MARCKS is a natively unfolded protein with an inaccessible actin-binding site: evidence for long-range intramolecular interactions

Hazel Tapp, Iman M. Al-Naggar, Elena G. Yarmola, Alexis Harrison, Gerry Shaw‡, Arthur S. Edison§ and Michael R. Bubb

The Departments of Medicine, ‡Neuroscience, and §Biochemistry and Molecular Biology, University of Florida College of Medicine, Gainesville, Florida 32610 USA and The Research Service, Malcom Randall Department of Veterans Affairs Medical Center, Gainesville, Florida 32608 USA.

Running Title: Evidence for long-range intramolecular interactions in MARCKS.

Correspondence should be addressed to: Dr. Michael R. Bubb Box 100221 Department of Medicine University of Florida Gainesville, FL 32610 Tel: (352) 392-4681 Fax: (352) 374-6170 Email: hubbmr@medicine.ufl.edu

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Summary

MARCKS is an unfolded protein that contains well-characterized actin-binding sites within the phosphorylation-site domain (PSD), yet paradoxically, we now find that intact MARCKS does not bind to actin. Intact MARCKS also does not bind as well to calmodulin as does the PSD alone. Myristoylation at the N-terminus alters how calmodulin binds to MARCKS, implying that in spite of its unfolded state, the distant N-terminus influences binding events at the PSD. We show that free PSD binds with site specificity to MARCKS, suggesting that long-range intramolecular interactions within MARCKS are also possible. Because of the unusual primary sequence of MARCKS with an overall isoelectric point of 4.2 yet a very basic PSD (overall charge of +13), we speculated that ionic interactions between oppositely charged domains of MARCKS were responsible for long-range interactions within MARCKS that sterically influence binding events at the PSD and explain the observed differences between properties of PSD and MARCKS. Consistent with this hypothesis, chemical modification of MARCKS that neutralize negatively charged residues outside of the PSD allow the PSD to bind to actin and increase the affinity of MARCKS for calmodulin. Similarly, both myristoylation of MARCKS and cleavage of MARCKS by calpain are shown to increase the availability of the PSD so as to activate its actin-binding activity. Since abundant evidence supports the conclusion that MARCKS is an important protein in regulating actin dynamics, our data imply that post-translational modifications of MARCKS are necessary and sufficient to regulate actin-binding activity.
Introduction

Myristoylated alanine rich C kinase substrate\(^1\) (MARCKS) is a well-characterized, charge polarized, natively unfolded molecule (1-3) with a centrally located active site known as the phosphorylation site domain (PSD). Consistent with the paradigm for natively unfolded proteins, MARCKS is thought to interact with several ligands so as to integrate information from various signal transduction pathways to produce an output signal that regulates cell motile and contractile function. Numerous studies of MARCKS protein have utilized a peptide with a sequence that corresponds to the PSD peptide as a substitute for studying interactions between the intact protein and its multiple ligands (3, 4). While this approach intuitively appears to be logical, given the unfolded state of the native protein, the substitution of PSD peptide for intact protein has never been rigorously justified. In fact, there are several reported experiments that imply that the PSD peptide behaves differently than intact MARCKS. Nonphosphorylated PSD peptide is known to have extended structure and to nucleate polymerization and to cross-link F-actin filaments (5-7), presumably because of two binding sites with site specific \(K_d\) of ~0.5 \(\mu\)M for F-actin (8). Although the PSD of MARCKS and its homologue MARCKS-related protein, have both been shown to bind to actin with similar affinity, intact recombinant MARCKS-related protein, with or without myristoylation, exhibits a lower affinity for actin (much greater than 1 \(\mu\)M), and does not cross-link F-actin or induce G-actin polymerization (9). While full length MARCKS has been shown to bind and bundle F-actin (4,10), the published data are only semi-quantitative. Comparison of the available data also suggests that intact MARCKS binds to vesicles containing acidic phospholipids with \(10^4\)-fold lower affinity than does the PSD peptide alone (11).
Calcium-dependent interactions between the PSD peptide and the Ca\(^{2+}\)-binding protein calmodulin have been extensively characterized and a crystallographic structure is available that reveals that the phenylalanine residues of the PSD are buried in a hydrophobic tunnel of calmodulin and the highly charged termini of the peptide interact with patches of opposite charge on the surface of calmodulin (12). Once again the PSD peptide is said to interact with higher affinity than the intact protein (\(K_d\) of 3.8 versus 12.7 nM, respectively, in 0.1 M KCl) with this ligand (13). Recently it has been speculated that myristoylation of MARCKS adds a second, low affinity, calmodulin binding site to MARCKS without evidence of cooperativity (13). Importantly, the addition of a second non-cooperative binding site cannot explain the long-known result that myristoylated MARCKS binds to calmodulin with higher affinity than non-myristoylated MARCKS (14) unless both binding sites can interact with a single calmodulin molecule simultaneously or myristoylation itself changes the accessibility of the PSD to bind to calmodulin. Independent binding would change the stoichiometry of the interaction to two calmodulins per MARCKS, but could not significantly increase the apparent affinity unless the calmodulin ligand was multivalent (oligomerized or attached to a bead). A recent crystallographic structure of a myristoylated peptide bound to calmodulin (15), with the myristoyl group in the same hydrophobic tunnel and interacting with many of the same residues as the phenylalanines of the PSD peptide, suggests that both the N-terminal myristoyl and PSD binding sites could not simultaneously interact with calmodulin without significant steric effects. However, the hydrophobic tunnel through calmodulin has been shown to be quite flexible and there are no experimental data to rule out the possibility that calmodulin could adjust to accommodate both putative binding regions. Of note, myristoylation of MARCKS is likely a dynamically regulated post-translational event (16). Non-myristoylated MARCKS has been
isolated from bovine brain (17) and a demyristoylase activity has been characterized (13), thus making the acronym somewhat of a misnomer. For the purposes of this study, except in instances in which there may be some confusion, we refer to MARCKS as the non-myristoylated protein, for which, due to its natively unfolded structure, there is reason to believe that the native protein is equivalent to recombinant protein.

Many in vivo studies implicate MARCKS in an actin regulating function (18-22), but the evidence is indirect and could in all cases be explained by invoking schemes in which MARCKS alters events that in turn regulate cytoskeletal dynamics. However, in several specific examples MARCKS co-localizes with F-actin and dissociation from actin is temporally associated with alterations in actin dynamics (22,23), or MARCKS localization is altered after treatment that disrupts actin-filaments (18,22). Such data are most readily interpreted as direct effects of MARCKS on actin. One target of MARCKS, phosphatidylinositol 4,5-bisphosphate (PIP$_2$), has been implicated in controlling the actin cytoskeleton by binding to many other actin-regulatory proteins such as N-WASP (24), suggesting one possible indirect mechanism of actin control by MARCKS. MARCKS may sequester PIP$_2$ in the plasma membrane by reversible PSD binding (25). However, it should be noted that published estimates of intracellular MARCKS concentration (12 µM) (26) are based on the yield of a bovine forebrain preparation, but the original data are probably more consistent with an intracellular concentration of 1.2 µM (27). Thus, while there may not be sufficient MARCKS to globally regulate PIP$_2$ metabolism, local pools of PIP$_2$ could potentially be regulated by MARCKS.

