Lateral Sequestration of Phosphatidylinositol 4,5-Bisphosphate by the Basic Effector Domain of Myristoylated Alanine-rich C Kinase Substrate Is Due to Nonspecific Electrostatic Interactions*

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A peptide corresponding to the basic (+13), unstructured effector domain of myristoylated alanine-rich C kinase substrate (MARCKS) binds strongly to membranes containing phosphatidylinositol 4,5-bisphosphate (PIP$_2$). Although aromatic residues contribute to the binding, three experiments suggest the binding is driven mainly by nonspecific local electrostatic interactions. First, peptides with 13 basic residues, Lys-13 and Arg-13, bind to PIP$_2$-containing vesicles with the same high affinity as the effector domain peptide. Second, removing basic residues from the effector domain peptide reduces the binding energy by an amount that correlates with the number of charges removed. Third, peptides corresponding to a basic region in GAP43 and MARCKS effector domain-like regions in other proteins (e.g. MacMARCKS, adducin, Drosophila A kinase anchor protein 200, and N-methyl-D-aspartate receptor) also bind with an energy that correlates with the number of basic residues. Kinetic measurements suggest the effector domain binds to several PIP$_2$. Theoretical calculations show the effector domain produces a local positive potential, even when bound to a bilayer with 33% monovalent acidic lipids, and should thus sequester PIP$_2$ laterally. This electrostatic sequestration was observed experimentally using a phospholipase C assay. Our results are consistent with the hypothesis that MARCKS could reversibly sequester much of the PIP$_2$ in the plasma membrane.

Phosphatidylinositol 4,5-bisphosphate (PIP$_2$)\textsuperscript{1} plays many important roles in cells (reviewed in Refs. 1–8). Not only is PIP$_2$ the source of 3 second messengers, its hydrolysis via phospholipase Cs (PLCs) (9, 10) produces inositol 1,4,5-trisphosphate and diacylglycerol (11, 12), and its phosphorylation via phosphoinositide 3-kinases produces phosphatidylinositol 3,4,5-trisphosphate (6, 13–15), but it also can be a second messenger itself (4, 8, 16, 17). Moreover, PIP$_2$ helps regulate cytoskeletal attachment (18–20), exo- and endocytosis (1–3), enzyme activity (21), and ion channel function (17, 22). Several groups (2, 8, 16, 23) have suggested that these myriad functions can be explained if there are different pools of PIP$_2$ in the plasma membrane. One hypothesis is that proteins act as reversible buffers to bind much of the PIP$_2$ and then release it locally in response to specific signals (8, 24, 25). These proteins would have to be present at a concentration comparable with PIP$_2$, be localized to the plasma membrane in quiescent cells. The binding of MARCKS to plasma membranes requires both hydrophobic insertion of its N-terminal myristate into the bilayer and electrostatic interactions between its effector domain and monovalent acidic lipids in the membrane (28). The membrane-bound basic effector domain produces a significant positive electrostatic potential that can act as a basin of attraction for multivalent acidic lipids such as PIP$_2$. The electrostatic sequestration of PIP$_2$ can be reversed either by the binding of calcium/calmodulin to the effector domain or by the PKC phosphorylation of the effector domain, which decreases the positive electrostatic potential (33).

MARCKS has an extended conformation in solution (34, 35) and may thus be classified as a “natively unfolded” protein (36, 37). MARCKS-(151–175), a peptide corresponding to the basic substrate; DAKAP200, Drosophila A kinase anchor protein 200; NMDA N-methyl-D-aspartate receptor; GAP43, growth-associated protein 43,000; CAP23, cortical cytoskeleton-associated protein of approximate molecular mass 25 kDa; N-WASP, neuronal Wiskott-Aldrich Syndrome protein; SCAMP2, secretory carrier membrane protein 2; AKAP79, A-kinase anchoring protein 79; PLD, phosphatidylcholine-specific phospholipase D; PLC, phosphoinositide-specific phospholipase C; PH, pleckstrin homology; PLC-PH, PH domain of PLC-


drich Syndrome protein; SCAMP2, secretory carrier membrane protein 2; AKAP79, A-kinase anchoring protein 79; PLD, phosphatidylcholine-specific phospholipase D; PLC, phosphoinositide-specific phospholipase C; PH, pleckstrin homology; PLC-PH, PH domain of PLC-δ1; PKC, protein kinase C; NEM, N-ethylmaleimide; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; NLPB, nonlinear Poisson-Boltzmann; MALDI, matrix-assisted laser desorption ionization; DMF, N,N-dimethylformamide; MOPS, 4-morpholinoethanesulfonic acid; NLPB, nonlinear Poisson-Boltzmann.

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‡ The abbreviations used are: PIP$_2$ or P1(4,5)P$_2$, phosphatidylinositol 4,5-bisphosphate; PIP$_3$, P(3,4,5)P$_3$, phosphatidylinositol 3,4,5-bisphosphate; IP$_3$, inositol 1,4,5-trisphosphate; PC, phosphatidylcholine; PS, phosphatidyserine; MARCKS, myristoylated alanine-rich C kinase substrate: MARCKS-(151–175), a peptide corresponding to residues 151–175 of bovine MARCKS; ∆C-MARCKS, a peptide lacking 3 Lys residues at the C terminus of MARCKS-(151–175); ∆N-MARCKS, a peptide lacking 5 Lys residues at the N terminus of MARCKS-(151–175); ∆N∆C-MARCKS, a peptide lacking 3 Lys residues at the C terminus and 5 Lys residues at the N-terminus of MARCKS-(151–175); FA-MARCKS, a peptide with all 5 Phe replaced by Ala in MARCKS-(151–175); Mac-MARCKS, macrophage-enriched myristoylated alanine-rich C kinase

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TABLE I

Sequences of peptides

| Peptide           | Sequence               | Ref. |
|-------------------|------------------------|------|
| MARCKS peptides   |                        |      |
| MARCKS-(151–175)  | CKKKKKRSSEKKSFLSLGESEKKKR | 26, 27 |
| ΔC-MARCKS         | CKKKKKRSSEKKSFLSLGESEKKKR |      |
| ΔN-MARCKS         | CRRFSSEKFLSLGESEKKKR    |      |
| ΔNAC-MARCKS       | CRRFSSEKFLSLGESEKKKR    |      |
| FA-MARCKS         | CRRKRRSKASSSKEKLSGASAKKK |      |
| Poly-Lys/Arg peptides |                    |      |
| Arg-13            | CRRRRRRRRRRRRRRR        |      |
| Lys-13            | CKKKKKKKKKKKKKKKKKKKK   |      |
| Lys-10            | CKKKKKKKKKKKKKKKKKKKK   |      |
| Lys-7             | CKKKKKKKKKKKKKKKKKKKK   |      |
| MARCKS-like peptides |                   |      |
| MacMARCKS-(87–110)| CKKKKKFKFSEKFLSLGESEKKKR | 86, 111 |
| Adducin-(717–734) | CKKKKKFKFSEKFSKKKKKKK   | 88   |
| DAKAP200-(119–141)| CRRKRRSKASSSKEKLSGASAKKK | 81   |
| NMDA-NRI-(875–898)| CRRKRRSKASSSKEKLSGASAKKK | 89, 90 |
| GMC family peptides |                   |      |
| GAP43-(30–56)    | CRRKRRSKASSSKEKLSGASAKKK | 93   |
| CAP25-(1–15)     | CRRKRRSKASSSKEKLSGASAKKK | 112  |
| Other peptides    |                        |      |
| PLD2-(554–575)   | CRRKRRSKASSSKEKLSGASAKKK | 113  |
| SCAMP2-(201–211) | CRRKRRSKASSSKEKLSGASAKKK | 114  |
| N-WASP-(181–197) | CRRKRRSKASSSKEKLSGASAKKK | 115, 117 |
| WASP-(225–232)   | CRRKRRSKASSSKEKLSGASAKKK |      |
| Syndecan-4-(188–194)|                    | 95   |

Effect domain, also in an extended form both in solution (38) and bound to membranes containing acidic lipids (e.g. phosphatidylserine (PS) or PIP₂) (39, 40). This effector domain peptide is a good model for studying the interaction of MARCKS with membranes, calcium/calmodulin, and PKC (29); most importantly, both MARCKS (41) and MARCKS-(151–175) (25, 41) inhibit the PLC-catalyzed hydrolysis of PIP₂.

