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Articles

Interactions Controlling the Membrane Binding of Basic Protein Domains: Phenylalanine and the Attachment of the Myristoylated Alanine-Rich C-Kinase Substrate Protein to Interfaces†

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ABSTRACT: Basic residues are known to play a critical role in the attachment of protein domains to membrane interfaces. Many of these domains also contain hydrophobic residues that may alter the binding and the position of the domain on the interface. In the present study, the role of phenylanine in determining the membrane position, dynamics and free energy of a peptide derived from the effector domain of the myristoylated alanine-rich C-kinase substrate (MARCKS) protein was examined. Deuterium NMR in membranes containing phosphatidylcholine (PC) and phosphatidylserine (PS) indicates that this peptide, MARCKS(151−175), partially penetrates the membrane interface when bound and alters the effective charge density on the membrane interface by approximately 2 charges per bound peptide. However, a derivative of this peptide in which the five phenylalanines are replaced by alanine, MARCKS-Ala, does not penetrate the interface when membrane-bound. This result was confirmed by depth measurements by electron paramagnetic resonance spectroscopy on several spin-labeled derivatives of the Phe-less derivative. In contrast to nitroxides on MARCKS(151−175), nitroxides on the derivative lacking Phe do not reside within the bilayer but are in the aqueous phase when the peptide is bound to the membrane. The Phe to Ala substitutions shift the position of the labeled side chains by approximately 10−15 Å. The side-chain dynamics of MARCKS-Ala are strongly influenced by membrane charge density and indicate that this peptide is drawn closer to the membrane interface at higher charge densities. As expected, MARCKS-Ala binds more weakly to membranes composed of PS/PC (1:9) than does the native MARCKS peptide; however, each phenylalanine contributes only 0.2 kcal/mol to the binding energy difference, far less than the 1.3 kcal/mol expected for the binding of phenylalanine to the membrane interface. This energetic discrepancy and the differences in membrane position of these peptides can be accounted for by a dehydration energy that is encountered as the peptide approaches the membrane interface. This energy likely includes a Born repulsion acting between the charged peptide and the low dielectric membrane interior. The interplay between the long-range attractive Coulombic force, the short-range repulsive force and the hydrophobic effect controls the position and energetics of protein domains on acidic membrane interfaces.

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Many water-soluble proteins that participate in cell signaling pathways are functional only when associated with the cytoplasmic surface of the plasma membrane. In general, these proteins become attached to the membrane surface as a result of protein acylation and/or the electrostatic interaction between a positively charged protein domain and the negatively charged membrane interface. This electrostatic interaction appears to be important for the membrane binding of a number of proteins, including the myristoylated alanine-rich C-kinase substrate, MARCKS[1] (*1*−*3*), protein kinase C (PKC) (*4*), neuromodulin (*2*, *5*, *6*), secretory phospholipase A_2 (*7*, *8*), the HIV gag protein (*9*), and members of the Src tyrosine kinase family (*10*−*12*). In addition, the phosphorylation of sites within these basic protein domains may serve as an "electrostatic switch" to control the membrane association and hence the activity of the protein (*13*).

The MARCKS protein is a major PKC substrate in many cell types, and it is phosphorylated during cellular processes such as phagocyte activation, secretion, and mitogenesis. To become attached to the membrane interface, both the N-terminal myristoylation of MARCKS and an electrostatic interaction between its highly basic effector domain and the acidic membrane surface are required (*3*, *14*, *15*). This effector domain, MARCKS(151−175), has a high affinity for calmodulin and includes three serines whose phosphorylation by PKC results in the translocation of MARCKS from the membrane to the cytoplasm (*13*, *14*). These three phosphorylation sites are located in a segment that contains five phenyalanine residues. Although the precise function of the MARCKS protein has not been determined, it has been proposed to act as a PKC-regulated calmodulin buffer, and it is likely to function in modulating PKC- and calmodulin-(CaM-) mediated signaling pathways that regulate actin−membrane interactions and actin cytoskeleton organization (*15*−*19*).

Several forces are thought to be important in promoting the electrostatic attachment of basic domains such as MARCKS(151−175) to the membrane interface. When a peptide corresponding to MARCKS(151−175) is examined, it fails to bind to membranes composed of phosphatidylcholine (PC) but it associates strongly with membranes containing acidic lipids such as phosphatidylserine (PS) (*2*). The same observation is made for a number of charged peptides, including simple peptides such as pentalysine (Lys_5) (*20*). Evidence obtained from ^2H NMR and measurements of monolayer surface pressure indicate that Lys_5 fails to penetrate the membrane interface (*21*, *22*), and indeed computational work examining the electrostatics of association of Lys_5 indicates that it should lie just on the aqueous side of the membrane when bound to the interface (*22*). However, the interaction of MARCKS(151−175) with the membrane interface is not purely electrostatic. EPR measurements on a series of spin-labeled peptides derived from MARCKS(151−175) as well as monolayer surface pressure measurements on this peptide provide strong evidence that MARCKS(151−175) partially penetrates the membrane interface with its five phenyalanines positioned several angstroms below the level of the lipid phosphate (*19*, *23*). The more highly charged N-terminal portion of this peptide, which resembles Lys_5, extends off the membrane interface and is found to reside in the aqueous phase. From these experimental and computation results, several competing forces are thought to contribute to the binding free energy and determine the position of the peptide when membrane-bound. One important force is the long-range electrostatic attraction between positive charge on the peptide and the negatively charged membrane interface. A second short-range repulsive force is thought to become important when these highly charged peptides approach close to the membrane interface. When close to the interface, these basic peptides experience a dehydration energy that includes the Born repulsion, generated by the difference in the dielectric between the aqueous phase and the membrane interior. The balance between this long-range attraction and short-range repulsion may account for the position of the free energy minimum for peptides such as Lys_5 several angstroms on the aqueous side of the membrane solution interface. In the case of MARCKS(151−175), the presence of five phenylalanine residues may provide an additional hydrophobic binding free energy that overcomes the short-range Born repulsion and positions the peptide at the membrane interface.