If the PSD of MARCKS is incompletely accessible to its many ligands in the intact protein, then two questions emerge that are addressed in the current studies. (1) How can a natively unfolded protein maintain the PSD in a buried, unavailable position? (2) Do
mechanisms exist to alter the availability of the PSD? Here we test a novel structural hypothesis related to the unusual charge distribution in the primary sequence of MARCKS and attribute physical significance to post-translational modifications that are now shown to regulate the actin-binding functions of MARCKS.
Experimental Procedures

Materials—Rabbit skeletal muscle actin was prepared from frozen muscle (Pel-Freeze Biologicals, Rogers AR) in buffer G (5.0 mM Tris-HCl, 0.2 mM ATP, 0.2 mM dithiothreitol, 0.1 mM CaCl₂, and 0.01% sodium azide, pH 7.8) (28) and pyrenyl-actin was prepared with 0.5-0.95 mol of label/mol of protein using the method of Kouyama and Mihashi (29). Peptides were synthesized by solid phase using FMOC chemistry at the University of Florida (8). The PSD peptide has the sequence KKKKKRFSFKSKFSFKLSGFSFKKSKK. The modified rhodamine labeled PSD peptide (Rh-PSD) (8) was N₁-terminal modified through an amide link with 5-(and 6)-carboxytetramethyl rhodamine succinimidyl ester and COOH-terminal labeled with Oregon green 488 succinimidyl ester (5-isomer). Calpain I purified from porcine erythrocytes was obtained from Calbiochem (San Diego CA). Calmodulin (CaM) isolated from bovine brain was purchased from Sigma-Aldrich (St. Louis MO). CaM-Sepharose beads were purchased from Amersham Pharmacia (Piscataway, NJ). Fluorescently labeled MIANS-CaM was a gift from J.M. Chalovich (East Carolina University, NC). It is wheat germ CaM (Mr 16,800) labeled at cysteine-27 with 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid.

Preparation of MARCKS and myristoylated MARCKS—Full length murine MARCKS DNA (GenBank accession number M60474) was inserted into a pMW172 vector (30) and transformed into *Escherichia coli* BL21(DE3) competent cells. Cells (300 ml) were grown overnight in LB media and used to inoculate 2 l of culture. After 3 h, protein expression was further induced by addition of IPTG (100 μM) for 1 h. Cultures were spun down and frozen at −80°C. Frozen cultures were resuspended in buffer consisting of 10 mM Tris-HCl, 100 μM EGTA, 5 mM β-mercaptoethanol, 2 mM EDTA, 100 μM phenylmethylsulfonyl fluoride, and 0.6 mM diisopropylfluorophosphate, pH 7.9. Resuspended cells were sonicated, heated to 85°C for 10
minutes, and centrifuged at 38,000 rpm for 1 h. The supernatant was loaded onto a DEAE column and fractions were collected with a 100-400 mM KCl gradient in 10 mM Tris-HCl, pH 7.9. Fractions containing MARCKS (as shown by SDS-PAGE and western blot using polyclonal goat anti-MARCKS antibody raised against a C-terminal synthetic peptide (Serotec Inc, Raleigh NC)) were combined and concentrated on a hydroxyapatite column, and further purified by gel filtration on HR Sephacryl 300. Concentration was determined by UV absorption at 258 nm ($e_M = 1100$ at 258 nm) or by amino acid analysis. MARCKS typically eluted as a monomer at 40 to 70 µM and was stored at -80° in the column buffer, 5 mM Tris-HCl, 5.0 mM β-mercaptoethanol, and 50 mM KCl, pH 7.9 (MARCKS buffer).

For myristoylated MARCKS, the E. coli strain BL21 was transformed with both the plasmid pBB131NMT (gift from Dr. J. Gordon, Washington University), which contains the gene for yeast N-myristoyltransferase (31), and the MARCKS-pMW172 plasmid described above, and selected in the presence of 50 µg/ml of both kanamycin and ampicillin. A frozen stock of transformed colonies was used to inoculate 200 ml of LB media containing 50 µg/ml kanamycin and ampicillin. The overnight culture (2 l) was grown to log phase, 400 mM IPTG was added to induce protein expression and the culture grown for 3 additional h. Myristoylated MARCKS was purified according to the protocol for MARCKS described above. Myristoylated MARCKS runs at ~83kDa on SDS-PAGE (slightly higher apparent molecular mass than MARCKS). It was characterized by mass spectroscopy with a peak at 29,874 Da (compared to 29,664 Da for non-myristoylated MARCKS), and these correspond to the predicted masses based on the sequence.

In order to test for the propensity of MARCKS to aggregate, gel-filtered monomeric MARCKS was concentrated to 300 µM in an Microcon filtration device (Millipore Corp.,
Billerica, MA) and then diluted to 100, 18 or 3 µM in MARCKS buffer. Samples were centrifuged at 150,000 x g for 15 minutes through a 20% sucrose cushion either 1 or 12 h after dilution. Pellets and supernatants were analyzed by SDS-PAGE with loading volumes inversely proportional to the protein concentration.

Assays of Actin-binding Function—Binding of MARCKS, covalently-modified MARCKS, myristoylated MARCKS or calpain-digested MARCKS to F-actin was detected by a high-speed centrifugation assay. Mg$^{2+}$-F-actin was prepared by converting Ca$^{2+}$-G-actin to Mg$^{2+}$-G-actin by addition of 0.125 mM EGTA and 0.05 mM MgCl$_2$ for ten minutes at room temperature, and then polymerizing with the addition of MgCl$_2$ to 2.0 mM final concentration. MARCKS (0-10 µM) was added to Mg$^{2+}$-F-actin (0 – 40 µM) in 100 µl total volume, and equilibrated for varying times (20 min to 24 h). The F-actin was pelleted at 140,000 x g in a tabletop ultracentrifuge for 1 h. Supernatants (60 µl) were removed and pellets were washed gently three times to remove trapped, unbound protein. Supernatant and pellets were then analyzed by SDS-PAGE or by fluorescence spectrometry to determine bound (pellet) or free (supernatant) MARCKS. For SDS-PAGE of covalently cross-linked MARCKS, 12% polyacrylamide gels were stained with SYPRO Ruby protein stain (Molecular Probes, Eugene OR) after the samples were concentrated in a filtration device. Actin filament cross-linking or bundling was assessed by a low-speed pelleting assay. Filament aggregates of either ordered bundles or isotropic networks of cross-linked filaments sediment at low g. Proteins or peptides were added to Mg$^{2+}$-F-actin (7 µM final concentration) to a final volume of 80 µl. After 10 minutes incubation, samples were centrifuged at 8000 x g for 20 minutes to pellet actin filament aggregates and any associated proteins. Supernatants (30 µl) were removed and loaded on 10 % SDS-PAGE. The presence of bundling or cross-linking is indicated by the depletion of actin from the supernatant. Effects of MARCKS
on the time course of actin filament polymerization were measured by the fluorescence change associated with the polymerization of pyrenyl-actin (29). Ca\(^{2+}\)-G-actin (3 \(\mu\)M, 4% pyrenyl-actin) was converted to Mg\(^{2+}\)-actin as before and polymerization was initiated by adjustment to 50 mM KCl and 2 mM MgCl\(_2\). (Experiments without KCl are specifically indicated in the text.) Seeded polymerization assays employed cross-linked oligomeric F-actin seeds (32) and 0.5 \(\mu\)M 4% pyrenyl-labeled Mg\(^{2+}\)-actin monomer. The assay was shown to be linear in response to seed concentration and actin monomer concentration with variation from these conditions. Polymerization in the presence or absence of MARCKS was measured using pyrene fluorescence, and the initial polymerization rates were determined using time course data which could be fit with a line without systematic deviation. The time course of actin filament depolymerization was assayed by dilution of 10 \(\mu\)M 10% pyrenyl-labeled F-actin polymerized with 2 mM MgCl\(_2\) to 0.1 \(\mu\)M into the same buffer containing 0 or 2.0 \(\mu\)M MARCKS.