In the work reported here, we investigated whether the binding of MARCKS-(151–175) to PIP₂ is due to nonspecific electrostatic interactions. We used three approaches to determine whether the binding depends on specific residues or just on the number of basic residues. We measured the binding of truncated versions of MARCKS-(151–175) (i.e. peptides missing basic residues from the N- and/or C-terminal regions) to PC/PIP₂ vesicles. We also measured the binding of peptides corresponding to basic regions in other proteins (macrophage-enriched myristoylated alanine-rich C kinase substrate (MacMARCKS), adducin, Drosophila A kinase anchor protein 200 (DAKAP200), N-methyl-b-aspartate (NMDA) receptor, and growth-associated protein of 117 kDa) to PC/PIP₂ vesicles to determine whether the binding correlates with the number of basic residues. We compared the binding of peptides with 13 Lys or 13 Arg residues to investigate whether the chemical nature of the basic residues affects the interaction with PIP₂.

We further tested the hypothesis that several PIP₂ diffuse together to form a binding site for MARCKS-(151–175) (8, 25, 39) by examining the effect of the mole fraction of PIP₂ in the membrane on the forward rate constant for the binding of the peptides to PC/PIP₂ vesicles. Finally, we determined the relative ability of MARCKS-(151–175) and other basic peptides to sequester PIP₂ laterally in membranes containing physiological concentration of PS by examining the ability of these peptides to decrease the PLC-catalyzed hydrolysis of PIP₂.

EXPERIMENTAL PROCEDURES

Materials—1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (PC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Alabaster, AL). The ammonium salt of l-α-phosphatidyl-d-myo-inositol 4,5-bisphosphate (PIP₂) was purchased either from Avanti Polar Lipids (Alabaster, AL) or Roche Molecular Biochemicals or purified from bovine brain extract (Sigma) as described elsewhere (42). Labeled [diloleoyl-1-14C]-l-α-diloleoylphosphatidylcholine ([14C]DOPC), [inositol-2-3H]-l-α-phosphatidyl-d-myo-inositol 4,5-bisphosphate ([3H]PIP₂), and [ethyl-1,2-3H]-ethylthymeleimide ([3H]NEM) were from PerkinElmer Life Sciences. Nonradioactive N-ethylmaleimide (NEM) was from Sigma. 6-Acryloyl-2-dimethylaminonaphthalene (acyrlydon) was from Molecular Probes, Inc. (Eugene, OR). Recombinant human PLC-δ₅ was purified from Escherichia coli as described elsewhere (43).

Peptides—Unless specified, all peptides (sequences listed in Table I) were obtained from American Peptide Co., Inc. (Sunnyvale, CA), and were determined to be >80% pure by reverse phase-high pressure liquid chromatography and MALDI-time-of-flight mass spectroscopy. Each peptide was blocked with an acetyl group at its N terminus and an amide group at its C terminus. A peptide corresponding to a basic region in secretory carrier membrane protein 2 (SCAMP2-(201–211)) was a generous gift from Prof. David Cai; its N terminus is unblocked, introducing an extra positive charge.

In the binding and kinetic measurements, we used peptides with an extra Cys at the N terminus, which permitted covalent attachment of either a radioactive ([3H]NEM) or a fluorescent (acyrlydon) label. In the i potential measurements, we used MARCKS-(151–175) and Lys-13 without the extra N-terminal Cys. In the PLC assays, we used MARCKS-(151–175) and a peptide corresponding to a basic region in phosphatidylcholine-specific phospholipase D2 (PLD2-(554–575)) without the extra N-terminal Cys, but GAP43-(30–56), ΔNAC-MARCKS, Lys-7, and SCAMP2-(201–211) had the extra N-terminal Cys. We added 1 mM diithiothreitol to the buffer to avoid the formation of disulfide bonds when the peptides contain the extra Cys. MARCKS-(151–175) with or without the extra Cys had the same effect on the PLC-catalyzed hydrolysis of PIP₂.

Peptide Labeling—We used a protocol modified from Molecular Probes (Conjugation with Thiol-reactive Probes) to label peptides with a thiol-reactive fluorescent acrylodon probe, as described in detail elsewhere (44). Briefly, we mixed 1 ml of ~1 mM peptide in 10 mM KH₂PO₄/KOH, pH 7.8, with acrylodon probe dissolved in DMP (mole ratio of 1.5:1 acrylodon/peptide) for 1 h, purified the labeled peptide using high pressure liquid chromatography, and checked its purity with MALDI mass spectrometry (CASM, State University of New York, Stony Brook).

We labeled peptides with radioactive [3H]NEM as described previously (45). Briefly, we placed 250 μCi of [3H]NEM in pentane on top of 20 μl of DMF, evaporated the pentane with argon gas, and then mixed the [3H]NEM in DMP with 1 ml of an ~1 mM peptide solution; this
procedure labeled ~1% of the peptide. We added to the solution containing the labeled peptide an excess of non-radioactive NEM (mole ratio of 1.5:1 NEM:peptide) to block the unlabeled Cys.

**Vesicle Preparations**—We used multilamellar vesicles (MLVs) for ζ potential measurements, 100 nm diameter large unilamellar vesicles (LUVs) for fluorescence measurements on LUVs, and centrifugation experiments as described in detail elsewhere (25, 46). In our experience, the protocol for preparing MLVs must be followed carefully to ensure a uniform distribution of PIP₃ in PC/PIP₃ (99:1) vesicles (either LUV or MLV). We measure the ζ potential of several individual vesicles to assess the uniformity of the MLV preparations. The protocol described below consistently produces a population of vesicles that move with similar velocity in an electric field, i.e., the ζ potentials due to the negatively charged PIP₃ are similar.

The critical step is to produce a dried lipid film in which PC and PIP₃ are mixed uniformly. We add solutions of PIP₂, it is important to use the ammonium salt, which is more soluble in chloroform than the sodium salt (47, 48)) and PC in chloroform (typically 500:1.4 mM ammonium salt, which is more soluble in chloroform than the sodium salt). We added to the solution containing the labeled peptide an excess of non-radioactive NEM (mole ratio of 1.5:1 NEM:peptide) to block the unlabeled Cys.

**Centrifugation Binding Measurements**—We measured the binding of [³H]H2O-labeled vesicles (Table I) to sucrose-loaded PC/PIP₃ LUVs using the centrifugation technique described previously (25, 46). Briefly, sucrose-loaded PC/PIP₃ LUVs were mixed with trace concentrations of [³H]H2O-labeled vesicles (typically 2–10 nmol). The mixture was centrifuged at 100,000 × g for 1 h. We calculated the percentage of peptide bound from the radioactivity of the peptide in the supernatant and in the pellet.

