molar partition coefficient, $K_m$, which is obtained in this fit is $\sim 3 \times 10^5 \text{ M}^{-1}$, which is close to that obtained previously (32).

It should be noted that neomycin has been reported to promote the transport of phosphoinositides across cell membranes (33). The mechanism leading to this transport is not understood, and experiments to determine whether neomycin can facilitate the transport of proxyl-PIP₂ in the model membrane systems used here are currently in progress.

**Multiple PI(4,5)P₂ Molecules Interact with MARCKS—**

Shown in Fig. 6A are EPR spectra of proxyl-PIP₂ in the presence and absence of MARCKS (151–175) in PC vesicles. MARCKS (151–175) has a high affinity for membranes containing PI(4,5)P₂ (14), and in Fig. 6A sufficient peptide has been added to bind all of the available proxyl-PIP₂ completely. As seen previously for neomycin and the PH domain, MARCKS (151–175) also produces a broadening of the proxyl-PIP₂ line shape. However, the broadening that is seen in the presence of MARCKS (151–175) is greater than that seen in Fig. 4 for neomycin or the PH domain.

At least two mechanisms may be acting to produce the line width changes seen in Fig. 6A. First, MARCKS (151–175) may diminish the motional averaging of the proxyl-labeled lipid in a manner similar to that seen for the PH domain or neomycin. Second, if MARCKS (151–175) binds multiple PI(4,5)P₂ molecules, an additional line width broadening might result from the proximity between spin labels. To determine whether some of the broadening is caused by the proximity between spin labels, the spectra shown in Fig. 6B were obtained under conditions where proxyl-PIP₂ was diluted with unlabeled PI(4,5)P₂. When the spin-labeled lipid is diluted by a factor of 1:3, the line shapes are still broadened upon the addition of MARCKS (151–175), but there is significantly less broadening than was seen in Fig. 6A. The effect of diluting the proxyl-PIP₂ suggests that multiple PI(4,5)P₂ species interact with MARCKS (151–175). If two or more proxyl-PIP₂ are brought sufficiently close to each other so that dipole-dipole or collisional exchange mechanisms take place, an additional line

---

**FIG. 5.** Titration of the central EPR resonance of proxyl-PIP₂ as a function of concentration of added neomycin. The total proxyl-PIP₂ concentration is 50 $\mu$M in PC vesicles at a lipid concentration of 20 mM. The data shown ( ) were obtained from two independent titration experiments. The solid line represents a nonlinear least squares fit through the data using Equations 3–6, which assume a 1:1 stoichiometry, where $K_m = 3 \times 10^5 \text{ M}^{-1}$. The agreement between these data and this fit is quite reasonable, and the accuracy of this fit is very sensitive to the parameters. The value of the molar partition coefficient, $K_m$, which is obtained in this fit is $\sim 3 \times 10^5 \text{ M}^{-1}$, which is close to that obtained previously (32).
width broadening will result. When these samples are rapidly frozen in LN₂ (data not shown), a dipolar broadened spectrum can be observed. Low temperature eliminates the effects of motion on the nitroxide EPR spectrum and allows the strength of the dipolar interaction to be estimated (34). Assuming a pairwise interaction, the EPR spectra indicate that proxyl-PIP₂8 are separated by distances on the order of 18–22 Å when bound to MARCKS (151–175) (35).

The EPR spectra shown in Fig. 6A were titrated as a function of the concentration of MARCKS (151–175), and Equation 6 was used to estimate the fraction of bound proxyl-PIP₂. The result is shown in Fig. 7 along with a curve generated using Equations 3–5 corresponding to a 1:1 binding of proxyl-PIP₂ to MARCKS (151–175). These data cannot be fit to a 1:1 binding, regardless of the choice of affinity constants. Also shown in Fig. 7 is a curve representing the best fit using the equilibrium given by Reaction 2 where both n and Kₐ are allowed to be adjustable parameters. A value of n = 3 produces the best fit to the data, but any stoichiometry (PI(4,5)P₂:MARCKS) in the range of 2.5–3.5 gives an acceptable fit to these data. The apparent molar partitioning obtained in this fit is 4 × 10⁵ M⁻¹, somewhat less than that expected based upon the binding measured under dilute conditions (14). The binding of MARCKS (151–175) is known to depend strongly on the surface charge density, and this slightly lower apparent affinity is not surprising given that the titration covers a range that saturates the free PIP₂ (and thus the negative surface charge density) in the bilayer. The equilibrium given in Reaction 2 is also simplified and cannot represent the actual molecular steps taking place. As a result the fit (solid line) shown in Fig. 7 must be viewed with some caution. Nonetheless, taken together with the data in Fig. 6, this titration provides strong evidence that multiple PI(4,5)P₂ molecules interact with the effector domain of MARCKS.