*Calmodulin-binding Assays*-Binding of PSD peptide or MARCKS to CaM-Sepharose beads to Rh-PSD was determined in a pull-down assay as previously described (33). Varying amounts of CaM-Sepharose suspended beads (total CaM 0-2 \(\mu\)M) were pipetted into 0.5 ml eppendorf tubes containing fixed amounts of Rh-PSD, (0.6 or 0.06 \(\mu\)M) in buffer (50 mM KCl, 0.1 mM CaCl\(_2\), 10 mM Tris-HCl pH 7.9,) to a final volume of 400 \(\mu\)l. Tubes were centrifuged at 7840 x g for 1 minute to pellet the CaM-Rh-PSD bead complex and the amount of free Rh-PSD was quantified by fluorescence spectroscopy using excitation 522 nm and emission 575 nm. The concentration of available CaM on the Sepharose beads was calibrated based on data obtained at 0.6 \(\mu\)M Rh-PSD, instead of using the manufacturer’s estimate which was ~20% higher than the value we obtained (see the results section for details). For full-length, recombinant MARCKS, the amount of MARCKS in the supernatant was quantified by Coomassie staining after SDS-
Since the fluorescence of Rh-PSD peptide increased by a factor of 2.3 upon binding to CaM, the increment of fluorescence could also be used to measure the fraction of bound Rh-PSD at varying concentrations of CaM in solution. Also, the fluorescence anisotropy of Rh-PSD peptide changed upon binding to CaM, providing another quantitative assay for binding that is discussed more completely below. Finally, wheat MIANS-CaM, labeled specifically with a fluorophore on its single cysteine residue at position 27 has been used successfully to generate a binding isotherm for calmodulin-binding proteins based on a large increment in fluorescence intensity (34). Saturation of MIANS-CaM by recombinant MARCKS, but not by PSD peptide, yielded a significant increase in fluorescence intensity with excitation at 322 nm and emission at 438 nm.

**Fluorescence Anisotropy**-Data were collected on a Photon Technology International (South Brunswick, NJ) spectrofluorimeter. Rh-PSD was excited with vertically polarized light at 546 nm. The horizontal, \( I_h \) and vertical \( I_v \) components of the emitted light were detected distal to a long-pass filter with a nominal cut-off of 570 nm (03 FCG 089; Melles Griot, Rochester NY) for ~20 s for each component. The total intensity of the Rh-PSD peptide fluorescence, \( I_v + 2G I_h \) was found to change proportionally upon MARCKS or CaM binding. The ratio, \( R \), of the fluorescence of the bound species divided by the free species, was calculated. The fluorescence anisotropy, \( r \), was calculated using \( r = (I_v-GI_h)/(I_v+2GI_h) \) (8). The \( G \) factor was determined for each experiment with the peptide in solution excited with horizontally polarized light and averaged over ~10 measurements. The experiments were performed in 0.3-ml samples in glass cuvettes. For competition assays, the direct binding assay was used to estimate an amount of MARCKS that bound ~2/3 of the Rh-PSD, and the anisotropy was measured at these
concentrations of MARCKS and Rh-PSD with varying concentrations of competing, unlabeled PSD.

The fitting procedure for the fluorescence anisotropy data included correction for the fact that the total fluorescence intensity changed upon binding of PSD CaM (Fig. 2B,C) or MARCKS (Fig. 4). Since bound MARCKS or CaM changed the fluorescence intensity of rhodamine-labeled PSD, the observed anisotropy \( r \) was a non-linear function of the fraction of PSD that is bound to MARCKS or CaM: \( r = \frac{r_f + (R \cdot r_b - r_f) \cdot b}{(1 + (R - 1) \cdot b)} \), where \( r_f \) is anisotropy of PSD, \( r_b \) is anisotropy of PSD bound to MARCKS or CaM, \( b \) is the fraction of PSD bound to MARCKS or CaM, and the ratio \( R \) is defined above.

**PSD-MARCKS Interactions**-MARCKS (5 µM) was covalently cross-linked to a rhodamine-labeled N-terminal PSD peptide with sequence KKKKKRFSFKK (25 µM) using 50 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), 16.5 mM N-hydroxysulfosuccinimide and 25 mM sodium phosphate dibasic, to give a final EDC concentration of 14 mM. Samples were incubated at room temperature for 30 minutes and cross-linked proteins separated by SDS-PAGE. This rhodamine-labeled peptide gave results indistinguishable from Rh-PSD in the anisotropy assay measuring competition with unlabeled PSD peptide for binding to MARCKS (data not shown). Binding of Rhodamine labeled N-terminal PSD peptide to MARCKS was also assessed in a gel overlay (far-western blot). After overnight transfer to PVDF membrane of purified recombinant murine MARCKS or a whole-cell extract prepared by addition of SDS gel buffer to washed, cultured macrophages (RAW 264.7 murine macrophage cell line), and blocking with 1 % BSA and 100 mM L-lysine, membranes were probed with peptide (5 - 10 µM) in MARCKS buffer for 1 hour. Excess probe was removed by washing in PBS, and the membrane was visualized and imaged by indirect fluorescence.
Preparation of EDC-modified MARCKS—As previously described for modification of the acidic residues of caldesmon (35), MARCKS (28 µM) in 100 mM MES, 400 mM ethanolamine pH 5.5 was incubated for 10 minutes at room temperature. Freshly prepared EDC (200 mM) was added to give varying final EDC concentrations (0 mM, 4 mM, 12 mM and 20 mM). Reactions were incubated at room temperature for 1 h, then stopped by addition of β-mercaptoethanol (100 mM), and then dialyzed into 5 mM Tris-HCl, 5 mM β-mercaptoethanol, pH 7.9. Aliquots of treated MARCKS from each reaction mixture were run on 12% SDS-PAGE to confirm modification by EDC as detected by an expected shift in electrophoretic mobility, as unmodified MARCKS runs anomalously as an ~80 kDa protein because of its unusual pI, and modification of the acidic residues shifts it towards its expected position at 30 kDa (35). Modification of the acidic but not basic residues was confirmed by amino acid analysis.

Calpain digestion of MARCKS—MARCKS was digested with calpain I (75 nM) in buffer (50 mM Tris-HCl, 100 mM NaCl and 2 mM CaCl₂, pH 7.9) for varying lengths of time. The reaction was stopped by adding SDS-PAGE sample buffer. For N-terminal sequencing of calpain digested MARCKS fragments, the digested fragments were first separated by SDS-PAGE and then transferred by electroblotting in 20% methanol and 10 mM MES, pH 6.0 to PVDF membrane and stained in 0.02% Coomassie R-250 in 40% methanol and 5% acetic acid for 30 s followed by destaining (40% methanol, 5% acetic acid) and air drying. Amino acid sequence was obtained by Edman degradation on a Procise Instument (Applied Biosystems 494 HT).

Results

Full-length Recombinant MARCKS Fails to Interact with Actin—MARCKS does not bind to F-actin as shown by SDS-PAGE after pelleting F-actin by ultracentrifugation (Fig. 1A). Neither
a dose dependent increase in MARCKS in the pellets nor a dose dependent decrease in MARCKS in the supernatants was observed. There were no reproducible differences in the amount of actin in the supernatants as a function of MARCKS concentration, i.e. MARCKS had no detectable effect on the critical concentration of actin. In spite of a different interpretation, these results are qualitatively similar to those reported by Hartwig et al. (5) in which pelleting assays were assayed by western blot and the molar ratio of bound MARCKS to actin was ~0.01 to 0.02 at saturation by MARCKS. Also, Wohnsland et al. (7) reported that only 1-5% of the total amount of MARCKS-related protein was associated with F-actin upon pelleting at 100,000 x g. The results reported here by us and elsewhere by others are either consistent with very low affinity ($K_d > 100 \mu M$) or with a very low stoichiometry of MARCKS to actin at saturation by MARCKS. Although several F-actin-binding proteins do exhibit low stoichiometry to actin, known examples such as capping proteins or proteins that use repetitive actin-binding sequences to associate with multiple subunits as a linear lattice have much different actin-binding properties than the PSD of MARCKS. Alternative theoretical explanations can be imagined to explain the low stoichiometry of binding. A MARCKS binding site could be buried within F-actin so that only a few sites can be simultaneously accessed or a MARCKS binding site could be created by two adjacent actin filaments in a filament bundle aligned so that only a few of the F-actin protomers have the correct orientation for binding. However, these explanations are not readily made consistent with binding data for PSD peptide alone showing 1:1 stoichiometry with F-actin subunits at saturation by PSD (8).