We use a molar partition coefficient K (49, 50) to describe the binding of the peptide to lipid vesicles without making assumptions about the absorption mechanism. The molar partition coefficient K is defined by the equation: \[ \frac{[P]_m}{[L]_m} = K\frac{[P]}{[L]} \] where \([P]_m\) is the molar concentration of peptide partitioned onto the membrane, \([P]\) is the molar concentration of free peptide in the bulk aqueous phase, and \([L]\) is the molar concentration of lipid accessible to the peptide. Under our conditions, \([L] >> [P]_m\). Thus \([L]\) does not change significantly after the peptide binds and is approximately one-half of the total lipid concentration for the LUVs because the peptide interacts only with the outer leaflet of the bilayer (the peptides are added to a solution of preformed vesicles). Combining the definition of K with the equation \([P]_m = [P] + [P]\), we get Equation 1.

\[
\frac{[P]_m}{[L]_m} = K(L + 1)
\]

Note that this equation for the molar partition coefficient K has the same form as the equation for the association constant if we assume (incorrectly) that the peptide forms a 1:1 complex with a lipid (51) (for different definitions of partition coefficients, see Refs. 49 and 52). In the biochemical literature it is conventional (e.g. see page 186 in Ref. 53) to define the binding free energy \(\Delta G - RT \ln K\) where the standard state is one in which all reactants have a concentration of 1 M. As discussed in detail elsewhere (49, 52), the relationship between the partition coefficient and the binding energy depends on the units used for the concentration and the definition of the standard state. To facilitate comparison with other studies where different standard states may be used and the free energy may differ by a cratic term (e.g. see Refs. 49 and 52 and paper in Ref. 54), we consistently use the change in binding free energy \(\Delta G K\), which is independent of the standard state (Equation 2).

\[
\Delta G K = -RT \ln K
\]

where \(K_1\) is the molar partition coefficient for the binding of the first peptide; \(K_2\) is the molar partition coefficient for the binding of the second peptide; \(R\) is the gas constant; and \(T\) is the absolute temperature. At room temperature (\(T = 295\) K), a 10-fold increase in \(K\) corresponds to an increase of \(\Delta G K\) ~1.4 kcal/mol.

Several experiments (data not shown) provide important controls for the results we obtained using the centrifugation assay. First, the small standard deviation of the values obtained for PC/PIP₃ vesicles indicates that each vesicle has the same fraction of PIP₃. Second, binding experiments using both radioactive [³H]H2O and [¹⁴C]PC as tracers showed the ratio of PC to PIP₃ did not change during the preparation of LUVs. Third, we obtained similar results for the binding of Lys-10 to PC/PIP₃ vesicles using bovine brain PIP₃ from three different sources; thus, it is unlikely that the results obtained for particular PIP₃ vesicles are affecting the results significantly. Furthermore, the MALDI mass spectra of these PIP₃ lipids are similar to those reported in literature (55). The concentration of PIP₃ in the chloroform stock solution was routinely determined by drying a known volume of the lipid solution and measuring its weight on a microbalance from Cahn Instruments, Inc. (Cerritos, CA); this procedure was checked occasionally with a phosphate buffer. Fourth, electron microscope and light scattering experiments showed that the low concentrations (2–10 nmol) of peptide we routinely used in the centrifugation assay did not cause significant vesicle aggregation. Fifth, adding 100 µM EDTA to the buffer had no effect on the binding of Lys-10 to PC/PIP₃ (99:1) vesicles, which suggests that PIP₃ was not chelated to a significant degree by cations (e.g. Ca²⁺, Mg²⁺). We measured the binding of Lys-10 to PC/PIP₂ (99:1) vesicles using two different buffers (1 mM MOPS (used routinely) and 10 mM HEPES), which suggests the buffer is not affecting the measurements.

Finally, the molar partition coefficient K should not depend on the peptide when \([L] >> [P]_m\); under these conditions the peptide does not bind a significant fraction of the acidic lipid in the vesicle. K was independent of the peptide concentration for the binding of peptides to 5:1 PC/PS vesicles (data not shown). If the prewarming step noted above was deleted during the preparation of PC/PIP₃ vesicles, however, the MLVs often had widely divergent ζ potentials, and the K value measured with a population of LUVs did depend on the peptide concentration.

Despite all these precautions, the results we obtained for the binding of peptides to PC/PIP₃ vesicles were still less reliable than the results we obtained for the binding of the same peptide to PC/PS vesicles (presumably because of variations in the PIP₃ content of the LUVs). For example, we measured the molar partition coefficient of Lys-10 peptide to PC/PIP₃ vesicles with ~20 sets of vesicles. For measurements on each set of vesicles, K has a small standard error. Comparisons of measurements on different sets of vesicles, however, usually produced much larger fold variability in the molar partition coefficient. K varied by only a factor of 2 in measurements with different sets of PC/PS vesicles. We measured the binding of other peptides using at least three sets of vesicles, and we used a least squares fit of the combined data to Equation 1 to obtain the molar partition coefficients, K, reported below. The error bars in Figs. 1, 3, 5, and 8 represent the standard deviations of the K values calculated from individual measurements using Equation 1 (e.g. the 21 measurements represented by circles for MARCKS-151–175 data shown in Fig. 1A).

**Fluorescence Binding Measurements**—We used a centrifugation-independent fluorescence assay (56) to measure the binding of acrylodan-labeled Lys-7 to PC/PIP₃ (99:1) LUVs. Acrylodan is an environment-sensitive fluorescent probe; when it moves from an aqueous solution (high dielectric) to a lipid bilayer (low dielectric), its emission peak blue shifts from ~520 to ~460 nm, and its fluorescence increases significantly. Thus we can calculate the binding from the change in the fluorescence.

**Binding Measurements Using Equilibrium Dialysis**—We used a third binding assay, equilibrium dialysis (57), to measure the binding of peptide to vesicles. Each Teflon dialysis cell from Harvard Bioscience, Inc. (Holliston, MA) contains two half-cells separated by a piece of polycarbonate dialysis membrane. We loaded the vesicle solution to one half-cell and the radioactively labeled peptide solution to the other one. After a 24-h equilibrium dialysis, we counted the radioactivity of the peptide in both half-cells and calculated the binding. We obtained the same results by loading the mixture of the peptide and the vesicles into one half-cell and loading buffer to the other one.

We routinely used the centrifugation assay for most of our measurements because it requires only a low peptide concentration (2–10 nmol) and takes only a short time (~1 h). It is especially useful for measuring the strong binding of peptide (e.g. MARCKS-151–175) or Lys-13 to PC/PIP₃ (99:1) vesicles where >10 nm peptide changes the charge of the vesicle. The fluorescence assay using the acrylodan probe typically
requires higher peptide concentrations (≥50 nM) for a good signal/noise ratio, and the probe introduces some binding energy because of its hydrophobic insertion (44). The equilibrium dialysis assay requires a longer time (≈24 h), and the loss of peptide to the dialysis membrane and the cell is significant. It requires peptide concentrations ≥200 nM in the acceptor vesicles as described elsewhere (44).

Kinetic Measurements—Kinetic measurements of MARCKS-(151–175) binding were performed on an SLM-Aminco spectrofluorometer with a stopped-flow attachment as described previously (44). Briefly, 100 nM of acrylodan-MARCKS-(151–175) was mixed rapidly with varying concentrations of PC/PIP2 LUVs (diameter 100 nm) in a stopped-flow chamber. Because the fluorescence of acrylodan-MARCKS-(151–175) increases when it binds to the vesicles, we measured the time trace of the fluorescence and calculated the relaxation time (τ) for this binding process. The relationship between the relaxation time (τ) and the association rate constant (k on) is described in Equation 3 (44).

\[ \tau = k_{on}[V] + k_{off}[L]/\nu + k_{off} \]  
(Eq. 3)

where [V] is the vesicle concentration; k off is the dissociation rate constant; [L] is the lipid concentration of the outer leaflet of the vesicle; \( \nu \) is the number of lipid molecules on the outer leaflet per vesicle (\( \nu = 4\pi r^2n \)), where \( r \) is the radius of the LUVs, is 100 nm, and \( n \) is the area per lipid molecule, is 0.7 nm² (44). Thus we could calculate the association rate constant (k on) and (much less accurately) the dissociation rate constant (k off) from the plot of 1/τ versus the lipid concentration.