Despite the importance of protein−membrane electrostatic interactions in cell signal transduction, there is relatively little molecular information currently available on the interplay between the forces that dictate the structure, position, and energetics of a protein bound to a lipid membrane surface. To test the importance of these interactions, we synthesized and investigated several peptides derived from the effector domain of MARCKS that lack phenylalanine (MARCKS-Ala) and compared their membrane position and binding with that of the native MARCKS peptide [MARCKS (151−175)] as well as pentalysine (Lys_5). Both ^2H NMR and EPR techniques were employed to measure the position of these peptides when membrane-bound, and EPR was used to investigate the dynamics and binding free energy of the MARCKS-derived peptide lacking phenyalanine. The results indicate that the Phe residues in MARCKS(151−175) provide the additional free energy necessary to attach the peptide to the membrane interface and that without them

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[1] Abbreviations: CaM, calmodulin; CD, circular dichroism; EPR, electron paramagnetic resonance; DCM, dichloromethane; DMF, dimethylformamide; HPLC, high-performance liquid chromatography; LAD, lithium aluminum deuteride; LUV, large unilamellar vesicle; MALDI TOF, matrix-assisted laser desorptionionization time-of-flight; MARCKS, myristoylated alanine-rich C-kinase substrate; MARCKS-Ala, the 25-amino acid myristoylated alanine-rich C-kinase substrate peptide (residues 151−175) with the five wild-type phenylalanine residues replaced by alanines; MBHA, *p*-methylbenzhydrylamine; MOPS, morpholinopropanesulfonic acid; MTSSL, methanethiosulfonate spin label; NiEDDA, nickel ethylenediaminediacetic acid; myr-Src (2−16), myristoylated N-terminal sequence of the $pp60^{src}$ gene product; NM, neuromodulin; NMM, *N*-methylmorpholine; PC, phosphatidylcholine; PKC, protein kinase C; POPC, palmitoyloleoylphosphatidylcholine; POPS, palmitoyloleoylphosphatidylserine; PS, phosphatidylserine; PyBOP, [(benzotriazolyl)oxy]tripyrrolidinophosphonium hexafluorophosphate; TFA, trifluoroacetic acid; TPS, 2,4,6-triisopropylbenzenesulfonyl chloride.

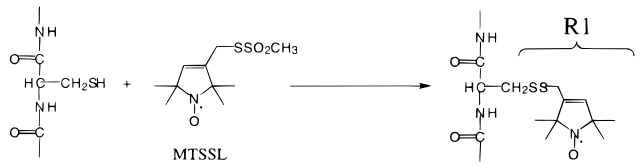
[2] The rotational correlation times calculated here and shown in Table 2 are rigorously correct only for a label having isotropic motion. Since the labels here exhibit anisotropic motion, this estimated rate is approximate and is intended only to provide a comparison of the rates of motion between different segments of the peptide.

Table 1: MARCKS-Derived Peptides[a]

peptide	sequence
MARCKS(151−175)	Ace-KKKKKRFSFKKSFKLSGFSFKKNKK-NH_2
MARCKS-Ala	Ace-KKKKKRASAKKSAKLSGASAKKNKK-NH_2
MARCKS-S8C	Ace-KKKKKRACAKKSAKLSGASAKKNKK-NH_2
MARCKS-L15C	Ace-KKKKKRASAKKSAKCSGASAKKNKK-NH_2
MARCKS-N23C	Ace-KKKKKRASAKKSAKLSGASAKKCKK-NH_2

[a] The peptides lacking phenylalanine, MARCKS-S8C, MARCKS-L15C and MARCKS-N23C, were derivatized with the methanethiosulfonate spin label (Scheme 1) to produce peptides with a single spin-label side chain (R1) at the single cysteine position.

Scheme 1



these peptides bind in a fashion similar to that observed for Lys_5 and other hydrophilic basic peptides. The data indicate that the interplay between dehydration effects, long-range electrostatic attraction, and the hydrophobic effect determine the membrane-bound position and dynamics of charged peptides.

EXPERIMENTAL PROCEDURES

Materials. Palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoylphosphatidylserine (POPS), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (POPA), headgroup spin-labeled PC, and spin-labeled doxyl phosphatidylcholines [1-palmitoyl-2-stearoyl(*n*-doxyl)phosphatidylcholines, $n = 5$, 7, 10, 12] were obtained from Avanti Polar Lipids (Alabaster, AL) and used without further purification. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine-α-d_2 (POPC-α-d_2) was synthesized as described below. The methanethiosulfonate spin label, *S*-(1-oxy-2,2,5,6-tetramethylpyrroline-3-methyl) (MTSSL), was purchased from Reanal (Budapest, Hungary). Nickel ethylenediaminediacetic acid (NiEDDA) was synthesized by following a protocol that was developed and generously donated by Dr. Christian Altenbach. Lithium aluminum deuteride (LAD) was purchased from MSD Isotopes (Montreal, Canada). *N*,*N*-Dimethylglycine ethyl ester, methyl iodide, and sodium tetraphenylborate were from Aldrich (Milwaukee, WI), and pentalysine and 2,4,6-triisopropylbenzenesulfonyl chloride (TPS) were from Sigma (St. Louis, MO).

Peptide Synthesis. A series of peptides (see Table 1) based on the MARCKS(151−175) sequence were synthesized and purified by the Biomolecular Research Facility at the University of Virginia (Charlottesville, VA). The identity of each peptide was confirmed by mass spectrometry. Both the wild-type MARCKS(151−175) sequence and a peptide where the five Phe residues were replaced with Ala residues were produced. For the alanine mutant MARCKS-Ala, three additional peptides having a single cysteine substitution were produced and derivatized by the SH-specific methanethiosulfonate spin label (Scheme 1) to produce a peptide with the side chain R1.

POPC-α-d_2 Synthesis. Headgroup-deuterated palmitoyloleoylphosphatidylcholine (POPC-α-d_2) was synthesized as described previously (*24*), beginning with the synthesis of choline-α-d_2 tetraphenylborate. The second step to produce this deuterated lipid involved coupling the choline salt to palmitoyloleoylphosphatidic acid (POPA) by use of triisopropylbenzenesulfonyl chloride (TPS) This second step and the purification of the lipid were carried out by Avanti Polar Lipids (Alabaster, AL). Deuteration of this position places the label at the position α to the phosphate group.

Multilamellar Vesicle Preparation for ^2H NMR. To produce multilamellar vesicles (MLVs) suitable for ^2H NMR, the appropriate amounts of lipids were dissolved in chloroform and dried in a rotary evaporator to get a thin film of lipid, which was further dried under vacuum for a minimum period of 4 h. The dry lipid was hydrated with a buffer containing 10 mM HEPES at pH 7.4. In cases where peptides were added to the MLVs, the appropriate amount of the peptide was dissolved in the hydration buffer, and the suspension was gently warmed and freeze−thawed five times to ensure homogeneity. Vesicles containing PC were made with POPC-α-d_2, and POPS was added to obtain the appropriate concentration of PS.

