The Effector Domain of Myristoylated Alanine-rich C Kinase Substrate Binds Strongly to Phosphatidylinositol 4,5-Bisphosphate*

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Both the myristoylated alanine-rich protein kinase C substrate protein (MARCKS) and a peptide corresponding to its basic effector domain, MARCKS-(151–175), inhibit phosphoinositide-specific phospholipase C (PLC)-catalyzed hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) in vesicles (Glaser, M., Wanaski, S., Buser, C. A., Boguslavsky, V., Rashidzada, W., Morris, A., Rebecchi, M., Scarlata, S. F., Runnels, L. W., Prestwich, G. D., Chen, J., Aderem, A., Ahn, J., and McLaughlin, S. (1996) J. Biol. Chem. 271, 26187–26193). We report here that adding 10–100 nM MARCKS-(151–175) to a subphase containing either PLC-C or -B inhibits hydrolysis of PIP$_2$ in a monolayer and that this inhibition is due to the strong binding of the peptide to PIP$_2$. Two direct binding measurements, based on centrifugation and florescence, show that ~10 nM PIP$_2$ in the form of vesicles containing 0.01%, 0.1%, or 1% PIP$_2$, binds 50% of MARCKS-(151–175). Both electrophoretic mobility measurements and competition experiments suggest that MARCKS-(151–175) forms an electroneutral complex with ~4 PIP$_2$. MARCKS-(151–175) binds equally well to PI(4,5)P$_2$ and PI(3,4,5)P$_3$. Local electrostatic interactions of PIP$_2$ with MARCKS-(151–175) contribute to the binding energy because increasing the salt concentration from 100 to 500 mM decreases the binding 100-fold. We hypothesize that the effector domain of MARCKS can bind a significant fraction of the PIP$_2$ in the plasma membrane, and release the bound PIP$_2$ upon interaction with Ca$^{2+}$/calmodulin or phosphorylation by protein kinase C.

Although phosphatidylinositol 4,5-bisphosphate (PIP$_2$) constitutes only ~1% of the lipids in the plasma membrane of a typical mammalian cell, it plays many important roles in signal transduction and cell biology (reviewed in Ref. 1). For example, PIP$_2$ is the source of three second messengers: diacylglycerol, inositol 1,4,5-trisphosphate (IP$_3$), and phosphatidylinositol 3,4,5-trisphosphate (reviewed in Refs. 2–4). PIP$_2$ also recruits proteins containing pleckstrin homology (PH) (reviewed in Ref. 5) and other domains (reviewed in Ref. 6) to the plasma membrane and is a cofactor for the activation of phospholipase D (reviewed in Refs. 7, 8). Finally, it is required for exocytosis (reviewed in Ref. 9) and activates several different ion channels (reviewed in Ref. 10). How can PIP$_2$ play so many different roles? The pool of PIP$_2$ hydrolyzed by phosphoinositide-specific phospholipase C (PLC) may be sequestered in cholesterol-enriched lipid "rafts" (11), or the enzymes that synthesize PIP$_2$ may be concentrated in specific regions of the plasma membrane (4). Alternatively, proteins may reversibly bind much of the PIP$_2$ in the plasma membrane, blunting changes in the level of free PIP$_2$ that would otherwise occur. We explore here the hypothesis that myristoylated alanine-rich protein kinase C substrate (MARCKS) not only binds a significant fraction of the PIP$_2$ in the plasma membrane, but also releases it upon binding by Ca$^{2+}$/calmodulin or phosphorylation by protein kinase C (PKC).

MARCKS (reviewed in Refs. 12 and 13) is a ubiquitous PKC (14) substrate, present at high concentration in many cell types; for example, its concentration in brain is ~10 μM (13, 15), comparable to the concentration of PIP$_2$. It has two conserved regions required for membrane binding: a myristoylated N terminus and a basic effector domain (residues 151–175). The mechanism by which MARCKS binds to the plasma membrane is well understood (reviewed in Refs. 16 and 17); the N-terminal myristate inserts into the bilayer and the cluster of basic residues in the effector domain interacts electrostatically with acidic lipids. When the effector domain of MARCKS binds to Ca$^{2+}$/calmodulin or is phosphorylated by PKC, its interaction with acidic lipids is reversed (reviewed in Refs. 12, 13, 16, and 17).

Previous work suggested that the effector domain of MARCKS interacts with PIP$_2$; both MARCKS and its effector domain peptide, MARCKS-(151–175), inhibit the PLC-catalyzed hydrolysis of PIP$_2$ in vesicles, and this inhibition is reversed by Ca$^{2+}$/calmodulin binding or PKC phosphorylation of MARCKS-(151–175) (18). Although the simplest interpretation of these results is that the effector domain binds strongly to PIP$_2$, it is difficult to rule out potential artifacts because MARCKS-(151–175) also aggregates the vesicles. For example, the putative large lateral domains formed in membranes by MARCKS-(151–175) (18, 19) are probably an aggregation-related artifact (20).