**MARCKS Does Not Change Position or Structure When Bound to PI(4,5)P₂-containing Membranes**—Previous work on MARCKS (151–175) demonstrated that this peptide assumed an extended structure when bound to PC:PS with its five phenylalanine residues buried within the interface (22). We find no evidence for significant structural changes in MARCKS (151–175) when complexed to PI(4,5)P₂. Shown in Table I are the central line widths for the EPR spectra of five single spin-labeled and five double spin-labeled MARCKS (151–175) in PC:PS (3:1) or PC:PI(4,5)P₂. In all cases, except one, the EPR line shapes are identical (within experimental error) when bound to PC:PS or PC:PI(4,5)P₂. There is a slight decrease in line width at a position closest to the N-terminal end of the peptide. This highly charged end of the peptide is positioned off the membrane interface, and the change in line shape may represent a slight shift in its position when these two lipid mixtures are compared. For the double labeled peptides, the increased line widths for i, i+3 and i, i+7 compared with the single labeled species are caused by interactions between the nitroxides (either dipole-dipole or spin exchange). These line widths should be strongly dependent upon the average separation between nitroxides and hence any change in average shape or secondary structure. These line widths are identical in the presence and absence of PI(4,5)P₂.

Shown in Fig. 8 are the EPR spectra for spin-labeled MARCKS (151–175) bound either to PC:PS- or PC:PI(4,5)P₂-containing membranes. Shown in Fig. 8A are two single labeled spectra for MARCKS (151–175), whereas Fig. 8B shows EPR spectra for two peptides with nitroxide pairs separated by i, i+3 and i, i+7, respectively. The spectra bound to PC:PS are unchanged compared with the case where the peptide is bound to PC:PI(4,5)P₂ membranes. The single labeled spectra arise from nitroxides that have a correlation time of about 3–4 ns, consistent with their attachment to an extended flexible peptide. In PC:PI(4,5)P₂, depth measurements were made for several singly spin-labeled MARCKS peptides and compared with depths obtained previously in PC:PS (3:1). The data are shown in Table II. In the central and C-terminal region of the peptide the label is at a depth of ~7 Å below the level of the lipid phosphate in PC:PI(4,5)P₂, and at the N terminus, the label is in the aqueous phase several Å from the lipid phosphate in this lipid mixture. These depths are identical, within experimental error, to the depths obtained previously in PC:PS (22).

Taken together, these data indicate that binding by PI(4,5)P₂ produces no significant conformational change in the membrane-bound structure of the effector domain of MARCKS, consistent with the less direct findings of CD measurements (14); in addition, PI(4,5)P₂ binding does not change the position of the MARCKS domain at the membrane interface. For all of the labeled residues examined, PI(4,5)P₂ binding does not significantly alter the dynamics of the spin-labeled side chain.

**DISCUSSION**

The work that is described here was carried out with several objectives in mind. First we wanted to investigate a spin-labeled derivative of PI(4,5)P₂ to determine whether it would provide a probe for protein-polyphosphoinositide head group interactions within the plane of the bilayer. Second, we wanted to characterize the interactions between the effector domain of MARCKS and PI(4,5)P₂. The proxyl-PIP₂ spin label synthesized here readily dissolves in chloroform or aqueous solution, presumably as inverted or normal micelles as discussed above. As a result, it was possible to incorporate reproducibly the probe into one or both leaflets of a lipid vesicle.

The EPR spectrum of the proxyl-PIP₂ broadens when it binds to either neomycin or the PH domain from PLC-61, and the change in line shape appears to be the result of a change in the dynamics of the proxyl label, so that the extent of motion averaging of the magnetic interactions of the nitroxide is reduced. When the EPR signal of proxyl-PIP₂ is titrated with
neomycin, the expected 1:1 stoichiometry is revealed. Thus, this probe is sensitive to interactions with macromolecules that are known to bind the PI(4,5)P₂ head group. In addition, the probe is sensitive to the local clustering of PI(4,5)P₂, which gives rise to spin-spin interactions between nitroxide labels. This sensitivity is potentially extremely useful for the examination of naturally occurring PI(4,5)P₂, and it does not resemble the diacylglycerol moiety of this lipid.