MARCKS does not have actin-regulatory functions previously associated with the PSD. We previously identified two potential actin-binding sites within the PSD that could explain how MARCKS can induce aggregation of actin filaments (8). In the presence of PSD peptide, Mg$^{2+}$-
actin filaments pellet at low centrifugal forces (8,000 x g), but the filaments remain in the supernatant in spite of a large excess of intact MARCKS (Fig. 1B). The results in Fig. 1B, in which the concentration of MARCKS exceeds that of actin, could possibly be explained by saturation of the actin-binding sites for MARCKS, leaving no free binding sites for MARCKS to exert a cross-linking effect. However, lower concentrations of MARCKS are also unable to produce any detectable filament bundling or cross-linking (data not shown). In the presence of 4 µM MARCKS, the time course of actin filament polymerization for Mg\(^{2+}\)-actin in 50 mM KCl and 2 mM MgCl\(_2\) was similar to control (Fig. 1C). The PSD peptide showed a ~4-fold increase in the rate of actin filament polymerization over the first 100 seconds at the same concentration (4 µM) as intact recombinant MARCKS. The same experiment performed in the absence of KCl showed a much greater discrepancy between MARCKS and PSD peptide, again with no effect attributable to MARCKS, but a ~30-fold increase in the rate of polymerization by PSD peptide (Fig. 1C, inset). Because the effects of KCl on this particular assay in the absence of PSD were undetectable, this result is consistent with prior reports of a significant difference in activity of the PSD in the presence and absence of KCl (6).

**Binding of the PSD to CaM-** Three different assays were used to investigate the binding of PSD to CaM. First, Rh-PSD was bound to CaM-Sepharose beads and the amount bound was determined from the observed decrease in supernatant fluorescence after pelleting Rh-PSD bound to CaM-Sepharose beads (Fig. 2A). The experiment was done at two different concentrations of Rh-PSD peptide (0.6 µM and 0.06 µM), one of which was substantially higher than the \(K_d\), so as to provide a more precise estimate of the concentration of CaM oriented on the bead appropriately for binding to PSD. The \(x\)-intercept of the essentially linear data for 0.6 µM Rh-PSD and varying amounts of CaM-Sepharose beads in Fig. 2A indicates a theoretical point at
which the concentrations of Rh-PSD and CaM are equal assuming a stoichiometry of 1 to 1. The amount of CaM-Sepharose beads required to bind 0.6 µM Rh-PSD was then assumed to provide an effective concentration of 0.6 µM CaM, and this result was used to calibrate all data using CaM-Sepharose beads. Binding isotherms for both sets of data are consistent with a $K_d$ of 10 nM for the binding of Rh-PSD to CaM. Second, CaM (in solution; not bead-bound) was equilibrated with Rh-PSD peptide and a binding curve was obtained based on the change in fluorescence that is observed upon the binding of Rh-PSD to CaM (Fig. 2B). At saturation by CaM, the maximal increase in fluorescence intensity was 2.3-fold. Assuming that the change in fluorescence intensity varied linearly with the fraction of bound Rh-PSD, then the data for two different concentrations of Rh-PSD can be globally fit to a $K_d$ of 9 nM. As long as the stoichiometry of the interaction between PSD and CaM is 1:1 and the PSD interacts in a site-specific manner with CaM, then this assumption is very likely correct. Published crystallographic data for the complex of PSD with CaM provide direct support for both of these claims (12). When unlabeled PSD peptide was added to a complex of Rh-PSD peptide and CaM, the PSD competed directly with the Rh-PSD for binding to CaM, so that at high concentrations of PSD, the fluorescence intensity of Rh-PSD mixed with CaM was about the same as that of Rh-PSD alone (Fig. 2B, inset). The theoretical fit to these competitive-binding data show that the rhodamine label on the PSD does not significantly influence the binding of PSD with CaM.

The third assay was steady-state fluorescence anisotropy of Rh-PSD, which varied from 0.06 in the free state to 0.18 in the bound state (bound to CaM). Anisotropy data were collected from the same samples used in Fig. 2B for steady-state fluorescence measurements. Because of the increment in fluorescence intensity upon binding CaM, equivalent molar quantities of Rh-PSD-CaM contributed 2.3 times as much to the measured value of fluorescence anisotropy as did
free Rh-PSD, necessitating correction of the data by this weighting factor (Fig. 2C). As observed with the fluorescence intensity measurements, unlabeled PSD competed with Rh-PSD, so that at high concentrations of unlabeled PSD, the anisotropy of a solution of Rh-PSD plus CaM was nearly identical to that of Rh-PSD alone (Fig. 2C, inset). With the assumption that the change in fluorescence intensity varied linearly with the fraction of bound Rh-PSD, a global fit of all the fluorescence and the anisotropy data for direct and competitive binding yields a $K_d$ of $9 \pm 4$ nM for Rh-PSD and $6 \pm 2$ nM for unlabeled PSD (Fig. 2A-C). These results show that Rh-PSD and unlabeled PSD bind to the same site on CaM with similar affinity.

**MARCKS binds with Lower Affinity than PSD Peptide to CaM and Myristoylation of MARCKS Alters Affinity for CaM**-Based on the CaM-Sepharose binding assay, a $K_d$ of 1.3 µM was calculated for MARCKS binding to CaM in 0.1 mM CaCl$_2$, compared to a $K_d$ of 10 nM for PSD under the same conditions (Fig. 2A). Schleiff et al (36) have shown that maximum binding of MARCKS-related protein to CaM occurs in 0.1 mM CaCl$_2$ and that the affinity of the complex is decreased 6-fold as the concentration of Ca$^{2+}$ is increased to 1 mM. Using the increment of fluorescence of MIANS-CaM to determine binding of MARCKS to CaM, we found a qualitatively similar dependence on calcium for recombinant MARCKS. Our data are consistent with a $K_d$ of 2.5 µM for binding MARCKS to MIANS-CaM in 0.6 mM CaCl$_2$, but the measured $K_d$ drops to 0.1 µM in 0.1 mM CaCl$_2$ (Fig. 3). Titration of MIANS-CaM with CaCl$_2$ alone had an insignificant effect on fluorescence intensity, thus ruling out the possibility that the change in fluorescence upon addition of MARCKS was caused indirectly by binding of MARCKS to Ca$^{2+}$ with a secondary decrease in free Ca$^{2+}$. While the assays are different, the measured values for $K_d$ are all significantly higher than those obtained for the PSD peptide at either 0.1 or 0.6 mM CaCl$_2$. We believe that the CaM-Sepharose bead assay gave a lower
affinity for MARCKS than the MIANS-CaM assay because the bead pull-down assay is performed under non-equilibrium conditions, and in this low-affinity interaction, the MARCKS protein detached from the beads before the supernatant was recovered for electrophoresis, thus giving a falsely elevated estimate of $K_d$. Qualitatively, however, this assay uniquely provides a straightforward comparison of the binding of MARCKS relative to PSD peptide. Unfortunately, the other available assays could not be performed on both MARCKS and the PSD peptide because only the PSD peptide was covalently modified with rhodamine and interactions between PSD and MIANS-CaM did not alter the fluorescence intensity of MIANS-CaM. These results independently confirm that the interaction of PSD with CaM is attenuated by the presence of the N- and C-termini in spite of the natively unfolded structure of full length MARCKS (13).