The objective of this study was to test our hypothesis more rigorously. First, we investigated whether MARCKS-(151–175) inhibits the PLC-catalyzed hydrolysis of PIP$_2$ in

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1 The abbreviations used are: PIP$_2$, phosphatidylinositol 4,5-bisphosphate; MARCKS, myristoylated alanine-rich protein kinase C substrate; MARCKS-(151–175), peptide corresponding to residues 151–175 of bovine MARCKS; PLC, phosphatidylycholine; PS, phosphatidylserine; PC, phosphatidylcholine; PIP$_2$, phosphatidylinositol 4,5-bisphosphate; PI(4)P, phosphatidylinositol 4-phosphate; NEM, N-ethylmaleimide; IP$_3$, inositol 1,4,5-trisphosphate; PLC, phosphoinositide-specific phospholipase C; PH, pleckstrin homology; PKC, protein kinase C; K, molar partition coefficient (Equation 1); K, apparent association constant for PI,1 binding (Equation 2); LUV, large unilamellar vesicle; MLV, multilamellar vesicle; CD, circular dichroism; EPR, electron paramagnetic resonance; MOPS, 4-morpholinopropanesulfonic acid; N, newton/s.
phospholipid monolayers, a system that eliminates the possibility of artifacts due to vesicle aggregation. Second, we measured directly the binding of MARCKS-(151-175) to bilayers containing PIP$_2$ using two independent techniques. Next, we conducted competition and electrophoretic mobility experiments to investigate the stoichiometry of the complex formed between MARCKS-(151-175) and PIP$_2$. Finally, we examined the importance of nonspecific electrostatic interactions by comparing the specificity of MARCKS-(151-175) for PI(4,5)P$_2$ versus PI(3,4)P$_2$ and measuring the effect of salt on the binding. The results all support the hypothesis that the effector domain of MARCKS binds with high affinity to PIP$_2$ in membranes; under "Discussion," we consider the biological implications of this strong interaction.

**EXPERIMENTAL PROCEDURES**

**Materials—**1-Palmitoyl-2-oleoyl-sn-glycerol-3-phosphatidylcholine (PC), 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphatidylethanolamine (PE), 1-stearoyl-2-arachidonoyl-sn-glycerol-3-phosphatidylcholine, phosphatidylglycerol (PG), and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). The ammonium salt of l-α-phosphatidylcholine, from Roche Molecular Biochemicals (Mannheim, Germany) or purified from bovine brain extract (Sigma) as described elsewhere (21). Dipalmitoyl phosphatidylglycerol 4-phosphate (PI(4)P$_2$) was from Calbiochem (La Jolla, CA). The ammonium salt of dipalmitoyl phosphatidylglycerol 4,5-bisphosphate (PI(4,5)P$_2$) was either purchased from Roche Molecular Biochemicals (Mannheim, Germany) or purified from bovine brain extract (Sigma) as described elsewhere (21). Dipalmitoyl phosphatidylglycerol 4-phosphate (PI(4)P$_2$) was from Calbiochem (La Jolla, CA). The ammonium salt of dipalmitoyl phosphatidylglycerol 4,5-bisphosphate (PI(4,5)P$_2$) and dipalmitoyl phosphatidylcholine 3,4-bisphosphate (PI(3,4)P$_2$) were from Matreya (Pleasant Gap, PA). t-+s-n-1-0-[1-6-[6-[7-[7-Nitrobenz-2-oxa-1,3-diazol-4-ylamino]hexanoyl]amino]hexanoyl]-2-n-hexanoylphosphatidylcholine 4,5-bisphosphate (NBDD-PIP$_2$) was from Echelon Research Laboratories Inc. (Salt Lake City, UT). Labeled (dioloyl-1-13C)-t-o-dioleoylphosphatidylcholine, linoleoyl-2-13C-t-o-phosphatidylcholine and t-o-phosphatidylglycerol 4,5-bisphosphate (NBDD-PIP$_2$) was from Echelon Research Laboratories Inc. (Salt Lake City, UT). Labeled (dioloyl-1-13C)-t-o-dioleoylphosphatidylcholine, linoleoyl-2-13C-t-o-phosphatidylcholine and t-o-phosphatidylglycerol 4,5-bisphosphate (NBDD-PIP$_2$) was from Echelon Research Laboratories Inc. (Salt Lake City, UT).