Lipid Vesicle Preparation for EPR. Lipid mixtures containing the desired mole ratio of POPC and POPS were produced by mixing the appropriate lipid solutions in chloroform, removing the chloroform by vacuum desiccation overnight, and hydrating the lipid film by the addition of 100 mM KCL and 10 mM MOPS, pH = 7.0. Unilamellar vesicles were produced by freeze−thawing this suspension five times followed by extrusion of the mixture through polycarbonate filters with a 0.1 μm pore diameter (Poretics, Livermore, CA) by use of a LiposoFast extruder (Avestine, Ottawa, Canada). For the EPR power saturation measurements, stock lipid solutions contained a 125 mM total lipid concentration while 10 mM stock solutions were produced for the lipid titration measurements.

^2H NMR Spectroscopy. Deuterium NMR spectra were obtained at a magnetic field of 8.5 T (360 MHz for ^1H and 55.49 MHz for ^2H) with a horizontal coil probe using the quadrupolar echo sequence described previously (*25*). The 90° pulse width, which was calibrated with a sample of D_2O, was 3.5 ms, and 8K or 16K scans were recorded for each sample. The time domain data were apodized with a 50 Hz exponential function and Fourier-transformed. All spectral processing was performed with Felix970 (MSI, Scranton, CA) running on a Silicon Graphics R4000 computer.

EPR cw Partition Coefficient Measurements. The membrane binding affinity of the spin-labeled MARCKS-derived peptides was determined by an EPR technique similar to that described previously (*26*). Briefly, 100 μL of a particular nitroxide spin-labeled peptide solvated in aqueous buffer (at pH 7.0 with 100 mM KCl and 10 mM MOPS) was placed in the sample reservoir. A Varian E-line Centuries series spectrometer outfitted with an X-band loop−gap resonator (Medical Advances, Milwaukee, WI) with a standard two-

loop, one-gap configuration was employed to make the binding measurements. By use of a plunger, approximately 10 μL of sample was loaded into and out of a round (0.5 × 0.7 o.d.) quartz capillary (VitroCom, Mt. Lakes, NJ) that was firmly secured within the loop−gap resonator. The peak-to-peak amplitude of the $m_I = -1$ EPR resonance of peptide was then measured as a function of the concentration of lipid. The peptide sample was titrated with lipid until the resulting EPR spectrum indicated that the peptide was fully bound to lipid. For each spectrum taken during the lipid titration, the faction of the peptide bound to the membrane, f_B, was determined from the peak-to-peak amplitude change of the high-field EPR resonance in a manner similar to that described previously (*27*). The molar partition coefficient with units of M^{-1}, K_p, was determined by assuming that the binding behavior of the peptide can be described by

$$f_B = \frac{K_p[\text{lipid}]}{1 + K_p[\text{lipid}]} \quad (1)$$

where [lipid] represents the molar concentration of lipid in the sample (*3*).

EPR Power Saturation Measurements. Continuous-wave (cw) power saturation measurements were performed with the same spectrometer and resonator configuration described above. The samples contained 100 μM peptide in the presence of lipid vesicles at a total phospholipid concentration of 100 mM. The high concentrations of lipid used ensured that the peptide was fully bound to membrane. The samples were held within the EPR resonator by a gas-permeable TPX capillary tube (Medical Advances, Milwaukee, WI), and the microwave power was varied from roughly 0.24 to 200 mW, with the exact range depending upon the particular peptide being examined. For each power setting, the peak-to-peak amplitude of the $m_I = 0$ resonance, A, of the nitroxide spin-labeled peptide was measured. The dependence of A on the incident microwave power, P, can be expressed as

$$A_{m_I=0} = I\sqrt{P}\left[1 + (2^{1/\epsilon} - 1)\frac{P}{P_{1/2}}\right]^{-\epsilon} \quad (2)$$

where I is a scaling factor, $P_{1/2}$ is the microwave power required to reduce the resonance amplitude to half its unsaturated value, and ϵ is a measure of the homogeneity of the saturation of the resonance (*28*). By allowing I, ϵ, and $P_{1/2}$ to be adjustable parameters in a fit of the data to eq 2, a characteristic $P_{1/2}$ is obtained. Values for $P_{1/2}$ are then generated for each sample under three different sets of conditions: (1) equilibrated with N_2, (2) equilibrated with air (20% O_2), and (3) equilibrated with N_2 in the presence of 20 mM NiEDDA. A depth parameter, Φ, was then calculated from the three $P_{1/2}$ values:

$$\Phi \equiv \ln\left[\frac{P_{1/2}(O_2) - P_{1/2}(N_2)}{P_{1/2}(\text{NiEDDA}) - P_{1/2}(N_2)}\right] \equiv \ln\left[\frac{\Delta P_{1/2}(O_2)}{\Delta P_{1/2}(\text{NiEDDA})}\right] \quad (3)$$

Since Φ is directly related to the difference in the standard-state chemical potentials of O_2 and NiEDDA and varies as a function of depth in the lipid bilayer, Φ provides an estimate of the relative nitroxide depth in the lipid bilayer (*28*).

To convert the Φ values into an absolute distance with respect to the membrane surface, calibration curves were obtained with the same lipid compositions used for power saturation measurements on the various MARCKS peptides. Each calibration curve was obtained by employing a series of nitroxide-labeled PCs: a headgroup spin-labeled PC and spin-labeled PCs with doxyl nitroxides at positions 5, 7, and 10 along the alkyl chain. While still suspended in chloroform, these spin-labeled lipids were codissolved with the desired mixture of nonlabeled lipids. This composite lipid mixture was then dried, hydrated, and extruded to form vesicles in the same manner as described above. The subsequent EPR power saturation measurements, obtained in the absence of all peptides, were made with a total lipid concentration of 100 mM and a spin-labeled lipid concentration of 250 μM.

RESULTS

MARCKS(151−175) Alters the Surface Charge Density and Penetrates the Membrane Interface As Detected by ^{2}H NMR. Figure 1A shows a series of ^{2}H NMR spectra for POPC-α-d_2 in membranes where the concentration of negatively charged POPS is altered. As the surface charge density is increased, the residual quadrupolar splitting for the α-position increases, and the response of $\Delta\nu_Q$ is plotted as a function of the POPS content in Figure 1B. This behavior in $\Delta\nu_Q$ is well documented and is a result of a change in the geometry of the lipid headgroup that occurs due to the local electrostatic field produced by the addition of POPS (*29*). The linear behavior shown in Figure 1B allows an estimate to be made of the effective membrane surface charge density at the level of the lipid headgroup. When ^{2}H NMR spectra are obtained for bilayers formed from POPC/POPS 2:1 with and without pentalysine (Figure 2A,B), we obtain identical spectra with a value of 8.5 kHz for $\Delta\nu_Q$ even when pentalysine is added at a concentration that should fully neutralize the negative charge density on the bilayer surface (lipid charge:peptide charge ratio of 1:2). These observations are identical to those from earlier experiments done under slightly different conditions (*21*). A large fraction of the pentalysine is bound under the conditions of these experiments, and this lack of change in the ^{2}H NMR spectrum has been taken as evidence that the peptide does not penetrate the interface but lies several angstroms off the membrane surface when bound (*21*).