Consistent with earlier observations that myristoylated MARCKS binds better than non-myristoylated MARCKS to a calmodulin-affinity column (14) and subsequent quantification of this effect (13), myristoylated MARCKS exhibited higher affinity to MIANS-CaM by a factor of 3 in 0.6 mM CaCl$_2$ and a factor of $>10$ in 0.1 mM CaCl$_2$ (Fig. 3). A simple explanation for the attenuation of binding by full-length MARCKS relative to PSD peptide and augmentation by myristoylation, is that the PSD is less accessible in full-length MARCKS due to intramolecular interactions within MARCKS that are modulated by myristoylation. Moreover, the unusual inverse relationship between CaM affinity and increasing calcium concentration over higher ranges of calcium could have a similar explanation. Others have surmised that calcium likely interacts with an acidic portion of MARCKS-related protein (36), and if calcium interacts similarly with an acidic portion of MARCKS (the N- or C-terminus), then calcium would not be expected to influence binding between PSD-CaM unless the acidic termini control binding events occurring at the PSD.
Investigating Intramolecular Interactions within MARCKS: Binding of Externally Added PSD to MARCKS-Based on the hypothesis that the positively charged PSD of MARCKS associates intramolecularly with one or more sites on the negatively charged ends of MARCKS, we tested for evidence that a synthetic peptide corresponding to the PSD may associate intermolecularly with MARCKS. Such binding could be site-specific and/or specific to the PSD sequence. Also, depending on the flexibility of MARCKS and the position of binding site(s) on the primary sequence of MARCKS, any intramolecular binding between PSD and a site on a oppositely charged termini would be expected to have complicated equilibrium kinetics. The consequences of localization of a binding site for PSD on a single MARCKS molecule (creating a high, effective local concentration of ligands) would be to some degree mitigated by any structural restraints imposed by physical position and intrinsic flexibility. The resulting equilibrium association reaction for intramolecular binding therefore might be either lower or higher affinity than experimentally observed for intermolecular PSD peptide-MARCKS interactions. Moreover, the intermolecular binding events measured experimentally using a synthetic PSD peptide would occur in competition with intramolecular binding, so the intermolecular equilibrium kinetics may also be expected to be complicated. Using Rh-PSD peptide, we observed saturable binding by MARCKS based on the changes in fluorescence amplitude and anisotropy that were consistent with association of Rh-PSD to MARCKS (Fig. 4A). Unlabeled PSD was able to displace Rh-PSD from MARCKS as determined by a return of the amplitude of fluorescence intensity and the anisotropy to levels measured for free Rh-PSD peptide (Fig. 4A, inset). Qualitatively, the decline in anisotropy as a function of unlabeled PSD is steep, suggesting that unlabeled PSD has high affinity for MARCKS.
Because of the aforementioned potential complications, the quantitative analysis of these data is quite speculative. In our analysis we attempted to globally fit the data for both samples in which variable amounts of MARCKS were added to Rh-PSD peptide and samples in which variable amounts of unlabeled PSD peptide were added to a fixed mixture of MARCKS and Rh-PSD. The straightforward assumption that the intramolecular PSD of MARCKS competes equivalently with unlabeled and labeled PSD peptides for a single binding site on MARCKS was employed. Based on this assumption, the results are consistent with the $K_d$ for binding of the PSD (labeled or unlabeled) to the binding site on MARCKS of $\approx 40$ nM, and the apparent (effective) concentration of the intrinsic PSD was estimated as $0.3 - 0.8$ µM. The fitting algorithm for the anisotropy data included a correction for the larger fluorescence contribution of free Rh-PSD relative to bound Rh-PSD. The effective local concentration of the intramolecular PSD depends on distance between the PSD and its binding site on MARCKS, and on steric factors that reflect limitations in relative orientations and conformations of MARCKS protein. Based only on geometric considerations and no steric constraints, the theoretical maximum effective concentration is $\sim 50$ µM (this assumes a 20 nm radius). While the data fit excellently based on the assumption of site-specific binding, the results do not rule out the possibility that combinations of complicating factors, including non-site specific binding, could explain our results equally well.

Experiments using a short oligomer of polylysine, a $ \sim 24$-mer with low polydispersity (32), to displace rhodamine-PSD peptide (44 nM) from MARCKS (0.25 µM) also are strongly suggestive of site-specific binding, but also point to a lack of sequence specificity (Fig. 4A, inset). Qualitatively, it is clear from the steep, nearly stoichiometric dependence of the competitive-binding isotherm on polylysine that polylysine is interacting with a single or at most
a very few sites on MARCKS; otherwise polylysine would have to saturate its several sites and require high stoichiometry relative to MARCKS to be an effective competitor. The relative high affinity of polylysine (≈ 14 nM) is likely related to the polyvalency of this ligand, as it likely can bind to its target in any of several registers. The lack of sequence specificity is presumvably compensated for in vivo by the high effective local concentration of the intramolecular PSD.

MARCKS (5 µM) can be successfully cross-linked to a synthetic rhodamine-labeled PSD peptide (25 µM) using the zero-length cross-linking reagent EDC (Fig. 4B), indicative of close proximity of the peptide and MARCKS in solution. Also, a small fraction of the EDC-modified MARCKS exhibits decreased electrophoretic mobility (lanes 2 and 3 of Fig. 4B), perhaps because of an intermolecular cross-link consistent with limited self-association, as discussed later in relation to Fig. 4D. The same rhodamine-labeled peptide binds to a band on PVDF membrane (after SDS-PAGE and transfer) that is at the same position as MARCKS (Fig. 4C). Not only does the peptide bind to purified recombinant protein, but it also demonstrates apparent selectivity for MARCKS as it recognizes a band at the expected position for MARCKS in whole cell extracts made from RAW 264.7 macrophages. The position of the murine MARCKS band for both recombinant protein and whole cell extract after transfer was identified by traditional western blotting. The faint doublet seen on one western blot is consistent with expectations for incompletely myristoylated MARCKS (14), but may also reflect other post-translational modifications. In this assay, the PSD appears to be recognizing only the band that would correspond to post-translationally modified MARCKS, but since the western blots are non-quantitative (and in this instance, demonstrate some variation even when using the same samples), this may either be due to the relative absence of unmodified MARCKS or due to selectivity for modified MARCKS.
If free PSD can bind to MARCKS, then perhaps MARCKS can also self-associate into homo-oligomers. Indeed, we found evidence of reversible MARCKS aggregation after concentration to 300 µM. High speed pelleting of concentrated samples occurred with dilution from 300 µM to 100 µM for one h and possibly a small amount of material was depleted from the supernatant after dilution to 18 µM, but none was depleted from the 3 µM sample (Fig. 4D). After 12 h, only the 100 µM sample showed any evidence of pelleting (data not shown). These results are consistent with competition between intermolecular and intramolecular binding sites for the PSD, with aggregation due to intermolecular binding only when the concentration of MARCKS molecules is much higher than the effective local concentration of the intramolecular binding site.