Recombinant human PLC-δ1 expressed in Escherichia coli, a generous gift from Dr. Mario J. Rebetschi, was purified as described elsewhere (22). Recombinant PLC-δ1 expressed in Sf9 cells, a generous gift from Dr. Suzanne Scarlata, was purified as described elsewhere (23).

**Peptide Preparations—**The peptide corresponding to the basic effector domain of bovine MARCKS-(151-175), to phospholipid monolayers, a system that eliminates the possibility of artifacts due to vesicle aggregation. Second, we measured directly the binding of MARCKS-(151-175) to bilayers containing PIP$_2$ using two independent techniques. Next, we conducted competition and electrophoretic mobility experiments to investigate the stoichiometry of the complex formed between MARCKS-(151-175) and PIP$_2$. Finally, we examined the importance of nonspecific electrostatic interactions by comparing the specificity of MARCKS-(151-175) for PI(4,5)P$_2$ versus PI(3,4)P$_2$ and measuring the effect of salt on the binding. The results all support the hypothesis that the effector domain of MARCKS binds with high affinity to PIP$_2$ in membranes; under "Discussion," we consider the biological implications of this strong interaction.

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MARCKS Binds Strongly to PIP₂

FIG. 1. MARCKS-(151–175) inhibits the PLC-catalyzed hydrolysis of PIP₂ in monolayers. A, MARCKS-(151–175) (concentrations indicated in the figure); PLC-δ₁ (0.1 nM), and CaCl₂ (free [Ca²⁺] ~ 2 μM; t = 0) were added sequentially to the subphase of a PC/PS/[³H]PIP₂ (68:5:33:0.5) monolayer and the percentage of PIP₂ hydrolyzed was measured at the times indicated. B, same experimental protocol using 0.1 nM PLC-β₁; instead of PLC-δ₁. 10–100 nM MARCKS-(151–175) inhibits the hydrolysis of PIP₂ catalyzed by both PLC isoforms. The data shown are representative of three sets of experiments. The lines show linear fits to the data obtained in the first 6 min. The slopes of the lines are the initial rates of hydrolysis of PIP₂ (% PIP₂ hydrolyzed/min) listed in Table I.

A control experiment showed that MARCKS-(151–175) has a similar effect on the zeta potential of 98:2 PC/dipalmitoyl PI(4,5)P₂ and 98:2 PC/native bovine brain PI(4,5)P₂ MLVs (data not shown).

RESULTS

MARCKS-(151–175) Inhibits the PLC-catalyzed Hydrolysis of PIP₂ in a Monolayer—Fig. 1 illustrates the effects of MARCKS-(151–175) on the PLC-catalyzed hydrolysis of PIP₂.

We deposited a mixture of lipids containing [³H]PIP₂ at the air-water surface; added MARCKS-(151–175), PLC, and CaCl₂ sequentially to the subphase; collected samples of the subphase at the indicated times; and measured the [³H]IP₃ to determine the percentage of [³H]PIP₂ hydrolyzed as a function of time (Fig. 1).

In the absence of MARCKS-(151–175), PLC-δ₁ produces rapid hydrolysis of PIP₂ following addition of CaCl₂ to the subphase (free [Ca²⁺] ~ 2 μM; ~25% of the PIP₂ in the monolayer is hydrolyzed in 5 min (open circles in Fig. 1A). Similar results were reported previously (28, 29, 35). Addition of low concentrations of MARCKS-(151–175) inhibits this hydrolysis; 50 nM MARCKS-(151–175) produces ~50% inhibition, and 150 nM produces almost complete inhibition (decrease in initial rate of hydrolysis or slope of line in Fig. 1A). (The measurements were done at a surface pressure of 25 mN/m to minimize the amount of PLC required. Control experiments at π = 30–35 mN/m, at which the area of a lipid in a monolayer corresponds to that of a lipid in a bilayer (see Footnote 2 in Ref. 28), produced qualitatively similar results (data not shown) to the data shown in Fig. 1A.) Fig. 1B shows that MARCKS-(151–175) also inhibits hydrolysis of PIP₂ catalyzed by PLC-β₁, a different isoform of PLC; 10 nM MARCKS-(151–175) produces ~50% inhibition, and 50 nM produces almost complete inhibition.

The monolayer results (Fig. 1) are consistent with those reported previously using phospholipid vesicles if one notes that higher concentrations of peptide were required to produce inhibition in the vesicle experiments because most of the peptide was bound to the vesicles (18). Specifically, ~10 μM MARCKS-(151–175) produced 90% inhibition of PLC-catalyzed hydrolysis in vesicles containing ~10 μM PIP₂. As expected, reducing the concentration of lipid decreased the concentration of peptide required for inhibition in the vesicle experiments (18). In the monolayer experiments shown in Fig. 1, there is much less PIP₂ than peptide, so most of the peptide is free in the subphase. (If the PIP₂ in a monolayer containing...
0.5% of this lipid were dispersed uniformly through the subphase, it would be present at a concentration of ~1 nm).