We determined the dissociation rate constant (k off) more accurately by measuring the relaxation time of peptide transfer from donor to acceptor vesicles as described elsewhere (44).

ζ Potential Measurements—As described previously (25, 58), we measured the electrophototropic mobility (velocity/field) of MLVs with or without the addition of basic peptides and calculated the ζ potential, the electrostatic potential at the shear plane, using the Helmholtz-Smoluchowski Equation 4 (59, 60).

\[ \zeta = u \cdot e / \epsilon \]  
(Eq. 4)

where ζ is the ζ potential of a vesicle; \( u \) is the velocity of the vesicle in a unit electric field; \( e \) is the viscosity of the aqueous solution; \( \epsilon \) is the dielectric constant of the aqueous solution; and \( \epsilon_r \) is the permittivity of free space. The ζ potential is proportional to the surface charge density and thus to the number of charged peptides that absorb to the vesicles.

PLC Monolayer Assay—We measured the effect of peptides on the PLC-catalyzed hydrolysis of PIP2 in monolayers as described in Refs. 25, 59, and 60. Briefly, we mixed PC, PS, and [3H]PIP2 in chloroform to form a 55:45:10 weight ratio, and then evaporated (10 min) to form a 55-Å-thick monolayer. Once the chloroform had evaporated, we measured the distribution for its atoms from similar functional groups in the CHARMM parameterization set.

Electrostatic Equipotential Calculations—We built an atomic model of MARCKS-(151–175) (Protein Data Bank code 1MAI) (65). We assumed that the head group of PIP2 is perpendicular to the surface, similar to the orientation of phosphatidylinositol (66, 67). We derived a detailed partial charge distribution for its atoms from similar functional groups in the CHARMM parameterization set.

Fig. 9 was generated by displaying the electrostatic potentials of the MARCKS-(151–175)/membrane systems in GRASP (68), a program for the visualization of the structural and biophysical properties of biological macromolecules. GRASP, however, is capable of solving only the linearized version of the Poisson-Boltzmann equation. In order to reproduce the non-linear electrostatic properties of the MARCKS-(151–175)/membrane systems, we solved the nonlinear Poisson-Boltzmann (NLBP) equation (33, 51, 64, 69, 70) for atomic models of these systems in 100 mK KC1 on a finite difference grid of size 65³ and used focused boundary conditions (51) to a final resolution of 0.5 grid/Å. The resulting potential maps as well as the atomic coordinates for the MARCKS-(151–175)/membrane models were then read into GRASP and displayed on an SCI Octane Workstation. The net charge used in the calculations are 0 for PC, –1 for PS, and –4 for PIP2. The calculations were performed initially using the partial charge sets for PC and PS described previously (64) and for PIP2 as described above. Essentially identical results were obtained for the –25 mV potential profiles >0.2 nm from the surface of the bilayer when a simpler charge set was assigned to the lipids. Specifically, we assigned a charge of –0.25 to each of the 4 oxygen atoms forming bonds with the phosphorus atom in PS, a charge of –0.33 to each of the 12 oxygen atoms forming bonds with the 3 phosphorus atoms in PIP2, and a charge of 0 to the remaining atoms in PC, PS, and PIP2. This removes the –25 and –25 mV potential profiles that extend between the positive and negative charges on the zwitterionic PC lipids, which tend to clutter up the diagram.

RESULTS

The Binding of Truncated MARCKS-(151–175) Peptides to PC/PIP2 Vesicles Correlates with the Number of Basic Residues—We used truncated MARCKS-(151–175) peptides to determine how the binding depends on the number of basic residues. These peptides (sequences listed in the first 4 lines of Table I) include the effector domain peptide (MARCKS-(151–175)), a peptide lacking 3 Lys residues at the C terminus (ΔC-MARCKS), a peptide lacking 5 Lys residues at the N terminus (ΔN-MARCKS), and a peptide lacking all 8 Lys residues (ΔNΔC-MARCKS). We measured the binding of these radioactively labeled peptides to PC/PIP2 vesicles using the centrifugation assay, and we calculated the molar partition coefficient K (Equation 1). The molar partition coefficient K is equal to the reciprocal of the lipid concentration that produces 50% peptide binding. As shown in Fig. 1A, deleting the 5 Lys residues at the N terminus of MARCKS-(151–175) decreases the binding –40-fold; deleting 8 Lys residues decreases the binding –1,000-fold. Fig. 1B shows that the change in the binding energy (Equation 2) correlates with the change in the number of basic residues in the peptide. Each basic residue we removed appears to contribute about 0.5 kcal/mol to the binding energy.

The Binding Does Not Depend on the Chemical Nature of the Basic Residues—If the binding is driven mainly by electrostatics, it should not depend on the chemical nature of the basic residues. Thus we examined the binding of peptides consisting of either 10 Lys or 19 Arg residues to PC/PIP2 vesicles. Fig. 2A shows that both Lys-13 and Arg-13 (●) bind with the same affinity to 99:1 PC/PIP2 vesicles. Fortuitously, both Lys-13 and Arg-13 bind to PC/PS vesicles with the same affinity as MARCKS-(151–175) in 100 mK monovalent salt.

The Aromatic Residues and Length of the Peptide Affect the Binding—To investigate the effect of aromatic residues on the binding, we replaced each of the 5 Phe in MARCKS-(151–175) with Ala (peptide defined as FA-MARCKS in Table I); we chose Ala because experiments with model peptides suggest this residue is neither attracted to nor repelled from the interface (71). We measured the binding of the peptides to PC/PIP2 or PC/PS vesicles using the centrifugation assay. Fig. 2A shows that FA-MARCKS (△) binds 100-fold less strongly to 99:1 PC/PIP2 vesicles than MARCKS-(151–175) (○). The observation that the 5 Phe residues contribute significantly (–3 kcal/mol) to the binding energy is consistent with previous observations that...
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The simplest explanation is that the length is affecting the binding. Previous experiments have shown that inserting 1 or 2 Ala between each of the basic residues in peptides containing 7 Lys (56), 5 Lys, or 5 Arg (73) residues decreases the binding of the peptides to PC/PS or PC/PG vesicles. The mechanism is not understood; the effect could arise because the local positive potential is reduced or because an extra entropy loss occurs on binding of the longer peptides to the interface (see Ref. 74). For both 99:1 PC/PIP_2 (Fig. 2A) and 5:1 PC/PS (Fig. 2B) vesicles, FA-MARCKS binds ∼100-fold less strongly than Arg-13 even though both FA-MARCKS and Arg-13 have 13 basic residues. The simplest explanation is that the length is affecting the binding.

We note in passing that although Lys-13 and Arg-13 bind with similar affinity to PC/PIP_2 vesicles (Fig. 2A), Lys-13 binds to 5:1 PC/PS vesicles about 10-fold less strongly than Arg-13.