**Effect of EDC-neutralization of Negative Charges on Interactions of MARCKS with Actin and Calmodulin**

Treatment with EDC in the presence of excess amine (400 mM ethanolamine) at low pH is expected to modify acidic residues but not basic residues, nor to induce intra-intermolecular cross-links (35). We treated conservatively with EDC for only one h at various concentrations of EDC. Amino acid analysis showed that at the highest concentration of EDC (20mM), only an average of 6 of the 59 acidic residues were modified, while an average of 5 and 2 residues were modified at 12 and 4 mM EDC, respectively. Prior reports suggest that the anomalously low electrophoretic mobility of proteins with very low PI is due to a failure to become uniformly charged in SDS, and that neutralization of these charges with EDC will cause such a protein to migrate as expected relative to mass (35). While SDS-PAGE shows that the electrophoretic mobility of MARCKS generally increases with neutralization (Fig. 5A, and experimental repeats not shown), the band width also increases, presumably because of heterogeneity in the covalent modifications (Fig. 5A). High-speed pelleting of 24 µM F-actin
and 16 µM EDC-treated MARCKS results in depletion of MARCKS from the supernatant, in contrast to the results for untreated MARCKS (compare Figs. 1A and 5A). Thus, EDC-modification of regions of MARCKS outside of the PSD convert MARCKS to an F-actin-binding protein.

Affinity of MARCKS for MIANS-CaM is similarly increased by EDC treatment (Fig. 5B). The data for untreated MARCKS are superimposable from Figs. 3 and 5C, but are shown on different scales. Treatment of MARCKS with EDC causes an increase in the fluorescence increment of MIANS-CaM that is associated with saturation by MARCKS, implying a greater influence of bound EDC-treated MARCKS on the fluorophore environment, and a change in the shape of the binding isotherm consistent with augmented affinity. Untreated or mock EDC-treated MARCKS gives a $K_d$ of 2.5 µM, while 4, 12, and 20 mM EDC of EDC-treated MARCKS give $K_d$ of 1.01, 0.65, and 0.37 µM respectively. These data imply that incremental neutralization of negative charges on the C- and N- termini of MARCKS allows binding of the PSD to actin or CaM.

MARCKS treated with 20 mM EDC enhanced the rate of filament polymerization in 2.0 mM MgCl₂, whereas 12mM EDC-treated MARCKS had only a very small effect (Fig. 5C). These data suggest that the dose response to EDC-treatment not only varies with the activity assayed, but also is not closely proportional to the extent of amino-acid modification. This is not particularly surprising given that prior reports have shown that the effects of the PSD on actin vary non-monotonically with PSD concentration (37), so that activation of MARCKS to less than 100% of the activity of PSD may be expected to cause either no effect or activity opposite of that seen for PSD alone, depending on the precise extent of activation. Moreover, different modifications of the PSD have been shown to have different effects on specific actin-binding
functions (8), so that partial activation of MARCKS might not be expected to produce the full spectrum of PSD-related functions. In spite of these arguments, the inhibition of actin depolymerization by EDC-treated MARCKS in 2.0 mM MgCl₂ has a very similar dose dependence as does the effect on polymerization, with only the 20 mM sample having substantial activity (Fig. 5D). In this assay, 2.0 µM of 20 mM EDC-treated MARCKS has activity roughly equivalent to 0.2 µM of PSD alone. A prior report described the observation that the PSD slows depolymerization and attributed this effect to a lower critical concentration in the presence of PSD (9). Alternatively, barbed end capping activity might be expected to produce the observed effect.

Modification of Full-length MARCKS by Myristoylation Enhanced the Rate of Actin-Filament Polymerization - A dose-dependent increase in the rate of actin filament polymerization was seen in the presence of myristoylated MARCKS in 2.0 mM MgCl₂ (Fig. 6). As discussed in the prior section, the PSD of MARCKS affects multiple parameters that alter the time course of actin polymerization, including effects on nucleation rates, filament capping, and bundling. Measurements of the time course of polymerization alone do not delineate which of these variables has been modified to produce the overall variation relative to control. Thus it is possible that myristoylation is selectively activating only some of the actin-binding properties of MARCKS. In this context, it should be noted that no evidence of bundling or significant binding of myristoylated MARCKS to F-actin was seen using the assays employed for MARCKS in Fig. 1 (data not shown). Also, there was no effect on elongation rates in a seeded polymerization assay (data not shown), and the implication is therefore that myristoylated MARCKS alters the time course of polymerization only by an effect on nucleation rate.
MARCKS-actin Interactions May Be Regulated by Proteolysis-inhibition of calpain has been reported to result in the accumulation of MARCKS in myogenic cells in culture (38) and rat hippocampal slices (39), and to be functionally related to myoblast migration and fusion (40, 41). MARCKS is cleaved in vitro by calpain, resulting in several bands on SDS-PAGE (Fig. 7A). The most prominent band identified by western blot using a goat anti-MARCKS antibody that recognizes the C-terminus (Serotec Inc, Raleigh NC) was isolated for N-terminal sequencing. Consistent with the known epitope recognized by the antibody, the sequence SPKAEDGAA identifies this proteolytic fragment as a 17.7 kDa MARCKS C-terminal cleavage product. The N-terminus of this fragment begins 17 residues before the PSD at residue Ser-134, so that the MARCKS cleavage site has the sequence, SSTS~SPKAE (~ represents scissile peptide bond). The primary structure of the substrate around the scissile bond has recently been confirmed as a recognition site for calpain cleavage, and interestingly, is identical to a calpain cleavage site previously identified in human protein kinase C-gamma (42). Cleavage at this site leaves only three acidic residues N-terminal to the PSD.

After digestion of MARCKS by calpain for times points up to one hour, MARCKS increased the rate of actin polymerization, with the extent of the increase correlating with the duration of digestion (Fig. 7B). When compared to SDS-PAGE results for the same MARCKS samples used to stimulate polymerization, there was no obvious candidate that increased in abundance in a manner that correlated temporally with the effect on the rate of actin polymerization. Some proteolytic fragments may be increasing in concentration, yet be unidentifiable because they may not stain well, so it is uncertain which, if any, of the bands on the gel identify MARCKS peptides that stimulate polymerization. In any case, these results suggest that cleavage of MARCKS by calpain may be a mechanism of activation of MARCKS.
that exposes the PSD by digestion and removal of the N-terminus (presumably by cutting at Ser-134) and/or the C-terminus of MARCKS.

**Discussion**

Initial experiments revealing the absence of actin-binding activity in recombinant MARCKS were unexpected. Yet we have documented that the DNA sequence of the MARCKS clone is correct, that the recombinant protein is recognized specifically by anti-MARCKS antibody, that it has the expected anomalous electrophoretic mobility on SDS-PAGE, that it has the absence of significant secondary structure by circular dichroism spectroscopy, that it yields distinctive, correct amino acid analysis results, that it has the expected mass by mass spectroscopy, and that it is not aggregated as assessed by analytical ultracentrifugation or gel filtration chromatography. All of these results support the conclusion that the recombinant protein is authentic MARCKS. Paradoxically, our results therefore imply that a natively unfolded protein has an inaccessible actin-binding site and a restricted CaM binding site. While our findings do not definitively provide an explanation, they clearly document the evidence confirming that such a paradox exists by demonstrating that the PSD is less available to bind to actin and CaM in the full length protein than in a peptide that corresponds to the PSD alone. The observation that neutralization of charged residues outside of the PSD can alter PSD-CaM and PSD-actin interactions shows that the acidic termini influence binding events at the PSD, and not surprisingly, that these effects are charge-dependent. Direct binding of PSD peptide to MARCKS shows that intramolecular interactions are likely to occur in MARCKS.