The simplest interpretation of the results illustrated in Fig. 1 is that MARCKS-(151–175) decreases the rate of hydrolysis by binding to PIP_2 in the monolayer and competing with the catalytic domain of PLC for PIP_2. The PLC assay system, however, is complicated. We considered three other possible explanations for our results. First, the inhibition could be due to the interaction of MARCKS-(151–175) with PLC. However, we could obtain no evidence for such an interaction using fluorescently labeled MARCKS and PLC (data not shown). Second, the inhibition could be due to the neutralization of the monovalent acidic lipids in the monolayer by MARCKS-(151–175); the rate of the PLC-catalyzed hydrolysis of PIP_2 decreases as the mole fraction of monovalent acidic lipids decreases (28), and addition of 50 nM MARCKS-(151–175) almost neutralizes the 2:1 PC/PS surface (44). However, we obtained similar results for both PLC-δ1 and PLC-β1 using PC/PIP_2 (99.5:0.5) monolayers (Table I); these monolayers are essentially electrically neutral prior to the addition of MARCKS-(151–175). Thus, the inhibition is not due to a decrease in the electrostatic surface potential of the monolayer. (We also observed similar effects using PS/PIP_2 (99.5:0.5) monolayers (Table I), so the inhibition does not depend strongly on the mole fraction of monovalent acidic lipids in the monolayer.) Third, the inhibition could be due to irreversible denaturation/complexation of PLC by a trace contaminant in the peptide solution. This is unlikely because addition of calmodulin reverses the inhibition induced by MARCKS-(151–175) in the monolayer system (data not shown), as was observed previously in the vesicle system (18).

Experiments measuring the effects of other molecules on the PLC-catalyzed hydrolysis of PIP_2 support our interpretation that MARCKS-(151–175) inhibits the reaction by binding to PIP_2. Neomycin, a molecule that forms a 1:1 complex with PIP_2 with an equilibrium dissociation constant in the 1–10 μM range (45), also inhibits the PLC-catalyzed hydrolysis of PIP_2; 2 μM neomycin produces a significant inhibition, and 20 μM neomycin produces ~50% inhibition of the PLC-δ1-catalyzed hydrolysis of PIP_2 (data not shown). Conversely, simple polypeptides such as pentalysine and heptalysine, which do not bind with high affinity to PIP_2 (46), do not inhibit the PLC-catalyzed hydrolysis of PIP_2. For example, 10 μM heptalysine, which binds sufficiently strongly to a 2:1 PC/PS membrane to reduce the zeta potential ~50% from ~50 to ~30 mV (20), has little effect on the hydrolysis of PIP_2, under conditions similar to those described in Fig. 1 (data not shown).

**TABLE I**
Effect of MARCKS-(151–175) on the PLC-catalyzed hydrolysis of PIP_2

| [Pep] | PLC-δ1 | % inhibition | [Pep] | PLC-β1 | % inhibition |
|-------|--------|--------------|-------|--------|--------------|
|       | PC/PIP_2 | (99.5:0.5) |       | PC/PS/PIP_2 | (66.5:33:0.5) |
| 50 nM | ND      | 50%          | 10 nM | 70%     | 70%          |
| 100 nM| 70%     | 80%          | 25 nM | 80%     | 80%          |
| 150 nM| 80%     | 80%          | 50 nM | ND      | 90%          |

a Data from Fig. 1A.
b Data from Fig. 1B.

MARCKS-(151–175) binds strongly to PIP_2. The PLC assay system, [3H]PIP_2, and the percentage of PIP_2 hydrolyzed was measured as in Fig. 1. [Pep] indicates the concentration of MARCKS-(151–175) added to the subphase of the monolayer. % inhibition ~ 100% × (initial rate of hydrolysis without peptide rate with peptide)/rate without peptide. This was determined from experiments similar to those shown in Fig. 1. The data are representative results from at least two sets of experiments.