Fig. 1. Removing basic residues from the MARCKS effector domain peptide decreases the binding to PC/PIP_2 (99:1) vesicles. A, the percentage of peptide bound at different lipid (PC/PIP_2, 99:1) concentrations. The symbols ○, △, and □ represent the binding data for peptides MARCKS-(151–175), ΔN-MARCKS, and ΔNAC-MARCKS, respectively. The curves are the least squares fits of Equation 1 to the data, which yield the value for the molar partition coefficient K. The sequencs and molar partition coefficients of the peptides are listed in Tables I and II. B, bar representations of molar partition coefficients from A and Table II (ΔC-MARCKS). Note that the change in the binding energy (see Equation 2) correlates with the change in the number of basic residues in the peptide. The K values for the binding of the peptides to PC vesicles are less than 1 × 10^{-6} M^{-1} (data not shown). Binding measurements were done with sucrose-loaded LUVs and trace concentration of peptides (2–10 nM) in a solution containing 100 mM KCl, 1 mM MOPS, pH 7.0, using the centrifugation technique. The error bars in Figs. 1, 3, 5, and 8 represent the standard deviations in the estimates of K.

Fig. 2. The effect of aromatic residues, the chemical nature of the basic residues, and the length of the peptide on the binding of peptides with 13 basic residues to PC/PIP_2 (99:1) or PC/PS (5:1) vesicles. A, binding of trace concentrations (2–10 nM) of MARCKS-(151–175) (○, data from Fig. 1A), Lys-13 (■), Arg-13 (▲), and FA-MARCKS (△) to 99:1 PC/PIP_2 vesicles. The sequences and molar partition coefficients of the peptides are listed in Tables I and II. Note that Lys-13 and Arg-13 bind with the same affinity to the PC/PIP_2 vesicles; the affinity is independent of the chemical nature of the basic residues and is identical to that of MARCKS-(151–175). Replacing the 5 aromatic Phe residues with 5 Ala residues (FA-MARCKS, △) reduces the binding of the MARCKS effector domain peptide (○) ∼300-fold. The two curves are drawn with molar partition coefficients K = 2 × 10^{-6} and 8 × 10^{-6} M^{-1}, respectively. Shortening the peptide increases the binding: although both Lys-13 and FA-MARCKS have 13 basic residues, Lys-13 (13 residues) binds to PC/PIP_2 vesicles ∼300-fold more strongly than FA-MARCKS (25 residues). B, binding of MARCKS-(151–175) (○), Arg-13 (▲), and FA-MARCKS (△) to 5:1 PC/PS vesicles. Note that replacing the aromatics with Ala has the same ∼300-fold effect on the binding to either PC/PS or PC/PIP_2 vesicles. Shortening the peptide also has the same effect. Binding data in A and B also show that MARCKS-(151–175) (○), Arg-13 (▲), and FA-MARCKS (△) bind with similar affinities to vesicles containing either 1% PIP_2 or 17% PS. The curves in B are drawn with molar partition coefficients 2 × 10^{-6} and 7 × 10^{-6} M^{-1}. The K values for the binding of the peptides to PC vesicles are less than 1 × 10^{-6} M^{-1} (data not shown). Binding measurements were done as described in the legend to Fig. 1.

the Phe residues of MARCKS-(151–175) penetrate the polar head group region of the membrane (40, 45, 72). The aromatic residues contribute to the binding of MARCKS-(151–175) not only to PC/PIP_2 vesicles but also to PC/PS vesicles. Fig. 2B shows that FA-MARCKS (△) binds 100-fold less strongly to 5:1 PC/PS vesicles than MARCKS-(151–175) (○). Our results are consistent with the work of Wiselmy and White (71), which showed that if a Phe on a neutral peptide inserts completely into the polar head group region of a membrane, it contributes ∼1 kcal/mol to the binding energy. Thus 5 Phe could maximally contribute 5 kcal/mol, which corresponds to ∼1,000-fold increase in the molar partition coefficient (Equation 2). There are several possible explanations for the less-than-a-maximal effect observed with our charged peptides. For example, a decrease in free energy due to penetration of aromatics into the bilayer is accompanied by an increase in Born/dehydration free energy required to move the charges closer to the interface, as discussed in detail elsewhere (33, 45).

The length of the peptide also appears to affect the binding. Previous experiments have shown that inserting 1 or 2 Ala between each of the basic residues in peptides containing 7 Lys (56), 5 Lys, or 5 Arg (73) residues decreases the binding of the peptides to PC/PS or PC/PG vesicles. The mechanism is not understood; the effect could arise because the local positive potential is reduced or because an extra entropy loss occurs on binding of the longer peptides to the interface (see Ref. 74). For both 99:1 PC/PIP_2 (Fig. 2A) and 5:1 PC/PS (Fig. 2B) vesicles, FA-MARCKS binds ∼100-fold less strongly than Arg-13 even though both FA-MARCKS and Arg-13 have 13 basic residues. The simplest explanation is that the length is affecting the binding.

We note in passing that although Lys-13 and Arg-13 bind with similar affinity to PC/PIP_2 vesicles (Fig. 2A), Lys-13 binds to 5:1 PC/PS vesicles about 10-fold less strongly than Arg-13.
This is consistent with the observation that Lys-5 binds to 4:1 PC/PG vesicles ~10-fold less strongly than Arg-5 (75), for reasons not understood.

Fig. 2 and Table III show that most of the basic peptides we studied bind with similar affinity to 99:1 PC/PIP₂ and 5:1 PC/PS vesicles. The Δψ potential (average electrostatic potential adjacent to the surface, reviewed in Ref. 59) of 5:1 PC/PS vesicles (−30 mV; see Ref. 76) is more negative than that of 99:1 PC/PIP₂ vesicles (−8 mV; see Ref. 25). All the experimental data we have obtained suggest the driving force for the binding of the peptides we have studied to PC/PIP₂ vesicles is mainly electrostatic in nature; hence, we conclude that the local potential the adsorbed peptide experiences is more negative than the average potential. The simplest explanation is that when a basic peptide (e.g. MARCKS-(151–175)) binds to PC/PIP₂ vesicles, several PIP₂ must diffuse into the neighborhood of the adsorbed peptide and produce a high local negative potential, as discussed in more detail below.

**Binding of Poly-Lys Peptides to PC/PIP₂ Vesicles Also Correlates with the Number of Basic Residues in Each Peptide—**MARCKS-(151–175) contains both basic and aromatic residues. Its binding to PC/PIP₂ vesicles depends on a complicated interplay of at least two factors that drive adsorption, electrostatic attraction of basic residues for the surface and hydrophobic insertion of aromatics into the polar head group region, and two factors that oppose adsorption, Born/dehydration repulsion and the entropy price required to pull several PIP₂ lipids together. These factors (except the entropy price) have been discussed elsewhere (33, 45). We investigated a simpler system, measuring the binding of peptides that contain only basic residues. Fig. 3A shows the binding of Lys-7, Lys-10, and Lys-13 to PC/PIP₂ vesicles. Fig. 3B shows that the change in the binding energy (Equation 2) correlates with the change in the number of basic residues. Specifically, adding 6 Lys residues to Lys-7 increases the molar partition coefficient 10³-fold, i.e. increases the binding energy −4 kcal/mol (Equation 2). (Or each basic residue contributes about 0.7 kcal/mol to the binding energy.) The simplest interpretation is that the binding energy increases because the peptides with more basic residues can bind more PIP₂ molecules (probably 3 or 4 for Lys-13 and MARCKS-(151–175)); EPR and electrophoretic data suggest MARCKS-(151–175) forms an electroneutral complex with several PIP₂ (25, 39).