Shown in Figure 2C is the ^{2}H NMR spectrum for 2:1 PC/PS lipid membranes in the presence of MARCKS(151−175) where the PS/peptide charge ratio is 1:2. In contrast to the addition of Lys_5, the spectrum shows a measurable change in the quadrupolar coupling constant (ν_e) with the addition of MARCKS(151−175), and from the calibration shown Figure 1B, the 0.9 kHz decrease in the quadrupolar splitting corresponds to a 38% reduction in the anionic surface charge experienced by the choline headgroup in the membrane. When the peptide charge:lipid charge ratio is lowered to 1:1, the quadrupolar splitting changes by 0.7 kHz, representing a 16% reduction in the effective anionic surface charge density. At this lower concentration, the 16% reduction corresponds to a contribution of about two positive charges

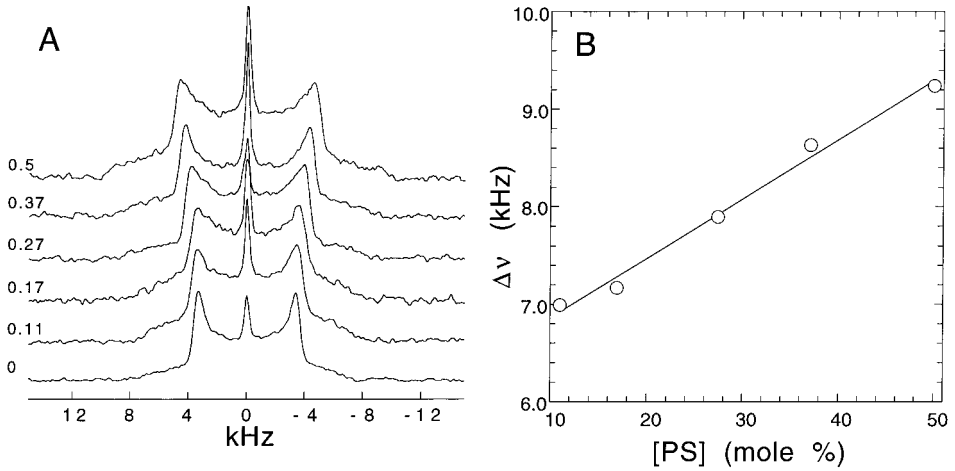


FIGURE 1: (A) Deuterium NMR spectra obtained from lipid membranes incorporating POPC-α-d_2 and increasing mole percent of POPS. The quadrupolar coupling constant (ν_e) of the POPC-α-d_2 increases with increasing anionic surface charge. Each sample had 10 mg of POPC-α-d_2, the appropriate amounts of POPS, and 50 mL of pH 7.4 buffer containing 10 mM HEPES. Typical spectral accumulation times were approximately 1 h. (B) Calibration of the changes in the quadrupolar coupling constant (ν_e) of POPC-α-d_2 as a function of PS mol % concentration. The displayed data were extracted from the ^{2}H NMR spectra illustrated in panel A. In this sample, the quadrupolar splittings increase linearly with PS mol % with a slope of 0.06 kHz/mol % and an intercept of 6.2 kHz.

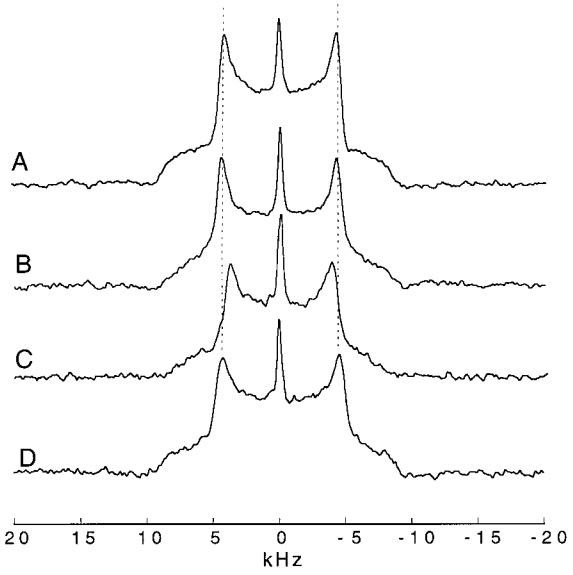


FIGURE 2: Deuterium NMR spectra of 2:1 PC/PS lipid membranes (A) alone, (B) with pentalysine added, (C) with MARCKS(151−175) added, and (D) with MARCKS-Ala added. Sufficient peptide is added so that the ratio of lipid to peptide charge is 1:2. For spectra A, B, and D, the quadrupolar splitting is approximately 8.5 kHz. Only the addition of MARCKS(151−175) (spectrum C) produces a significant change in the quadrupolar splitting of POPC-α-d_2. The addition of MARCKS(151−175) decrease the residual quadrupolar splitting by 0.9 kHz, corresponding to a 38% reduction in the anionic charge experienced by the choline headgroup in the lipid membrane.

from each peptide if it is assumed that all of the peptides are fully bound. Thus, the effect of this peptide is different than that observed for Lys_5 and suggests that the peptide lies much closer to the membrane interface so the change in charge density is detected by the POPC headgroup. This observation is consistent with previous work with EPR and surface pressure measurements indicating that the MARCKS-(151−175) segment is partially inserted into the bilayer interface (*19*, *23*).

The difference between the behavior of Lys_5 and MARCKS(151−175) could be the result of the five phenylalanine residues in MARCKS(151−175), which lie within the membrane interface when the peptide is membrane-bound (*23*). To determine whether these residues are responsible for the interfacial attachment of this peptide, a MARCKS-(151−175) mutant, MARCKS-Ala, was synthesized in which each of the five wild-type phenylalanine residues were replaced with an alanine. Figure 2D shows the ^{2}H NMR spectra of 2:1 PC/PS lipid membranes with MARCKS-Ala added to a POPS:peptide charge ratio of 1:2. Unlike MARCKS(151−175) peptide (Figure 2C), this phenylalanine-less analogue does not induce a significant change in the quadrupolar coupling constant (ν_e) and behaves in an identical fashion in this regard to pentalysine. This suggests that the equilibrium position of the MARCKS-Ala peptide, when membrane-associated, lies far enough from the water−membrane interface so that its positive charges do not significantly alter the negative charge density felt at the membrane headgroup by the deuterated POPC. This result also suggests that the five phenylalanines of MARCKS(151−175) are essential for the partial penetration of the wild-type peptide into the headgroup region of the lipid membrane.