**Fig. 2A** and a fluorescence technique (Fig. 2B) to measure the binding of MARCKS-(151–175) to vesicles containing PIP_2. Fig. 2A illustrates that [3H]NEM-MARCKS-(151–175) binds strongly to PC/PIP_2 vesicles. We reported previously that incorporation of 1% PIP_2 into PC vesicles enhances the binding of [3H]NEM-MARCKS-(151–175) to vesicles 10^4-fold (25). Fig. 2A extends the data to show the effect of lower mole fractions of PIP_2 on the binding. [3H]NEM-MARCKS-(151–175) binds weakly to PC vesicles (K ~ 10^2 m⁻¹), which can be explained by the hydrophobic insertion of the 5 Phe residues into the bilayer (25). The molar partition coefficient (Equation 1) increases as the mole fraction of PIP_2 in the membrane increases: K ~ 10^4, 10^5, and 10^6 m⁻¹ for PC/PIP_2 LUVs containing 0.1%, 0.1%, and 1% PIP_2, respectively. In other words, a solution containing ~10^-9 m PIP_2 in the form of PC/PIP_2 vesicles binds 50% of [3H]NEM-MARCKS-(151–175). In Fig. 2B, we use a fluorescence technique to measure the binding of acrylodan-MARCKS-(151–175) to PC/PS vesicles. The acrylodan probe is polarity-sensitive; when it binds to a lipid bilayer, its fluorescence signal increases and is blue-shifted. Fig. 2B shows that acrylodan-MARCKS-(151–175) binds to PC/PS/PIP_2 vesicles containing 0.1% and 1% PIP_2 with K ~ 10^5 and 10^6 m⁻¹, respectively, results comparable to those shown in panel A. Thus, measurements using both the centrifugation and the fluorescence techniques show that MARCKS-(151–175) binds strongly to PC/PS vesicles.

Incorporation of PIP_2 into PC/PS vesicles also increases the binding of MARCKS-(151–175) to these vesicles; the molar partition coefficient for 10:1 PC/PS vesicles, 6 × 10^3 m⁻¹ (25), increases ~100-fold to 5 × 10^5 m⁻¹ (data not shown) when the vesicles contain 1% PIP_2 (93:6:1 PC/PS/PIP_2; the mole fraction of PS was reduced to keep the electrostatic surface potential constant).

**MARCKS-(151–175) Binds More than One PIP_2—**Fig. 3 illustrates that, as the concentration of MARCKS-(151–175) increases, the percentage of MARCKS-(151–175) bound to PC/PIP_2 vesicles decreases (open circles). The curve illustrates the predicted effect assuming the peptide forms a 1:1 complex with PIP_2; for example, increasing the concentration of MARCKS-(151–175) from 2 nM to 10 nM should have little effect on the fraction of peptide bound because the accessible concentration of PIP_2 = 30 nM. If only 1:1 complexes were formed, ~50 nM MARCKS-(151–175) should be required to decrease the percentage of peptide bound from ~80% to 50%, but Fig. 3 shows that only 10 nM peptide produces this reduction. These data strongly suggest that one MARCKS-(151–175) binds to one (n > 1) PIP_2. It is difficult, however, to deduce with any confidence the stoichiometry of the peptide/lipid interaction from these data because the theoretical expressions are complicated and model-dependent (47).
show addition of MARCKS-(151-175) quenches the fluorescence, consistent with the peptide binding to several NBD-PIP₂ and inducing a local demixing (data not shown).

**Electrophoretic Mobility Measurements Show MARCKS-(151-175) Binds Equally Well to PI(4,5)P₂ and PI(3,4)P₂**—We measured the electrophoretic mobility (velocity/field) of PC/PIP₂ MLVs and calculated the zeta potential (potential 0.2 nm from surface) using Equation 3. The zeta potential is proportional to the surface charge density (42) and thus can be used to monitor the binding of positively charged MARCKS-(151-175) to negatively charged PC/PIP₂ MLVs. Fig. 4A illustrates that addition of MARCKS-(151-175) changes the zeta potential of PC/PIP₂ vesicles significantly. Specifically, 10⁻⁷ M MARCKS-(151-175) decreases the zeta potential of 98:2 PC/PIP₂ MLVs by ~50% (Fig. 4A, open squares). If we assume that the peptide forms electroneutral complexes with PIP₂, 10⁻⁷ M MARCKS-(151-175) binds ~50% of accessible PIP₂ in PC/PIP₂ MLVs. This agrees qualitatively with the results from the monolayer and binding measurements: 10⁻⁸ – 10⁻⁷ M MARCKS-(151-175) inhibits significantly the hydrolysis of PIP₂ in monolayers (Fig. 1 and Table I) and 10⁻⁸ M PIP₂ in the form of PC/PIP₂ vesicles binds ~50% of MARCKS-(151-175) in the direct binding experiments (Fig. 2). Fig. 4A also illustrates that MARCKS-(151-175) has a similar effect on the zeta potential of MLVs formed from 98:2 PC/PI(4,5)P₂ (open squares), and 98:2 PC/PI(3,4)P₂ (filled triangles). This lack of specificity between PI(3,4)P₂ and PI(4,5)P₂ is expected if the relatively unstructured peptide binds mainly through electrostatic interactions with the phosphoinositides.