**Different Assays Produce Similar Binding Data—**We also used a centrifugation-independent fluorescent assay to measure the binding of acrylodan-labeled Lys-7 to PC/PIP₂ vesicles. Fig. 4 shows that acrylodan-labeled Lys-7 binds weakly to PC vesicles but binds significantly to PC/PIP₂ (99:1) vesicles. This binding is ~10-fold stronger than that of radioactive NEM-labeled Lys-7 (Fig. 3), probably due to the effect of hydrophobic acrylodan enhancing the binding of Lys-7 to vesicles as noted previously with PC/PS (56). The third assay we used is equilibrium dialysis. The results (data not shown) agree with those obtained using the centrifugation assay (Fig. 3); radioactive NEM-labeled Lys-10 binds weakly to PC vesicles but binds with significant affinity to 99:1 PC/PIP₂ vesicles.

The binding of basic peptides to PC/PIP₂ vesicles also decreases with increasing ionic strength. A theoretical model explaining why the binding of basic peptides to membranes containing monovalent acidic lipids depends on salt concentration is given elsewhere (39, 51). Briefly, increasing the salt concentration screens the charges on the membrane interface and thus decreases the magnitude of the negative electrostatic potential experienced by the basic peptide at the membrane surface.

**One MARCKS-(151–175) Binds Several PIP₂—**Kinetic stopped-flow measurements of the interaction between MARCKS-(151–175) and PC/PS vesicles showed that the association constant kₐ remains diffusion-limited as the mole fraction of PS in the membrane decreases. For 5:1, 10:1, and 15:1 PC/PS vesicles, the 40-fold decrease in the partition coefficient...
ficient arises from an increase in the dissociation rate constant (29, 44). This result is consistent with theoretical (77) and experimental (78, 79) evidence that monovalent acidic lipids do not significantly redistribute when a basic peptide binds. There is, however, evidence that PIP2 redistributes when MARCKS-(151–175) binds to PC/PIP2 vesicles. Specifically, both ζ potential (25) and more direct EPR experiments (39) suggest that one MARCKS-(151–175) binds three or four PIP2 to form an electroneutral complex. If one MARCKS-(151–175) pulls together several PIP2 when it binds to a PC/PIP2 membrane (<1% PIP2), the association rate constant, $k_{on}$, should decrease as the mole fraction of PIP2 on the membrane decreases because the PIP2 must diffuse further to associate with the peptide. We did indeed observe this effect. Fig. 6 shows that $k_{on}$ for the interaction between MARCKS-(151–175) and 99:1 PC/PIP2 vesicles is $1 \times 10^{11}$ M$^{-1}$ s$^{-1}$, which is close to the diffusion-limited rate (assuming the diffusion coefficient of the peptide in the aqueous solution is $3 \times 10^{-6}$ cm$^2$ s$^{-1}$, see Ref. 44). When we reduce the mole fraction of PIP2 from 1 to 0.1%, $k_{on}$ (proportional to the slope) decreases ~10-fold and is no longer diffusion-limited (Fig. 6).

The molar partition coefficient $K$ also decreases ~10-fold (25). As expected from the measurements of the forward rate constant and equilibrium partition coefficient, the dissociation rate constant $k_{off}$ (from independent measurements described above) does not depend on the percentage of PIP2 in the vesicles and is $-1$ s$^{-1}$ (data not shown). Thus the change in $K$ is due only to the reduced association rate $k_{on}$. (The data we obtained are overdefined and self-consistent; the molar partition coefficient $K$ for the interaction between MARCKS-(151–175) and 99:1 PC/PIP2 vesicles deduced from kinetic measurements of $k_{on}$ and $k_{off}$ (25) is $2 \times 10^6$ M$^{-1}$ ($K = k_{on}/(v k_{off})$, see Ref. 44), which is consistent with the value of $K$ from direct binding measurements (Fig. 1).)

If one MARCKS-(151–175) pulls together several PIP2 when it binds to a PC/PIP2 membrane, $k_{on}$ should decrease not only if the distance the PIP2 lipids must diffuse increases (Fig. 6) but also if the viscosity of the membrane increases. As expected, increasing the viscosity by incorporating 30% cholesterol into 99:1 PC/PIP2 vesicles decreases the association rate constant significantly (~5-fold; data not shown).

The decrease in the forward rate constant illustrated in Fig. 6 probably is not due to a significant change in peptide conformation upon binding to the PC/PIP2 surface. Measurements with spin-labeled MARCKS-(151–175) peptides show that the peptide remains in an extended conformation and exhibits little α-helical structure when it binds to the PC/PIP2 membrane (39), a result consistent with our CD measurements (data not shown).

ζ Potential Measurements Suggest That Both MARCKS-(151–175) and Lys-13 Bind Strongly to PC/PIP2 Vesicles—The
to the effector domain of MARCKS, as illustrated in Table I. MacMARCKS (also known as MARCKS-related protein or F52) is a MARCKS family member highly expressed in macrophages (26, 27, 30). Adducin is a membrane skeletal protein that binds to F-actin and assists the recruitment of spectrin to F-actin (reviewed in Ref. 80). DAKAP200 is a Drosophila scaffolding protein that binds regulatory subunits of protein kinase AII (81). NMDA receptors act as glutamate-gated ion channels in the central nervous system (reviewed in Ref. 82). MARCKS (83–85), MacMARCKS (86, 87), DAKAP200 (81), adducin (88), and the NMDA receptor (89, 90) all bind with high affinity to calcium/calmodulin and can be phosphorylated by PKC. These proteins all contain basic regions that constitute calcium/calmodulin binding domains and also have serine residues that can be phosphorylated by PKC (Table I). GAP43 is present at high concentrations in neural tissue and plays important roles in nerve growth (reviewed in Refs. 91–93). Even though the basic region in GAP43 has little sequence homology to the MARCKS effector domain (see Table I), Laux et al. (24) have postulated that GAP43, like MARCKS, may bind a significant fraction of PIP2 in neural tissues.

Fig. 8 summarizes data (not shown) similar to those illustrated in Fig. 1A, Fig. 2A, and Fig. 3A for peptides corresponding to the basic regions in these proteins. All the peptides bind significantly to PC/PIP2 vesicles (K values also listed in Table II), and the binding correlates qualitatively with the number of basic (and aromatic) residues in the peptides, consistent with the hypothesis that the binding is mainly driven by local electrostatic interactions.

As summarized elsewhere, there is good evidence that a cluster of basic residues in the neuronal Wiskott-Aldrich syndrome protein (N-WASP) binds PIP2 (94). Clusters of basic residues in WASP (94), cortical cytoskeleton-associated protein of approximate molecular mass 23 kDa (CAP23) (24), and Syndecan-4 (95) may also interact with PIP2. Peptides corresponding to the basic regions in these proteins, however, bind only weakly to PC/PIP2 vesicles (sequences and binding data in Tables I and II). The result was expected because these peptides have ≤8 basic and 0 or 1 aromatic residues. More basic/aromatic residues appear to be required to bind an unstructured peptide strongly to a PC/PIP2 vesicle (see Fig. 1B, Fig. 3B, and Fig. 8). There are several reasons why a cluster of basic residues in a structured protein such as N-WASP might inter-