In consideration of hypotheses that could explain these results, one idea was originally postulated as an explanation for the low affinity of MARCKS for negatively-charged phospholipid vesicles (11). Perhaps the electrostatic potential of acidic residues of MARCKS
that are distant from the PSD reduce the effective charge of the PSD. This explanation is most believable with regard to supposedly non-specific interactions between anionic phospholipids and the PSD, and less believable for site-specific interactions between CaM (12) or actin (8) and the PSD. Even in the instance of non-specific binding, the contribution of distant acidic residues may be relatively small compared to the free energy contribution of the multiple electrostatic interactions occurring at the PSD-phospholipid junction. An analysis of distance and salt dependence of coulombic interactions in proteins by Lee et al. (43), directly applicable to the analysis of MARCKS-protein interactions, and likely applicable to electrostatic interactions between MARCKS and phospholipids, shows that in 0.01 M KCl, ΔG = 0.2 kcal/mol for charges separated by distances of = 15 Å and in 0.1 M KCl, ΔG = 0.1 kcal/mol for charges separated by distances of = 12 Å. Thus, the 5-6 acidic residues that are likely to fall into the range limits of 12 to 24 Å are likely to have no more impact on binding to phospholipids than do the five Phe residues within the PSD. Each phenylalanine individually contributes ~0.2 kcal/mol, and their complete replacement by Ala decreases binding by ~6-fold (44) rather than the 10^4-fold observed when comparing MARCKS relative to PSD peptide (11). In contrast, models of PSD-phospholipid structure show coulombic interactions occurring at a range of 3 to 5 Å (45, 46), comparable to the length of a protein-protein salt bridge. While the individual coulombic interactions that bind MARCKS to anionic phospholipids may contribute as little as ~0.6 kcal/mol, localization of the multiple charges in the vicinity of the ligand will have a major contribution to the overall free energy of interaction (47), and the free energy contribution of distant acidic residues would be expected to be insignificant in comparison.

An alternative possibility supported by our current results is that the acidic termini of MARCKS interact, either specifically or non-specifically, with the PSD so as to shield the PSD
from its ligands. The free energy difference between ligand binding to MARCKS and PSD peptide is related to the free energy required to displace the acidic chains to a distance at which electrostatic effects become negligible. If the intramolecular interaction between MARCKS and its PSD were entirely non site-specific, then the observed differences between binding myristoylated and non-myristoylated MARCKS to either CaM or actin would require still another explanation, as myristoylation has no effect on charge, and therefore should not have an effect on non-site specific ionic interactions between the PSD and the acidic termini. The hypothesis that site-specific intramolecular interactions between the MARCKS termini and the PSD regulate the availability of the PSD for its ligands is attractive from the perspective that the effects of myristoylation could be similarly explained - myristoylation may sterically inhibit the intramolecular interactions. The only data directly in support of this hypothesis are from Fig. 4, providing evidence of site-specific binding of PSD peptide to MARCKS that includes the observation that PSD competes directly with Rh-PSD peptide to bind to MARCKS. The salt dependence of nearly every equilibrium reaction studied here does not help to distinguish between these site-specific and non site-specific binding. While it is true in general that short range electrostatic interactions are less salt dependent than long range interactions (43), complicated interactions with multiple charges make it impossible to conclude that any particular reaction is too salt dependent to be a short-range interaction or insufficiently salt-dependent to be a long-range interaction.

Given the inaccessibility of the PSD, what mechanisms are available for activation? Based on the available data, myristoylation and proteolysis are two post-translational modifications that are potential candidates. The calpains are ubiquitous intracellularly located Ca$^{2+}$-dependant neutral cysteine proteases. Because the calpains typically function through
limited proteolysis that modifies rather than terminates substrate activity (48), calpains are implicated in several basic physiological functions related to the cytoskeleton such as activation of protein kinase C at the plasma membrane (49). The one specific cleavage site identified in this manuscript would serve to isolate the PSD from the N-terminus of MARCKS (Fig. 7), preventing intramolecular interactions that might block access to the PSD. Further degradation of MARCKS is clearly indicated by these data, and it is possible that additional proteolysis is necessary for the activation of actin-binding activity observed in Fig. 7. These data provide the first evidence that calpain-cleavage of MARCKS alters molecular function, and in a broader context, the idea that calpain could regulate intramolecular interactions in MARCKS could be functionally relevant for any ligand that targets the PSD.

Myristoylation already has a functional role relative to MARCKS, contributing to membrane localization as a “myristoyl-electrostatic switch” (50). Independent data that myristoylation could also regulate accessibility of the PSD are limited to the effects on CaM binding reported here and by others (14) and the observation that myristoylated MARCKS-related protein incorporates significantly more phosphate than non-myristoylated MARCKS-related protein upon in vitro phosphorylation by the catalytic subunit of protein kinase C (51). Here we show that myristoylation alters actin-binding functions of MARCKS. This and the prior observations are all consistent with the unifying concept that myristoylation increases access to the PSD.

MARCKS is potentially the target of a myriad of additional post-translational modifications, including phosphorylation by multiple kinases (52, 53), proteolysis by other enzymes (54), O-glycosylation (55), ADP ribosylation (56), and evidence of other non-covalent ligands (57). While we have shown that it is qualitatively possible to activate the MARCKS
PSD with specific post-translational modifications, thereby providing these modifications with potential functional significance, other post-translational modifications of MARCKS may be operative in vivo, either alone or in combination. Furthermore, these modifications may selectively regulate specific PSD ligands and their function.
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Footnotes

The abbreviations used are: MARCKS, Myristoylated alanine rich C kinase substrate; PSD, the phosphorylation site domain of MARCKS; Rh-PSD, PSD that is modified at the NH₂ terminus by an amide link to 5-(and 6)-carboxy-tetramethyl rhodamine succinimidyl ester; pyrenyl-actin, actin labeled on Cys-374 with N-(1-pyrene)iodoacetamide; PIP₂, phosphatidylinositol 4,5-bisphosphate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; CaM, calmodulin; MIANS-CaM, wheat germ calmodulin labeled on Cys-27 with 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid.

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Figure Legends

Fig. 1. **Comparison of the effects of MARCKS and PSD peptide on actin.** A, pellets of Mg²⁺-F-actin (30 µM, 20 µM and 10 µM) after incubation with MARCKS (8 µM) are shown along with respective supernatants of these actin-MARCKS mixtures including a control of MARCKS without actin. MARCKS fails to accumulate in the pellet and is not depleted from the supernatant. The extra band in the supernatant lanes from samples with actin is due to a small amount of non-pelleting contaminant that is most probably a digestion fragment of actin. B, SDS-PAGE of supernatants after low speed centrifugation of actin filaments (7 µM) alone (*first lane*) or incubated with PSD peptide (2 µM; *second lane*) or MARCKS (10 µM; *third lane*). The presence of actin bundles or cross-linked filaments induced by PSD peptide is indicated by depletion of actin from the supernatant. C, time course of actin filament polymerization (3 µM
Mg^{2+}-actin, 4% pyrenyl-actin) in 50 mM KCl and 2 mM MgCl₂ in the absence (closed squares) or presence of MARCKS (4 µM) (open squares) or PSD (4 µM) (triangles). The inset shows the same comparison in the absence of KCl with the same units and symbols.