**Valence of PIP₂**—A comparison of panels A and B in Fig. 4 illustrates that, in the absence of MARCKS-(151-175), MLVs containing 3% or 6% PS have similar zeta potentials to MLVs containing 1% or 2% PIP₂, respectively. These results suggest that, although the maximum valence of PIP₂ is ~5, the effective valence of PIP₂ is ~3 at pH 7.0, in agreement with previous results (48). NMR experiments show about 1 proton is bound to PIP₂ at pH 7 (48, 49). The results in Fig. 4 suggest that a K⁺ is also bound to PIP₂ in 100 mM KCl to yield an effective valence of ~3. However, we do not know the valence of PIP₂ when it is bound to a basic peptide such as MARCKS-(151-175); it could lose a bound proton or potassium ion.

**Stoichiometry of Complex from Zeta Potential Data**—When
MARCKS Binds Strongly to PIP_2

Fig. 4. Effect of MARCKS-(151–175) on zeta potential of vesicles. A, MARCKS-(151–175) produces significant changes in the zeta potentials of 99:1 PC/PI(4,5)P_2 (○), 98:2 PC/PI(4,5)P_2 (●), and 98:2 PC/PI(3,4)P_2 (▲) vesicles, but does not affect the zeta potential of PC (△) vesicles. B, MARCKS-(151–175) has little effect on the zeta potential of MLVs containing monovalent acidic lipids: 97:3 PC/PS (△), 94:6 PC/PS (●), 94:6 PC/PI (▲) or PC only (○). The zeta potential was calculated from the measured electrophoretic mobility of MLVs using Equation 3. Each point shows the average of at least 20 measurements in two different experiments; the error bars illustrate the standard deviations when they are larger than the size of the symbols.

Fig. 5. The binding of MARCKS-(151–175) to PC/PIP_2 LUVs depends on the salt concentration. The molar partition coefficients (K) deduced from binding measurements like those described for Fig. 2A are plotted versus the salt concentration of the bathing solution. Increasing the salt concentration 5-fold decreases the binding ~100-fold for PC/PIP_2 vesicles containing 1% (● and ○) and 0.1% (▲ and △) PIP_2. The filled and open symbols show the data obtained with 2 nM MARCKS-(151–175) and 5 nM MARCKS-(151–175), respectively.

the [lipid] ≫ [peptide], 10^{-8} \text{ M} PIP_2 binds 50% MARCKS-(151–175) (Fig. 2); thus, 10^{-8} \text{ M} MARCKS-(151–175) should bind significantly to PIP_2 when the [peptide] ≫ [lipid]. Assuming MARCKS-(151–175) forms a 1:1 complex with PIP_2, the valence of a 1:1 peptide:PIP_2 complex would be \sim +10, and the zeta potential would be positive for [peptide] > 10^{-6} \text{ M}. The data in Fig. 4A, however, show that the zeta potentials of 99:1 PC/PIP_2 or 98:2 PC/PI(4)P_2 MLVs remain negative after the addition of 10^{-8} – 10^{-7} \text{ M} MARCKS-(151–175), providing additional evidence that one MARCKS-(151–175) binds to several PIP_2 to form an electroneutral complex (i.e. one +13 valent MARCKS-(151–175) combines with four \sim 3 valent or three \sim 4 valent PIP_2). We stress that the results in Figs. 3 and 4 provide only indirect evidence about the stoichiometry of the complex; the question needs to be addressed using more direct experimental approaches.

MARCKS-(151–175) Bounds Only Weakly to Vesicles Containing Small Fractions of Other Acidic Lipids—Addition of MARCKS-(151–175) to 6% PS or 6% PI vesicles has little effect on the zeta potential of the vesicles (Fig. 4B). The peptide exerts an intermediate effect on the zeta potential of PC/PI(4)P vesicles; 10^{-7} \text{ M} MARCKS-(151–175) changes the zeta potential of 4% PI(4)P vesicles from \sim 18 \text{ mV} to \sim 15 \text{ mV} (data not shown).

Although MARCKS-(151–175) binds strongly to vesicles containing high molar fraction of monovalent acidic lipids (e.g. PS and PG) due to nonspecific diffuse double layer effects, it binds only weakly to vesicles containing a low molar fraction (<10%) of monovalent acidic lipids (Fig. 4B and Ref. 25). Thus, the strong binding of MARCKS-(151–175) to PC/PIP_2 vesicles apparent from the data in Figs. 2 and 4A is not due to the average electrostatic potential (zeta potential) of the vesicles.