### Table I

| Peptide                        | Net charge | Aromatic residues | K (M⁻¹) (PC/PIP2) |
|-------------------------------|------------|-------------------|-------------------|
| MARCKS peptides               |            |                   |                   |
| MARCKS-(151–175)              | +          | 5                 | 2 × 10⁶           |
| ΔC-MARCKS                     | +          | 5                 | 4 × 10⁵           |
| ΔN-MARCKS                     | +          | 5                 | 6 × 10⁵           |
| ΔNC-MARCKS                    | +          | 5                 | 3 × 10⁵           |
| FA-MARCKS                     | +          | 5                 | 6 × 10⁵           |
| Poly-Lys/Arg peptides         | +          | 0                 | 1 × 10⁶           |
| Arg-13                        | +          | 0                 | 1 × 10⁶           |
| Lys-13                        | +          | 0                 | 1 × 10⁶           |
| Lys-10                        | +          | 0                 | 3 × 10⁵           |
| Lys-7                         | +          | 0                 | 8 × 10⁵           |
| MARCKS-like peptides          |            |                   |                   |
| MacMARCKS-(87–110)            | +          | 4                 | 6 × 10⁵           |
| Adducin-(717–734)             | +          | 2                 | 3 × 10⁶           |
| DAKAP200-(119–141)            | +          | 3                 | 5 × 10⁵           |
| NMDA-NR1-(875–898)            | +          | 2                 | 9 × 10⁵           |
| GMC family peptides           |            |                   |                   |
| GAP43-(30–56)                 | +          | 1                 | 2 × 10⁵           |
| CAP23-(1–13)                  | +          | 5                 | <10²              |
| Other peptides                |            |                   |                   |
| PLD2-(554–575)                | +          | 4                 | 1 × 10⁶           |
| SCAMP2-(201–211)              | +          | 4                 | 9 × 10⁵           |
| N-WASP-(181–197)              | +          | 0                 | 2 × 10⁵           |
| WASP-(223–232)                | +          | 0                 | <10²              |
| Syndecan-4-(188–194)          | +          | 4                 | <10²              |
Electrostatic equipotential profiles adjacent to 5:1 PC/PS, 2:1 PC/PS, and 99:1 PC/PIP_2 membranes before and after MARCKS-(151–175) binds. We calculated the electrostatic potentials using the NLPB equation (33, 51, 64, 69, 70) in a solution containing 100 mM monovalent salt and displayed them using GRASP program (68). The –25 mV equipotential profile is shown in red, and the +25 mV equipotential profile is shown in blue. MARCKS-(151–175) and PIP_2 are shown in yellow-green and yellow, respectively. A, 5:1 PC/PS membrane. B, MARCKS-(151–175) bound to a 5:1 PC/PS membrane. C, 99:1 PC/PIP_2 membrane; the charge used for each PIP_2 is –4. D, one MARCKS-(151–175) sequesters 3 PIP_2 (1 in rear) on a 99:1 PC/PIP_2 membrane. E, 2:1 PC/PS membrane; the distance between the –25 mV equipotential profile and the membrane is ~10 Å. F, the positive local potential produced by MARCKS-(151–175) absorbed to a 2:1 PC/PS membrane can sequester negatively charged PIP_2. The equipotential profiles are displayed in a mesh mode except in A.

How PIP_2 Binds to the Basic Effector Domain of MARCKS

Electrostatic Equipotential Profiles of Membranes with or without MARCKS-(151–175)—To illustrate the role of electrostatic interactions in the binding of MARCKS to bilayers, we calculated the electrostatic equipotential profiles adjacent to 5:1 PC/PS, 99:1 PC/PIP_2, and 2:1 PC/PS membranes with or without a single membrane-associated MARCKS-(151–175) (Fig. 9). The electrostatic equipotential profiles (~25 and +25 mV) were calculated from finite difference solutions to the NLPB equation (33, 51, 64, 69, 70) for atomic models of the membrane and peptide, and assuming a solution containing 100 mM monovalent salt. Fig. 9A shows the –25-mV equipotential profile (red) adjacent to a 5:1 PC/PS membrane. When MARCKS-(151–175) binds to the membrane (Fig. 9B), a strong positive potential (+25 mV, blue) is produced in its vicinity. Theory (77) and experiments (78, 79) suggest that the acidic lipid PS is not significantly concentrated in the plane of the membrane when the basic peptide binds to the membrane surface. Fig. 9C shows a model with 4 PIP_2 lipids in a patch of membrane containing ~400 lipids, i.e., a bilayer containing 1% PIP_2. The equipotential profiles (~25 mV, red) of this 99:1 PC/PIP_2 membrane are discrete and centered around each PIP_2; to a first approximation they are hemispheres with a value about twice that calculated using Debye-Hückel theory (the potential doubles when charges are confined to the interface because of the image charge effect and another factor, as discussed in Refs. 59 and 99). Both experimentally (25, 76) and computationally, the average surface potential adjacent to a 99:1 PC/PIP_2 membrane (~8 mV; Fig. 9C) is significantly less negative than that adjacent to a 5:1 PC/PS membrane (~30 mV; Fig. 9A). Nevertheless, MARCKS-(151–175) and other peptides bind with similar affinity to these two membranes (Table III and Fig. 2). The binding is driven mainly by electrostatic interactions, implying that MARCKS-(151–175) experiences similar local electrostatic potentials in both membranes. This could happen if the peptide sequesters several PIP_2, as illustrated in Fig. 9D. EPR (39) and kinetic (see Fig. 6A above) data provide more direct evidence that MARCKS-(151–175) sequesters several PIP_2 lipids in its immediate neighborhood when it binds to a PC/PIP_2 membrane.

Is the electrostatic interaction sufficient to offset the entropy price required to sequester (i.e., concentrate laterally) the PIP_2? Calculations (as outlined in Ref. 35) using solutions to the NLPB equation suggest that the partitioning of a single PIP_2 from bulk membrane to a position adjacent to membrane-absorbed MARCKS-(151–175) produces a decrease of ~3 kcal/mol in the electrostatic free energy. This is sufficient to overcome the decrease in mixing entropy.

The phospholipids in the inner leaflet of the plasma membrane of a typical mammalian cell contain not only ~1% PIP_2 but also ~30% PS. Fig. 9E shows the essentially flat ~25-mV equipotential profile (red) adjacent to a 2:1 PC/PS membrane; this profile is located ~10 Å above the membrane. (The simpler Gouy-Chapman theory, which assumes the charges are smeared uniformly over the surface, predicts an essentially identical profile (64) that agrees well with experimental data (59).) MARCKS-(151–175) binds strongly to 2:1 PC/PS (Fig. 9F) via electrostatic interactions, even without PIP_2. What is the interaction between MARCKS-(151–175) and PIP_2 after the peptide binds to the membrane? Fig. 9F shows that the local potential of MARCKS-(151–175) remains positive when it has bound to the 2:1 PC/PS membrane. PIP_2 has a higher charge (about ~4, see Ref. 8 for discussion) than PS (~1), so its electrostatic sequestration by MARCKS-(151–175) is stronger than that of PS (8). Thus our computations predict that

**Table III**

| Peptide       | PC/PIP_2 | PC/PS, 5.1 |
|---------------|----------|------------|
| MARCKS-(151–175) | 2 x 10^6 | 2 x 10^6  |
| AC-MARCKS     | 4 x 10^6 | 2 x 10^6  |
| ΔN-MARCKS     | 6 x 10^6 | 6 x 10^6  |
| ΔNAC-MARCKS   | 3 x 10^6 | 2 x 10^6  |
| FA-MARCKS     | 8 x 10^7 | 8 x 10^7  |
| Poly-Lys/Arg peptides |          |            |
| Arg-13        | 1 x 10^9 | 1 x 10^9  |
| Lys-13        | 1 x 10^9 | 7 x 10^9  |
| Lys-7         | 8 x 10^4 | 2 x 10^2  |
Table IV

| Peptide   | Net charge | Aromatic residues | [Peptide] |
|-----------|------------|-------------------|-----------|
| MARCKS-(151–175) | 13+        | 5                 | 100 nM    |
| SCAMP2-(201–211) | 4+         | 4                 | 1 µM      |
| GAP43-(30–56) | 9+         | 1                 | 10 µM     |
| ΔNAC-MARCKS | 5+         | 5                 | 10 µM     |
| PL2D-(554–575) | 6+         | 4                 | 10 µM     |
| CAP23-(1–13) | 5+         | 1                 | >10 µM    |
| Lys-7     | 7+         | 0                 | >100 µM   |