Power saturation of the EPR spectrum of proxyl-PIP₂ indicates that the probe is sensitive to interactions with macromolecules that are known to bind the PI(4,5)P₂ head group. In addition, the probe is sensitive to the local clustering of PI(4,5)P₂, which gives rise to spin-spin interactions between nitroxide labels. This sensitivity is potentially extremely useful for the examination of naturally occurring PI(4,5)P₂, and it does not resemble the diacylglycerol moiety of this lipid.
gives rise to spin-spin exchange or dipolar broadening, indicating that more than one proxyl-PIP$_2$ is bound to the effector domain of MARCKS. The data obtained here are consistent with a stoichiometry of about 2.5–3.5 proxyl-PIP$_2$/MARCKS, a number that is in approximate agreement with that found based on competition and electrophoretic mobility measurements (14).

The association between MARCKS (151–175) and proxyl-PIP$_2$ is strong enough to slow the rotational rate of the labeled lipid and/or alter the dynamics of the proxyl moiety, but this binding does not appear to involve specific molecular contacts at the lipid concentrations used here (14). An aqueous peptide population would also be readily apparent from the EPR spectra of these spin-labeled peptides, and none is detected (38).

The association between MARCKS (151–175) and proxyl-PIP$_2$ is strong enough to slow the rotational rate of the labeled lipid and/or alter the dynamics of the proxyl moiety, but this binding does not appear to involve specific molecular contacts at the lipid concentrations used here (14). An aqueous peptide population would also be readily apparent from the EPR spectra of these spin-labeled peptides, and none is detected (38).

The data obtained here are consistent with a stoichiometry of about 2.5–3.5 proxyl-PIP$_2$/MARCKS, a number that is in approximate agreement with that found based on competition and electrophoretic mobility measurements (14).

The association between MARCKS (151–175) and proxyl-PIP$_2$ is strong enough to slow the rotational rate of the labeled lipid and/or alter the dynamics of the proxyl moiety, but this binding does not appear to involve specific molecular contacts at the lipid concentrations used here (14). An aqueous peptide population would also be readily apparent from the EPR spectra of these spin-labeled peptides, and none is detected (38).
affinity. Thus, MARCKS is likely to sequester other phosphoinositides based largely on their valence. It should be noted that interactions with proxyl-PIP₂ are not seen for all basic peptides. When we titrated PC:PIP₂-proxyl membranes with pentalysine under the same conditions used here for MARCKS (151–175), there was no significant change in line shape and no evidence for a high affinity interaction (data not shown), consistent with the finding that pentalysine does not bind to PC:PI(4,5)P₂ (39). Thus, although the N-terminal end of the MARCKS effector domain begins with a Lys₉ sequence, this sequence alone is not sufficient to sequester PI(4,5)P₂. Again, this is consistent with the idea that the ability of MARCKS effector domain to sequester PI(4,5)P₂ is driven by electrostatic interactions. Electrostatic fields are additive, and the distribution of ions around a charged site will depend upon the exponent of the valence of the site. As a result, peptides with a larger net positive charge will have a greater ability to alter the lateral distribution of negatively charged lipids such as PI(4,5)P₂. Discrete binding sites for PI(4,5)P₂ do not exist on the lateral distribution of negatively charged lipids such as PI(4,5)P₂.

In summary, a spin-labeled derivative of PI(4,5)P₂ has been synthesized and shown to report interactions between PI(4,5)P₂ and molecules that are known to bind the PI(4,5)P₂ head group within the plane of the bilayer. This probe is sensitive to changes in label motion which result from interactions at the membrane interface, and it can be used to determine the stoichiometry of protein-PI(4,5)P₂ interactions. The probe is also sensitive to local clustering, such as might be found in a PI(4,5)P₂-rich domain. Data obtained using this probe indicate that a peptide derived from the effector domain of MARCKS interacts with PI(4,5)P₂ with a stoichiometry that is greater than 1:1. These data support the hypothesis that MARCKS functions to sequester PI(4,5)P₂ within the plane of the bilayer.

Acknowledgments—We thank Karen Zaiger for the synthesis of several of the spin-labeled MARCKS peptides used in this study. The spin-labeled PI(4,5)P₂ was prepared with precursors provided in part by Echelon Research Laboratories, Inc. We also thank Stuart McLaughlin for helpful discussions during the course of this work.