Local Electrostatic Effects Are Important—We measured the effect of ionic strength on the binding of MARCKS-(151–175) to PIP_2. Fig. 5 shows that increasing [KCl] from 100 mM to 500 mM decreases the peptide’s molar partition coefficient \sim 100-fold for both 99:1 PC/PIP_2 and 99:9:0.1 PC/PI(4)P_2 vesicles; the average electrostatic potential at the surface of these vesicles is very small or negligible and cannot drive the strong binding of MARCKS-(151–175) to PIP_2. Specifically, the zeta potential (average potential 0.2 nm from surface) is only \sim 8 \text{ mV} (Fig. 4) or \sim 1 \text{ mV} for 1% PIP_2 or 0.1% PIP_2 vesicles, respectively. Note that the molar partition coefficient does not change when the peptide concentration is increased from 2 nM (Fig. 5, filled symbols) to 5 nM (Fig. 5, open symbols). This observation that the molar partition coefficient is independent of peptide concentration when the [lipid] ≫ [peptide] (see Equation 1), provides an important control against several potential artifacts (e.g. the binding sites are not saturated).

Three observations suggest that the binding of MARCKS-(151–175) to PIP_2 depends on a local electrostatic interaction. First, peptide binding decreases as the salt concentration increases (Fig. 5). Second, MARCKS-(151–175) binds with similar affinity to PI(4,5)P_2 and PI(3,4)P_2 (Fig. 4A). Third, MARCKS-(151–175) binding to the phosphonosistides (PI, PI(4)P, and PIP_2) increases with lipid charge (Fig. 4). The local positive electrostatic potential adjacent to a MARCKS-(151–175) peptide adsorbed to a bilayer containing 30% monovalent acidic lipid is illustrated in Fig. 3 of Ref. 17.

Structure of MARCKS-(151–175) Bound to PC/PIP_2 Vesicles—Both CD and EPR measurements indicate that MARCKS-(151–175) is in an extended nonhelical form when it is bound to PC/PS vesicles (50). Our CD measurements suggest that MARCKS-(151–175) also is in an extended form when it is
bound to PC/PIP_2 vesicles (data not shown). Thus, the picture (17) of an extended MARCKS-(151–175) with its aromatics penetrating the polar head group region derived previously from CD, EPR (50), and monolayer measurements (25) is probably also valid for the interaction of MARCKS-(151–175) with PC/PIP_2 vesicles.

**DISCUSSION**

Previous work showed that both the MARCKS protein and a peptide corresponding to its effector domain, MARCKS-(151–175), inhibit the PLC-catalyzed hydrolysis of PIP_2 in phospholipid vesicles containing physiological concentrations (33%) of the monovalent acidic lipid PS; interaction of the peptide with Ca^{2+}/calmodulin or phosphorylation by PKC reverse the inhibition (18). The results we report here show that this reversible inhibition is not an artifact related to the peptide-induced aggregation of the vesicles (20); MARCKS-(151–175) inhibits the PLC-catalyzed hydrolysis of PIP_2 in monolayers comprising a mixture of PC/PS/PIP_2 (Fig. 1). The simplest interpretation of these results is that the effector domain of MARCKS binds to PIP_2 with high affinity and competes successfully with the catalytic domain of PLC for this lipid. Our observation that ∼100 nM MARCKS-(151–175) produces 90% inhibition of PLC-catalyzed hydrolysis of PIP_2 (Fig. 1) thus provides information about the affinity of the peptide for PIP_2 presented in a physiologically relevant PC/PS/PIP_2 surface. This information is difficult to obtain by conventional binding techniques because the peptide also binds strongly to surfaces containing physiological concentrations of monovalent acidic lipids like PS (25).

Direct binding measurements show that incorporating PIP_2 into PC vesicles greatly enhances the binding of MARCKS-(151–175) to the vesicles (Fig. 2). Specifically, ∼10^{-8} μM PIP_2, in form of PC/PIP_2 vesicles containing either 0.01%, 0.1%, or 1% PIP_2, binds 50% of MARCKS-(151–175). This strong binding probably involves formation of an electroneutral complex consisting of one +13 valent MARCKS-(151–175) and three or four PIP_2 because peptide binding does not produce charge reversal of vesicles in the electrophoretic mobility experiments (Fig. 4A) and competition measurements suggest the peptide binds to several PIP_2 (Fig. 3). Two experiments suggest that local, nonspecific electrostatic interactions contribute strongly to the binding: MARCKS-(151–175) exhibits no specificity for PI(4,5)P_2 versus PI(3,4)P_2 (Fig. 4A), and increasing the salt concentration 5-fold decreases the binding 100-fold (Fig. 5).

The interaction of PIP_2 with MARCKS-(151–175) may be compared with its interaction with neomycin and the PH domain of PLC-δ_1, two other well characterized molecules that bind PIP_2 with high affinity. Addition of ∼10^{-5}, 10^{-6}, or 10^{-8} μM PIP_2 (in the form of PC/PIP_2 vesicles) binds 50% of neomycin (45), the PH domain (51), or MARCKS effector domain (Fig. 2), respectively. Neomycin and the PH domain form 1:1 complexes with PIP_2 (45, 51–53) whereas MARCKS-(151–175) probably interacts with several PIP_2 (this report).