**Discussion**

Several experimental results support our conclusion that the binding of MARCKS-(151–175) to PC/PIP2 membranes is driven mainly by local, nonspecific, electrostatic interactions between the 13 basic residues on the peptide and the multivalent PIP2 lipids. First, the binding of truncated MARCKS-(151–175) peptides to PC/PIP2 vesicles correlates with the number of basic residues in the peptides (Fig. 1). Second, the binding of peptides corresponding to MARCKS-like domains in other proteins also correlates with the number of basic (and aromatic) residues (Fig. 8). Third, the binding does not depend on the chemical nature of the basic residues; Lys-13 and Arg-13 bind with the same affinity to PC/PIP2 vesicles (Fig. 2). Fourth, MARCKS-(151–175) binds equally well to membranes containing PI(4,5)P2 and PI(3,4)P2 (25). Fifth, the binding decreases as the ionic strength increases (Fig. 6). The atomic model in Fig. 9D, where the potentials have been calculated from the NLPB equation, illustrates the electrostatic component of the binding.

Aromatic residues and the length of the peptide also affect the binding to PC/PIP2 membranes. Replacing 5 Phe with Ala in MARCKS-(151–175) decreased the binding 100-fold (Fig. 2), and shortening the peptide (25 to 13 residues) while keeping the same number of basic residues increased the binding 100-fold (Fig. 2).

Several experiments provide strong evidence that one MARCKS-(151–175) binds to several PIP2 in a PC/PIP2 bilayer. EPR measurements on spin-labeled PIP2 (39) provide perhaps the most direct evidence, supported by the kinetic data reported here (Fig. 6). Potential (Fig. 7) and competition experiments (25) also support this conclusion.

Finally, our PLC inhibition measurements suggest that peptides corresponding to the basic domains in MARCKS and GAP43 can laterally sequester PIP2 even when the monovalent acidic lipid PS is present at a 30-fold excess. These experiments provide support for the hypothesis discussed below that these domains can reversibly sequester PIP2 in the plasma membrane of a cell, where PS is typically present at a much higher concentration than PIP2.

Comparing the binding of MARCKS effector domain peptide and the well characterized PH domain of PLC-δ1 (PLC-PH) to PIP2 reveals several important differences. First, MARCKS-(151–175) lacks structure and is in an extended conformation in solution or when bound to a membrane (39, 40), and its binding to PIP2 is driven by nonspecific local electrostatic interactions. In contrast, PLC-PH has a well defined structure and its binding to PIP2 is mediated by 12 specific “lock and key” hydrogen bonds (65). This explains why MARCKS-(151–175) exhibits no specificity for PI(4,5)P2 over PI(3,4)P2, whereas PLC-PH binds more strongly to the former lipid, a specificity that is well understood from the crystal structure of its complex with IP3 (65). Second, one MARCKS-(151–175) binds to 3–4 PIP2, whereas PLC-PH forms a 1:1 complex with PIP2; this explains why MARCKS-(151–175) binds 100-fold more strongly to 99:1 PC/PIP2 vesicles than does PLC-PH (8). Third, MARCKS-(151–175) binds strongly to both PC/PS and PC/PIP2 membranes (Fig. 2), whereas PLC-PH binds with significant affinity only to membranes containing PIP2 (100). A corollary is that the targeting of MARCKS-(151–175) to membranes does not require PIP2, whereas the targeting of PLC-PH to membranes does require PIP2. Fourth, the functions of the two domains are different. The putative function of MARCKS is to reversibly sequester PIP2 in the plane of the membrane by nonspecific local electrostatic interactions, whereas the well established functions of PLC-PH include targeting the enzyme to the plasma membrane and increasing the local concentration of substrate PIP2 that catalytic domain experiences, which
allows the enzyme to act precessively (9, 10). The mechanism by which MARCKS binds to a phospholipid bilayer or to a plasma membrane is well understood and requires the electrostatic interaction of its basic effector domain with acidic lipids (28, 30). Our working hypothesis is that the basic effector domain sequesters much of the PIP_{2} in the membrane through nonspecific local electrostatic interactions. Specifically, the local potential adjacent to the effector domain of MARCKS is positive (Fig. 9, B and F). Because PIP_{2} has higher valence (–3 or –4) than PS (–1), it will be more strongly attracted to the positive potential adjacent to the cluster of basic residues (8). Our PLC experiments show that both MARCKS and its effector domain inhibit the PLC-catalyzed hydrolysis of PIP_{2} in vesicles or monolayers containing ~1% PIP_{2} and 33% PS (25, 41); earlier studies showed that calcium/calmodulin binding or PKC phosphorylation releases the effector domain of MARCKS from the membrane, restoring the rate of PLC-catalyzed hydrolysis of PIP_{2} (33, 41). Our results showing that peptides corresponding to the basic regions of other proteins (e.g. GAP43) inhibit the PLC-catalyzed hydrolysis of PIP_{2} support the hypothesis that they too may sequester PIP_{2} in the plasma membrane.

One important caveat is that, in most cell types, the concentration of MARCKS and other putative PIP_{2} buffers (e.g. GAP43) is not well established and may not be sufficiently high to buffer a significant fraction of the PIP_{2}. For example, although the concentration of MARCKS in brain tissue is sufficiently high (10 μM, see Refs. 27 and 32) to sequester most of the PIP_{2}, the concentration of MARCKS/MacMARCKS in quiescent macrophages is probably too low to perform this function. Upon activation of the macrophage, however, the concentration of MARCKS/MacMARCKS increases 20-fold to about 5–10 μM (calculated from the data in Ref. 101) and the concentration of MARCKS also increases (102).

Biological Corollaries—Caroni and colleagues (24) have reported evidence from cell biology experiments that supports the hypothesis MARCKS sequesters a significant fraction of PIP_{2} in the plasma membrane. For example, if the hypothesis is correct, overexpression of MARCKS might be expected to increase the synthesis of PIP_{2} to maintain a constant free level of PIP_{2}. Indeed, overexpression of MARCKS in PC12 cells does increase the production of PIP_{2} (24). Furthermore, MARCKS is not uniformly distributed in the plasma membrane of some cell types (103); it is concentrated in the membrane ruffles of fibroblasts (104) and, along with MacMARCKS, in the nascent phagosomes of macrophages (105, 106). If MARCKS sequesters PIP_{2}, this lipid should colocalize with MARCKS in these phagosomes of macrophages (105, 106). If MARCKS sequesters PIP_{2}, this lipid should colocalize with MARCKS in these phagosomes of macrophages (105, 106). If local electrostatic sequestration by MARCKS is also electrostatically sequestered by the basic effector domain of MARCKS. In summary, experiments from cell biology (24) and model systems (reviewed in Ref 8) provide complementary evidence that MARCKS (and possibly GAP43, see Ref. 24) could act to reversibly buffer PIP_{2} in the plasma membrane of many cell types.

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Lateral Sequestration of Phosphatidylinositol 4,5-Bisphosphate by the Basic Effector Domain of Myristoylated Alanine-rich C Kinase Substrate Is Due to Nonspecific Electrostatic Interactions
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