Alanine to Phenylalanine Substitution Produces a Large Shift in the Equilibrium Position of the MARCKS Domain As Detected by EPR. To complement the results obtained by ^{2}H NMR, a set of EPR experiments were conducted with the three nitroxide-labeled MARCKS-Ala peptide mutants shown in Table 1. Figure 3 shows the EPR spectra obtained for the three spin-labeled versions of the MARCKS-Ala peptide in buffer and in the presence of PC/PS (3:1) vesicles at a lipid concentration that is in excess of that required to bind all the peptide to the membrane. When bound to the membrane interface, the motion of the spin-labeled side chains is slowed by a factor of 3−4 compared to that in solution. The estimated values for the rotational correlation times (τ_c) from these spectra are shown in Table 2. A direct comparison of the line shapes for the native peptide, MARCKS(151−175), and MARCKS-Ala labeled at position 23 is shown in Figure 4. When bound to the membrane, the correlation times for the MARCKS-Ala derivative are roughly 3 times faster than that for the native MARCKS(151−175) peptide observed previously (*23*). Also

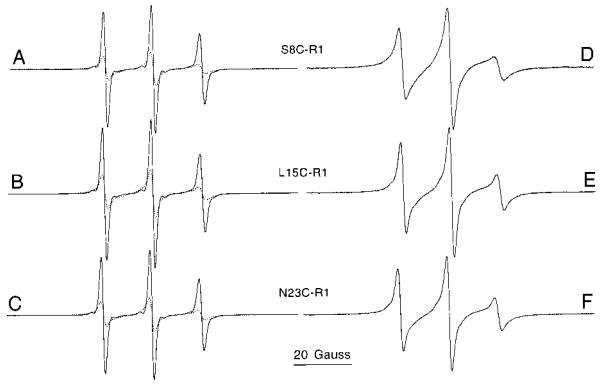


FIGURE 3: EPR spectra of the 25-amino acid MARCKS-Ala peptide mutants (A) S8C-SL, (B) L15C-SL, and (C) N23C-SL in an aqueous solution of 100 mM KCl and 10 mM MOPS, pH = 7.0. The dashed lines are spectra taken of the same spin-labeled peptides fully bound to 100 mM PC/PS (3:1) lipid vesicles of 1000 Å in diameter. The amplitudes of these spectra were normalized by total spin number. These membrane-bound spectra are shown expanded in panels D, E, and F for the S8C-R1, L15C-R1, and N23C-R1 derivatives, respectively. The spectra were taken at 25 °C with a scan width of 100 G, a scan time of 480 s, and a modulation amplitude of 1.0 G.

Table 2: EPR Parameters for Spin-Labeled MARCKS-Ala Peptides That Are Fully Bound to 1000 Å Diameter Lipid Vesicles Composed of PC/PS (3:1) at a Total Lipid Concentration of 100 mM

peptide	τ_c[a] (ns) (aqueous)	$\Delta P_{1/2}$-(air)[b]	$\Delta P_{1/2}$-(NiEDDA)[b]	Φ	τ_c (ns)	A_0 (G)
S8C-SL	0.41	5.5	18.6	−1.2	1.7	16.7
L15C-SL	0.35	4.6	24.6	−1.7	1.1	16.7
N23C-SL	0.31	4.8	22.3	−1.5	1.0	16.7
N23C-SL (native)		24.3	6.5	1.3	4.3	15.8

[a] Correlation times for aqueous MARCKS-Ala peptides in 100 mM KCl and 10 mM MOPS, pH = 7.0. [b] Values for $\Delta P_{1/2}$ are given in milliwatts. Air contains approximately 20% O_2, which is a hydrophobic paramagnetic species. NiEDDA, an uncharged hydrophilic paramagnetic species, was added to a final concentration of 20 mM. For comparison, data for one of the native peptides (not lacking the five phenylalanines) is shown (*23*).

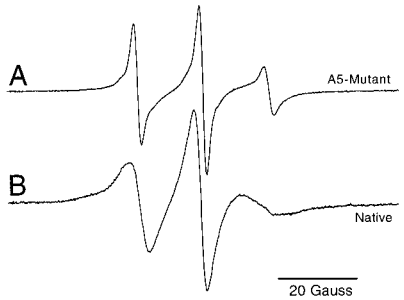


FIGURE 4: Comparison of the EPR spectra for MARCKS-derived peptides fully bound to 1000 Å lipid vesicles at a PC/PS ratio of 3:1: (A) The phenylalanine to alanine derivative, MARCKS-Ala, labeled at position 23 (N23C-R1), and (B) the native MARCKS-(151−175) also labeled at position 23 (data taken from ref *23*). The peptides are at a concentration of approximately 100 μM and the lipid is at a concentration of 100 mM.

shown in Table 2 are the averaged hyperfine coupling constants (A_0), which indicate that the environment in which the side chains of MARCKS-Ala are found is more polar than that for MARCKS(151−175). These line widths and rotational correlation times for MARCKS-Ala are similar to that obtained for a membrane-bound peptide derived from

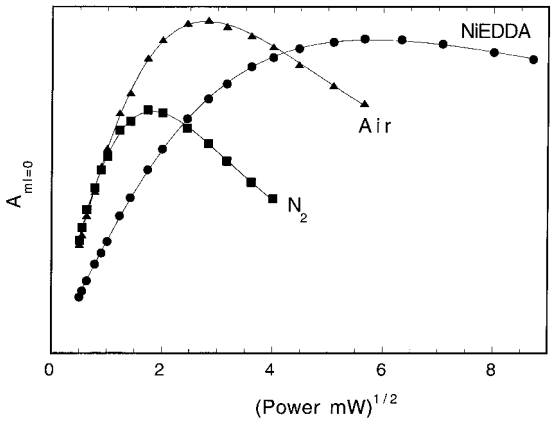


FIGURE 5: Power saturation curves obtained for the spin-labeled MARCKS-Ala peptide, L15C-SL, while fully bound to 1000 Å diameter PC/PS (3:1) lipid vesicles. The buffer contained 100 mM KCl and 10 mM MOPS, pH = 7.0, and all the spectra were taken at 25 °C with a modulation amplitude of 1.0 G. Amplitudes of the $m_I = 0$ line in the presence of N_2 alone (■), in the presence of air (▲), and in the presence of 20 mM NiEDDA (●) are illustrated as functions of the square root of the microwave power ($P^{1/2}$). The solid lines denote the curve fit to the data using eq 2. The $P_{1/2}$ values for this labeled peptide were 3.9, 8.5, and 28.5 mW for N_2, air, and NiEDDA, respectively. Two sets of $P_{1/2}$ values were obtained for each peptide and were used to determine the values of $\Delta P_{1/2}$ and the values of Φ listed in Table 2 (see Experimental Procedures).