REFERENCES

1. Berridge, M. J. (1987) Annu. Rev. Biochem. 56, 159–193
2. Vanhaesebroeck, B., Leevers, S. J., Khaterah, A., Timms, J., Katso, R., Dresdell, P. C., Woscholski, R., Parker, P. J., and Waterfield, M. D. (2001) Annu. Rev. Biochem. 70, 59–92
3. Toker, A. (1998) Curr. Opin. Cell Biol. 10, 254–261
4. Raucher, D., Stauffer, T., Chen, W., Shen, K., Gao, S., York, J. D., Sheets, M. P., and Meyer, T. (2000) Cell 100, 221–228
5. Czyczkin, M. P. (2000) Cell 100, 693–696
6. Martin, T. F. (1997) Curr. Opin. Neurobiol. 7, 331–338
7. Martin, T. F. (2001) Curr. Opin. Cell Biol. 13, 495–499
8. Kobrinsky, E., Mandahl, T., Zhang, H., Jin, T., and Logothetis, D. E. (2000) Nat. Cell Biol. 2, 507–514
9. Randazzo, P. A. (1997) J. Biol. Chem. 272, 6788–7692
10. Pike, L., and Miller, J. M. (1998) J. Biol. Chem. 273, 22298–22304
11. Glaser, M., Watanuki, S., Buser, C. A., Boguslavsky, V., Kashitza, W., Morris, A., Rebucci, M., Scarlata, S. F., Runnels, L. W., Prestwich, G. D., Chen, J., Aderem, A., Ahn, J., and McLaughlin, S. (1996) J. Biol. Chem. 271, 26187–26195
12. Arbuzaova, A., Wang, L., Wang, J., Hangyas-Mihalyne, G., and McLaughlin, S. A. (2001) J. Biol. Chem. 276, 5012–5019
13. Aderem, A. (1992) Cell 71, 715–716
14. Murray, D., Ben-Tal, N., Honig, B., and McLaughlin, S. (1997) Structure 5, 983–989
15. McIlroy, B. K., Walters, J. D., Blackshear, P. J., and Johnson, J. D. (1991) J. Biol. Chem. 266, 4959–4964
16. Verghese, G. M., Johnson, J. D., Vasulka, C., Haupt, D. M., Stumpo, D. J., and Blackshear, P. J. (1994) J. Biol. Chem. 269, 9361–9367
17. Laux, T., Fakami, K., Thelen, M., Golub, T., Frey, D., and Caroni, P. (2000) J. Cell Biol. 149, 1455–1471
18. Wang, J., Arbuzaova, A., Hangyas-Mihalyne, G., and McLaughlin, S. A. (2001) J. Biol. Chem. 276, 5012–5019
19. Blackshear, P. J. (1993) J. Biol. Chem. 268, 1501–1504
20. Kim, J. K., Blackshear, P. J., Johnson, D. J., and McLaughlin, S. A. (1994) Biochim. Biophys. Acta 121, 227–237
21. Prestwich, G. D. (1996) Acc. Chem. Res. 29, 503–509
22. Qin, Z., and Cafiso, D. S. (1996) Biochemistry 35, 2917–2925
23. Chen, J. J., Proft, A. A., and Prestwich, G. D. (1996) J. Org. Chem. 61, 6305–6312
24. Bartlett, G. R. (1959) J. Biol. Chem. 234, 466–468
25. Tall, E., Dorman, G., Garcia, P., Runnels, L., Shah, S., Chen, J. J., Proft, A. A., Gu, Q. M., Chaudhary, A., Prestwich, G. D., and Rebucci, M. J. (1997) Biochemistry 36, 7239–7248
26. Archer, S. J., Ellena, J. F., and Cafiso, D. S. (1991) Biophys. J. 60, 389–398
27. Victor, K., and Cafiso, D. S. (1998) Biochemistry 37, 3402–3410
28. Aaltenbach, C., Greenhalgh, D. A., Khorana, H. G., and Hubbell, W. L. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11286–11291
29. van Paridon, P. A., de Kruijff, B., Ouwerkerk, K., and Wirtz, K. W. A. (1986) Biochim. Biophys. Acta 877, 216–219
30. Victor, K. G., and Cafiso, D. S. (2001) Biophys. J. 81, 2241–2250
31. Pastor, R. W., Venable, R. M., Karplos, M., and Szabo, A. (1988) J. Chem. Phys. 89, 1128–1140
32. Gabev, E., Kastanowicz, J., Abbott, S., and McLaughlin, S. (1989) Biochim. Biophys. Acta 979, 105–112
33. Ozaki, S., DeWald, D. B., Shope, J. C., Chen, J., and Prestwich, G. D. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 11286–11291
34. Rabenstein, M. D., and Shin, Y.-K. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8439–8443
35. Gross, A., Columbus, L., Hidek, C., Aaltenbach, C., and Hubbell, W. L. (1999) Biochemistry 38, 10324–10335
36. Chattopadhyay, A., and London, E. (1987) Biochemistry 26, 39–45
37. Hubbell, W. L., Gross, A., Langen, R., and Lietzow, M. A. (1998) Curr. Opin. Struct. Biol. 8, 649–656
38. Victor, K., Jacob, J., and Cafiso, D. S. (1999) Biochemistry 38, 12527–12536
39. Denisov, G., Winnik, S., Luan, P., Glaser, M., and McLaughlin, S. (1998) Biophys. J. 74, 731–744