The binding of MARCKS-(151–175) to vesicles containing monovalent acidic lipids such as PS involves three forces: a long range Coulomb attraction, a short range Born/dehydration repulsion, and a short range hydrophobic attraction of the aromatic residues for the polar head group region of the bilayer (25). Although these three forces also must be important in mediating the interaction of the peptide with membranes containing PIP_2, this binding is not yet well understood at a molecular level. Additional experimental information is required to understand the role of the 5 Phe and other specific residues in the binding of MARCKS-(151–175) to PIP_2. Ongoing NMR experiments, EPR measurements with spin-labeled PIP_2,2 and our quenching measurements with fluorescent PIP_2 should provide useful information. The direct binding measurements we report here, however, are sufficient to show that the effector domain of MARCKS binds to PIP_2 with high affinity (Figs. 2–5) and sequesters the PIP_2 away from the catalytic domain of PLC (Fig. 1). Extrapolating from our observations on the MARCKS protein and effector domain peptide in model systems, we hypothesize that the effector domain of MARCKS reversibly sequesters a significant fraction of the PIP_2 in the plasma membrane of cells.

**Biological Implications of MARCKS Effector Domain Binding to PIP_2**—Fig. 6 illustrates what is known about the binding of MARCKS to membranes. Work from many different laboratories has shown the MARCKS protein binds to phospholipid vesicles—and by implication to plasma membranes in cells—through hydrophobic interaction of its N-terminal myristate with the interior of the bilayer and electrostatic interaction of its basic effector domain (residues 151–175) with acidic lipids (Refs. 54–58; reviewed in Refs. 16 and 17). Fig. 6 illustrates the 13 positively charged residues (blue plus signs) and 5 aromatic residues (green ovals) in the effector domain; much of what we know about the interaction of the effector domain with membranes comes from experiments with the MARCKS-(151–175) peptide, which appears to bind in a similar manner to the effector region in the intact protein (reviewed in Ref. 17). The regions of MARCKS flanking the effector domain contain negatively charged residues (red minus signs) and should be repelled from the negatively charged surface. What is new is the recognition that the effector region can bind PIP_2 (lipids with red circles in Fig. 6) with high affinity; in a PC/PIP_2 membrane, ∼4 PIP_2/peptide are bound. Biological membranes such as the plasma membrane also contain monovalent acidic lipids that may affect the binding stoichiometry, so the exact number of PIP_2 bound is not known. It must be significant, however, because both the peptide and protein inhibit PLC-catalyzed hydrolysis of PIP_2 on membranes that contain 33% monovalent acidic lipids. 

2 D. Cafiso, personal communication.
acids and phosphoinositides (see Fig. 1 and Ref. 18).

There are several corollaries to our hypothesis that MARCKS binds a significant fraction of the PIP2 in the plasma membrane and releases it upon interaction with Ca2+/calmodulin or phosphorylation by PKC. First, if MARCKS is to bind a significant fraction of the PIP2, it must be present in cells at concentrations comparable to PIP2; it is in many cell types (e.g., 10 μM in brain, Refs. 13 and 15). Second, if reversible binding of PIP2 by MARCKS is important for phosphoinositide function, overexpression of MARCKS should stimulate increased production of PIP2 to maintain a constant free concentration of PIP2 in the membrane; this has been observed in some cells (59). Third, in cells where MARCKS is distributed nonuniformly in the plasma membrane (presumably because of protein-protein interactions, possibly with actin), PIP2 should be colocalized with MARCKS; in fibroblasts, both MARCKS and PIP2 are concentrated in membrane ruffles (60–62). Fourth, the mechanism by which MARCKS is targeted selectively to the plasma membrane is unknown; the interaction of the effector domain with PIP2 documented here could contribute to this targeting, as could the less specific electrostatic targeting mechanism that Silvius and co-workers (63) recently proposed with respect to the targeting of K-ras4B to the plasma membrane. Fifth, and perhaps most importantly, local activation of PKC or calmodulin should produce a local increase in the free concentration of PIP2 in the plasma membrane; although this has been observed in model systems (see Fig. 3 in Ref. 18), it is not known if Ca2+/calmodulin and PKC can produce the release of PIP2 bound by the effector domain of MARCKS in biological cells. This putative function of MARCKS could be examined in cells using green fluorescent protein–MARCKS (64) and different fluorescent indicators for PIP2, such as fluorescently labeled neomycin (65) and green fluorescent protein–PH (66–69).

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