Src, which is in an extended structure and lies on the aqueous side of the membrane−solution interface (*12*). Together, this information suggests that the side chains of the membrane-bound MARCKS-Ala are not buried within the lipid interface and are in a more polar environment lying further away from the water−membrane interface. It should be noted that the MARCKS-Ala derivative, like the native MARCKS(151−175), appears to be an extended β-strand structure on the membrane interface as indicated by circular dichroism of the membrane-bound peptide (data not shown).

More quantitative information on the position of these spin-labeled side chain was obtained by power saturating the central line width of the EPR resonance obtained from these peptides. Figure 5 illustrates an example of a power saturation measurement obtained with the spin-labeled version of MARCKS-Ala, L15C-R1, and Table 2 contains a summary of the values calculated for $\Delta P_{1/2}$ and the Φ parameters for the different mutants. The values represent an average of several independent experiments and have an associated error of ±0.1 for both the $\Delta P_{1/2}$ and Φ parameters. Compared with data obtained previously for MARCKS(151−175) (*23*), the MARCKS-Ala peptide is significantly less accessible to the hydrophobic relaxation agent (O_2) while being significantly more accessible to the hydrophilic relaxation agent (NiEDDA). These results again indicate that the membrane-bound MARCKS-Ala peptide takes up a position further on the aqueous side of the membrane interface than the native MARCKS(151−175). The differences in accessibility to the two relaxation agents cannot be attributed to occlusion of the residue by the peptide structure, since in every case the line widths are consistent with the residue site being fully accessible to these reagents.

As briefly discussed above (see Experimental Procedures), the Φ parameter provides a quantitative measurement of the relative distance of the labeled side chain from the water−membrane interface. An absolute distance can then be

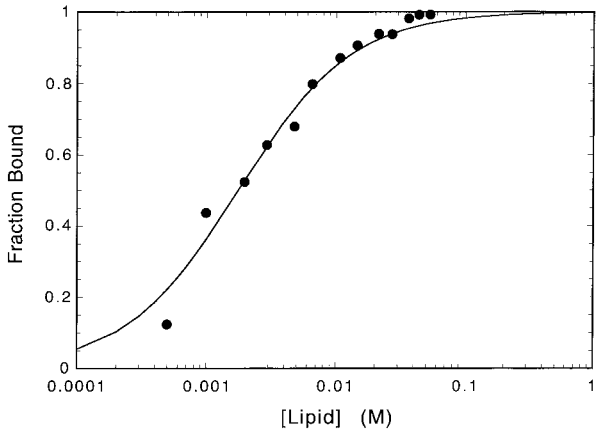


FIGURE 6: Titration of the MARCKS-Ala mutant, L15C-R1, with lipid 1000 Å diameter vesicles containing PC/PS (9:1). The buffer contained 100 mM KCl and 10 mM MOPS, pH = 7.0, and the peptide was at a concentration of less than 5 μM. The solid line represents a fit to the fraction of peptide bound, f_B, as a function of the total lipid concentration; see eq 1. K_p is the reciprocal molar partition coefficient for the peptide, and a value for K_p of 570 M^{-1} is obtained here. However, since the peptide has access to only one interface, the value of K_p in terms of the accessible lipid will be twice this value, or approximately 1140 M^{-1}.

assigned to the particular Φ value by a previously measured calibration (*12*). Previous work on the native MARCKS-(151−175) determined that the majority of the peptide side chains resided approximately 5−10 Å below the level of the phospholipid phosphate (*23*). However, the values of Φ obtained here indicate that the side chains of the MARCKS-Ala mutant are located ≥10 Å above the water−membrane interface, a change of at least 15 Å compared to labels in the central portion of MARCKS(151−175). It should be noted that although the measurement of Φ is highly reproducible, there is likely to be considerable uncertainty associated with distance determinations for residues lying significantly off the membrane surface. This occurs because the concentration gradients are small for both paramagnetic relaxation agents at distances more that 10−15 Å from the interface (*12*). Nonetheless, it is clear that the MARCKS-Ala peptide is localized at a position several angstroms on the aqueous side of the membrane-solution interface and that the five Phe residues on MARCKS(151−175) serve to localize this peptide at the water-membrane interface.

Each Phenylalanine Contributes Only 0.2 kcal/mol to the Binding of MARCKS(151−175). To examine the effect of these Phe residues on the free energy of binding, a series of lipid titration measurements were conducted to determine the molar partition constant (K_p) of the MARCKS-Ala mutant; Figure 6 displays an example of a titration of MARCKS L15C-SL. Similar experiments were also conducted with the other two versions of the MARCKS−F(151−175) peptide, S8C-SL and N23C-SL. The results of these experiments revealed a molar partition constant of 1100 ± 100 M^{-1} for the MARCKS-Ala mutant when associated with 1000 Å lipid vesicles contained 10 mol % PS in PC.

These binding measurements were carried out with approximately 5 μM spin-labeled peptide, which is near the lower limit needed for EPR. This minimal peptide concentration did not permit accurate EPR measurements of peptide binding at higher PS concentration or measurements of the native peptide. Higher binding constants associated with increased PS concentrations or with the native peptide would necessitate lower peptide concentrations so that the peptide does not produce a significant alteration in the membrane charge density. Previous measurements on the native peptide have been made by alternate methods, and the molar partition constant of MARCKS(151−175) was found to be $K_p = 7 \times 10^3$ M^{-1} under similar conditions by a sucrose-loaded LUV technique (*2*). Because spin labels on MARCKS-Ala do not penetrate the membrane interface, the labels are unlikely to have a significant effect on the partitioning of the peptide, and it is reasonable to make a comparison of the binding obtained here for MARCKS-Ala with data on the native peptide obtained by the sucrose loading technique.

From these binding constants, a estimate of the contributions that the phenylalanine residues make to the aqueous−membrane partitioning of the MARCKS(151−175) peptide can be made. This change in the free energy of binding $\Delta\Delta G_{F\rightarrow A}$ is given by

$$\Delta\Delta G_{F\rightarrow A} = -RT \ln\left[\frac{K_p(\text{MARCKS}(151-175))}{K_p(\text{MARCKS-A}_5)}\right] = -1.1 \text{ kcal/mol}$$

This implies that the free energy contribution to binding of each Phe residue in the MARCKS(151−175) peptide is $\Delta\Delta G_{F\rightarrow A} = -0.22 \pm 0.02$ kcal/mol of Phe compared to an Ala substitution. This experimentally derived value can be compared to the Wimley−White interfacial hydrophobicity scale developed to determine the free energy of membrane partitioning of oligopeptides in extended conformations (*30*). The MARCKS(151−175) peptide is longer than the pentamers used to develop the hydrophobicity scale, but MARCKS(151−175) also associates with the membrane in an extended, nonhelical conformation as do the pentamers (*23*). The Wimley−White scale indicates that the Phe-to-Ala substitution should yield $\Delta\Delta G_{F\rightarrow A} = -1.30 \pm 0.08$ kcal/mol of Phe, a result approximately 6 times greater than that measured for the substitutions in the MARCKS(151−175) peptide.

Membrane Charge Density Alters the Dynamics of MARCKS-Ala on the Membrane Interface. The motion of the nitroxide side chains on the peptide MARCKS-Ala when membrane-bound is strongly dependent upon the mole fraction of negatively charged lipid within the membrane interface. Shown in Figure 7 are EPR spectra for the L15C-R1 derivative in buffer and in the presence of 1000 Å extruded vesicles at a total lipid concentration of 100 mM, a concentration well in excess of that necessary to completely bind the peptide. The amplitudes of the spectra in Figure 7 are normalized in terms of the total spin concentration in each sample. At 10 mol % PS, the correlation time for the labeled side chain is lengthened approximately 40% compared to the aqueous peptide, but the relative motion of this side chain is still relatively rapid and isotropic. Increasing the charge density on the membrane interface produces a very dramatic increase in the correlation time, which is seen in Figure 7 as a broadening of the EPR line shape. The correlation time increases by about 200% when the charge density on the membrane interface is increased from 10 to 25 mol % PS. A reasonable interpretation of this result is that the peptide is drawn closer to the membrane interface

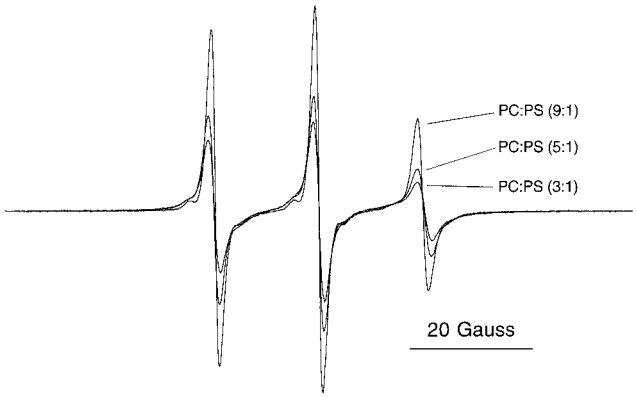


FIGURE 7: Effect of membrane charge density on the EPR spectra of the membrane-bound MARCKS-Ala peptide, L15C−R1. As the charge density is increased by increasing the mole fraction of POPS in POPC, the rotational correlation time for the nitroxide at this position increases. The rotational correlation times are 0.34 ns in buffer (not shown), 0.48 ns at 10 mol % PS, 0.65 ns at 17 mol % PS, and 0.85 ns in 26 mol % PS. The three spectra shown are normalized by the area of their respective second intergrals. The peptide is at a concentration of 50 μM, and the 1000 Å diameter vesicles are at a concentration of 100 mM lipid.

as the charge density is increased, which restricts the movement of the peptide side chains.

DISCUSSION

The central 14-residue portion of the MARCKS effector domain contains three phosphorylation sites, which are surrounded by five phenylalanine residues. When membrane-bound, this central region is localized at the membrane interface, with the more highly charged N-terminal portion localized in the aqueous phase (see Figure 8) (*23*). In the present study, we examined the role that these phenylalanine residues play in determining the position and dynamics of the peptide on the membrane interface. Unlike the peptide derived from the native sequence, the peptide lacking phenylalanine (MARCKS-Ala) fails to alter the headgroup angle when bound to the membrane as determined by ^{2}H NMR, and it does not appear to penetrate the membrane interface. As judged by EPR, spin-labeled side chains on the MARCKS-Ala analogue exhibit a faster rotational correlation time by a factor of approximately 3−4 compared with spin-labeled derivatives of the native MARCKS(151−175) peptide. The data presented here also shows that spin-labeled side chains on MARCKS-Ala are no longer localized within the membrane interface but are positioned in the aqueous phase. These side chains are shifted in position by at least 15 Å compared to the native peptide when bound to the membrane. These data provide strong evidence that these phenylalanines are essential for the localization of MARCKS to the membrane interface. Without them, these peptides still bind strongly to membranes containing acidic lipids, but they assume an equilibrium position that is several angstroms above the membrane-solution interface as shown in Figure 8.

The behavior of the MARCKS peptide lacking phenylalanine is similar to that observed for pentalysine. Although EPR experiments have not yet been carried out on this charged peptide, ^{2}H NMR experiments indicate that Lys_5 does not penetrate into the membrane interface (*21*). Peptides such as Lys_5 also fail to produce changes in monolayer surface pressure, and a computational analysis of the forces acting on this pentapeptide predict that it should assume an equilibrium position lying several angstroms above the membrane interface (*22*). This finding is consistent with previous EPR work on MARCKS(151−175) showing that the N-terminal end of the peptide, which begins with five lysine residues, lies several angstroms above the membrane−solution interface. An equilibrium position above the membrane interface is also found for peptides derived from the N-terminal end of the tryosine kinase pp60^{src} (*12*). These peptides, unlike peptides based on the native MARCKS sequence, are relatively hydrophilic and lack the hydrophobic residues found in the central portion of MARCKS(151−175).

It should be noted that for the native MARCKS-derived peptide, only a small portion of the charge on the peptide is detected by the POPC headgroup in membranes formed from POPC/POPS. Although this peptide has a net charge of +13, the effective charge detected by the PC headgroup by ^{2}H NMR was approximately +2. This large discrepancy could be explained if only the central portion of this peptide is closely associated with the membrane interface, as suggested by EPR measurements (*23*). Within the central 14-residue stretch of MARCKS(151−175) containing the five phenylalanine residues, the net charge is +3. The ^{2}H NMR measurement used here detects changes in headgroup angle produced by local changes in the electrostatics of the interface (*29*). Apparently charges that lie a few angstroms away from the interface in the aqueous phase have no effect on the headgroup as measured by this technique (*21*).

Why do highly charged hydrophilic peptides assume a position several angstroms above the membrane interface? These highly charged peptides are brought to the membrane interface as a result of the long-range Coulombic force acting between the peptide and a negatively charged membrane, and one might expect the peptide to assume a position that brings them within van der Waals contact of the membrane interface. Their failure to contact the surface is attributed to a dehydration effect that includes the Born repulsion, and this effect becomes important when the charged peptide approaches within a few angstroms of the membrane interface (*6*, *22*). At distances close to the membrane interface, the electrostatic field from charged residues, such as lysine, extends into the low dielectric of the membrane interior, and the energy associated with this field increases the energy of the peptide and opposes the long-range attractive force (*22*).

Several other observations are consistent with the idea that the equilibrium position of these peptides is a result of a balance between the Coulombic attraction and Born repulsion. When the charge density on the membrane interface is altered from 10 to 27 mol %, there is a dramatic change in line shape and side-chain motion of MARCKS-Ala, consistent with the peptide being brought closer to the membrane interface. These changes in charge density should represent a shift in the membrane surface potential (under the conditions used here) from about −24 to −45 mV (*31*). While this is a small electrical energy difference for a univalent species, it represents a substantial energy difference for a species having a valence of +13. Previous EPR work on MARCKS(151−175) showed that the highly charged N-terminus of the peptide resides on the aqueous side of the membrane−solution interface and that the distance of this

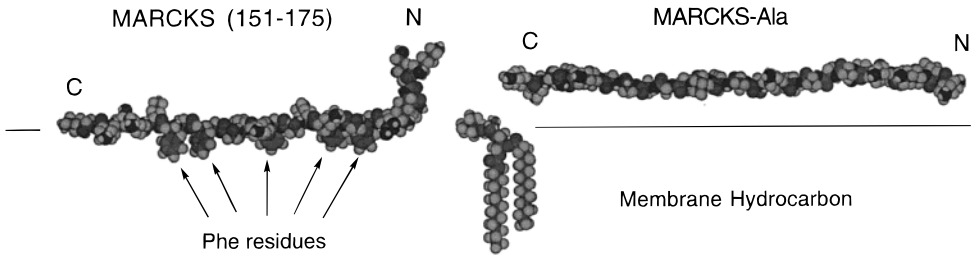


FIGURE 8: Models showing the position of the native effector domain of MARCKS, MARCKS(151−175), and the Phe → Ala mutant derivative of MARCKS, MARCKS-Ala. The position of MARCKS(151−175) is based on previous EPR work (*23*), and the position of MARCKS-Ala is based on CD and EPR measurements reported here. Without the five phenylalanine residues in the central portion of the peptide, the MARCKS effector domain binds strongly to membrane but fails to insert into the membrane interface.

segment from the membrane interface was also altered by the membrane charge density (*23*) (no effect of charge density on distance was observed for the central region of this peptide, which is partially inserted into the membrane interface). Thus, these results suggest that an increase in the strength of the long-range electrostatic attraction alters the equilibrium binding position for these peptides and brings them closer to the membrane interface.

The presence of the Phe residues in MARCKS(151−175) contributes a significant hydrophobic character to the peptide, and the additional hydrophobic binding energy is apparently sufficient to overcome the Born repulsion and attach the central domain of MARCKS(151−175) to the membrane interface. It is important to note that when the binding free energy of the native MARCKS(151−175) is compared to that for the Phe-less derivative, MARCKS-Ala, the removal of the five residues results in a loss in the free energy of binding of only 1.1 kcal/mol, which corresponds to approximately 200 cal/mole of Phe. A similar difference between the binding of MARCKS(151−175) and MARCKS-Ala was also seen in PC/PS (10:1) membranes with acrylodan-labeled peptides (Arbuzova and McLaughlin, personal communication). This difference is only 17% of 1.3 kcal/mol expected for the association of each phenylalanine residue with the membrane interface (*30*). This remarkable result is not unexpected and it suggests that a large portion of the free energy gained from the hydrophobic interaction of phenylalanine with the interface is expended in doing work against the dehydration energy that is encountered as the peptide is brought to the membrane interface.

The Wimley−White scale for interfacial side-chain hydrophobicities was obtained by measuring the partition free energies for a series of short model peptides (*30*). It is fully consistent with the hydrophobic effect and should be valid for comparing the binding of peptides and proteins provided they assume the same membrane topology. We believe that the discrepancy for phenylalanine seen here between this hydrophobicity scale and that determined from the binding of MARCKS(151−175) and MARCKS-Ala_5 is simply a result of the different positions assumed by these two peptides. A previous study using EPR showed that aliphatic amino acid side chains yielded similar membrane affinities independent of position (*32*). However, in this previous study, substitutions were made for groups already lying within the membrane interface, and the model peptide used was fully bound to bilayers in the absence of acidic lipid. The substitutions made also produced minor structural perturbations in the peptide. These results indicate that a comparison of the membrane binding free energy for peptides should be valid provided the substituted groups lie at or within the interface and provided that there are no major structural or positional changes in the peptide upon substitution.

The data shown here place the MARCKS-Ala_5 peptide side chains at a distance greater than 10 Å from the level of the lipid phosphate, a distance that is considerably further than expected on the basis of the computational analysis of peptides such as Lys_5 (*22*). This extended distance from the interface was also seen for peptides based on the N-terminus of the Src tyrosine kinase (*12*) and for the highly charged N-terminal tail of the native MARCKS peptide (*23*). There may be several explanations for this discrepancy. First, the EPR data is reporting the position of the side chain R1, and this does not necessarily represent the position of the a charged lysine residue or the peptide backbone. On the other hand, the side chain R1 is relatively hydrophobic and one would expect it to associate with the interface if the peptide backbone were within a few angstroms of the interface. Second, the computational work that has been performed assumes a smooth interface, while the EPR data is providing a time-averaged distance from an interface that is clearly not smooth (*33*). As a result, it may not be easy to estimate the effective distance dependence of the Born repulsion, because the interface is not well-defined and the effective dielectric transition would be difficult to accurately estimate.

In summary, the data presented here indicate that the five phenylalanines in the effector domain of MARCKS [MARCKS(151−175)] function to attach this domain to the membrane interface. Without these residues the peptide side chains assume a position that lies greater than 10 Å above the membrane−solution interface when the peptide is bound. The position of the MARCKS effector domain on the membrane interface is a result of a competition between several forces: a long-range Coulombic attraction, a hydrophobic interaction with the membrane interface (due to the five phenylalanines in this sequence), and a dehydration energy that includes the Born repulsion. Surprisingly, these five phenylalanine residues do not make a significant contribution to the free energy of membrane binding in the context of this peptide, a result that can be accounted for by the interplay between these forces. Thus, the interplay between the electrostatic attraction, hydrophobicity, and the Born repulsion generated by a peptide can modulate the specific location at which the peptide resides with respect to the water−membrane interface.

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