**FIG. 2. Binding of PSD peptide to calmodulin.** A, CaM-Sepharose suspended beads (0-2 µM) were equilibrated with Rh-PSD peptide of 0.6 µM (circles) or 0.06 µM (squares), the beads were pelleted, and the amount of free Rh-PSD remaining in the supernatant was measured by fluorescence intensity of the rhodamine label using absorption 522 nm and emission 575 nm. Both sets of data were fit simultaneously to give a $K_d$ of 10 nM for the binding of PSD to CaM, with the fit shown by the solid lines. The buffer included 50 mM KCl, 0.1 mM CaCl₂, and 10 mM Tris-HCl, pH 7.9. Inset, the same data are compared with those obtained for full-length recombinant MARCKS (open circles). CaM-Sepharose beads were equilibrated with 0.6 µM MARCKS in the same buffer as for the PSD, the beads were pelleted, and the amount of free MARCKS remaining in the supernatant was measured by quantification of Coomassie staining after SDS-PAGE. The solid line is the expected result for $K_d = 1.3$ µM. B-C, binding of Rh-PSD to CaM in solution (not bead-bound) was studied using fluorescence intensity ($B$) and fluorescence anisotropy ($C$) in samples at concentrations of Rh-PSD of 20 nM (circles) and 67 nM (triangles) with varying amounts of CaM. The buffer included 50 mM KCl, 0.6 mM CaCl₂, and 10 mM Tris-HCl, pH 7.9. Insets (B,C), competition of PSD with Rh-PSD (20 nM) for binding to CaM (16 nM). The fit to the anisotropy data ($C$) includes a correction that weights the bound and free Rh-PSD based on the increment of fluorescence intensity observed upon binding CaM in $B$. At saturation with CaM, this increment is a factor of 2.3, so that, for example, when the anisotropy change is 2.3/3.3 or 70% of the maximal anisotropy change, then ½ of the Rh-PSD is free and ½ bound. A global fit to the fluorescence and anisotropy data with the same set
of parameters yields $K_d$ of $6 \pm 2$ nM for binding of PSD peptide and $9 \pm 4$ for binding of Rh-PSD peptide to CaM (solid lines).

**Fig. 3. Binding of MARCKS and myristoylated MARCKS to MIANS-CaM.** The fluorescence intensity of 0.2 µM MIANS-CaM was determined in the presence of varying concentrations of MARCKS (squares), or myristoylated MARCKS (circles). At 0.6 CaCl$_2$, $K_d$ of 2.5 and 0.9 µM were calculated for MARCKS and myristoylated MARCKS binding to CaM, respectively, as shown by the solid lines. *Inset,* at 0.1 mM CaCl$_2$, the data were fit to $K_d$ of 0.1 µM for MARCKS (squares) and less than 9 nM for myristoylated MARCKS (circles).

**Fig. 4. Intramolecular interactions between Rh-PSD and MARCKS.** A, when MARCKS is added to Rh-PSD (44 nM) in 50 mM KCl, 0.6 mM CaCl$_2$, and 10 mM Tris-HCl, pH 7.9, the fluorescence of Rh-PSD (not shown) decreases with increasing MARCKS, and at saturation, the fractional decrease was calculated as 0.53. Fluorescence anisotropy was measured in the same samples, with an observed increase in anisotropy consistent with a direct interaction between MARCKS and the smaller fluorescent ligand. *Inset,* unlabeled PSD peptide is able to displace Rh-PSD (44 nM) from MARCKS (1 µM), consistent with site-specific binding and a one to one stoichiometry of MARCKS for PSD or Rh-PSD. When saturating amounts of unlabeled PSD peptide are included in the mixture of MARCKS and Rh-PSD, the anisotropy of Rh-PSD decreases to a level near that expected for free Rh-PSD (compare inset with origin of the main panel) and the level of fluorescence increases, also to baseline levels (not shown). A simultaneous fit to all of the data shown using the assumption that the intramolecular PSD of MARCKS competed equivalently with the intermolecular labeled and unlabeled PSD for a single binding site on MARCKS gives the $K_d$ in a range of 10 nM (shown by solid lines) with good fits obtainable for any $K_d = 40$ nM. Data consistent with site-specific binding are also obtained in
presence of increasing amounts of polylysine (*triangles*). The apparent \(K_d\) for polylysine is \(= 14\) nM. When one polylysine occupies that site, rhodamine-PSD is excluded. *B*, SDS-PAGE of untreated MARCKS (*lanes 1 and 4*), EDC cross-linked MARCKS (*lanes 2 and 5*) and EDC cross-linked MARCKS with N-terminal rhodamine-labeled PSD peptide (*lanes 3 and 6*) visualized by coomassie staining (*lanes 1-3*) or by indirect UV fluorescence of the rhodamine label (*lanes 4-6*). *C*, purified recombinant MARCKS (*lanes 1, 4, 6 and 8*) and two RAW 264.7 whole cell extracts (one in *lanes 2, 5, 7 and 9* and the other in *lanes 3 and 10*) were stained with coomassie (*lanes 1-3*) or transferred to PVDF membrane and western blotted for MARCKS (*lanes 4-7*) or for a gel overlay using N-terminal rhodamine-labeled PSD peptide as probe (*lanes 8-10*). The two western blots are of identical samples transferred on different days and reveal that the major immunoreactive band in the cell extracts has slightly lower electrophoretic mobility than does the recombinant MARCKS. The gel overlay was imaged by indirect fluorescence and the digital fluorescence image was electronically inverted. *D*, concentration-dependence of pelleting for MARCKS after high-speed centrifugation after dilution for 1 h from a 300 µM stock.

**Fig. 5.** **EDC-treated MARCKS binds to actin or CaM and accelerates the rate of actin filament polymerization.** *A*, F-actin (24 µM) high speed pelleting assay with EDC-treated MARCKS (16 µM; treated with either 0 mM, 4 mM, 12 mM or 20 mM EDC) in which supernatants of spun or unspun samples were equally concentrated and stained with SYPRO Ruby protein stain. *B*, the fluorescence intensity of 0.2 µM MIANS-CaM is shown as a function of untreated MARCKS (*closed squares*), or untreated MARCKS in EDC buffer (*open squares*), or EDC-treated MARCKS with 4 mM EDC, (*triangles*), 12 mM EDC, (*diamonds*) or 20 mM EDC, (*circles*). *C*, time course of actin filament polymerization (3 µM; 4% pyrenyl-actin) with
6.0 µM EDC-treated MARCKS (same symbols as in B) in 2.0 mM MgCl₂. Polymerization buffer alone is shown as an additional control (open circles). D, time course of actin filament depolymerization after dilution of 10% pyrenyl-labeled F-actin to 0.1 µM into the same polymerization buffer with or without 2.0 µM of EDC-treated MARCKS (same symbols as in C), or for comparison, with 0.2 (open stars) or 2 µM (closed stars) PSD.

**Fig. 6.** Myristoylated MARCKS increases the rate of actin filament polymerization. The time course of Mg²⁺-actin (3 µM, 4% pyrenyl-actin) polymerization with 2 mM MgCl₂ in the absence (squares) or presence of myristoylated MARCKS at 1 µM (triangles), or 2 µM (circles), or 2 µM PSD (diamonds) was monitored using pyrene fluorescence.

**Fig. 7.** Calpain digestion of MARCKS increases the rate of actin filament polymerization. A, SDS-PAGE of calpain-digested MARCKS reveals several bands after Coomassie staining. In a separate experiment in which MARCKS was calpain digested for 30 m (Coomassie stain shown in lane C), the most prominent band appearing after calpain digestion on a western blot using anti-MARCKS antibody directed against the C-terminus (lane W) has apparent electrophoretic mobility of 40 kDa (labeled with an asterisk) and was N-terminal sequenced, confirming that it corresponds to a C-terminal MARCKS peptide that begins 17 residues before the PSD. B, calpain digestion of MARCKS enhances the rate of growth of actin filaments comparable to the enhancement seen with PSD. The digested MARCKS is from the same samples (after various times of digestion) as those run on SDS-PAGE in A. Polymerization of Mg²⁺-actin (3 µM, 4% pyrenyl-actin) was initiated in 2.0 mM MgCl₂, 0.8 mM CaCl₂ and 40 mM NaCl with calpain alone (diamonds), or with 5 µM of calpain-digested MARCKS after 15 (closed triangles), 30 (open triangles), 45 (closed circles), and 60 (open circles) m of digestion, respectively, or 2 µM of calpain-digested PSD for 30 m (stars).
Figure 1

A

| Actin (µM) | pellets | supernatants |
|------------|---------|--------------|
| 30         | 20      | 10           |
| 30         | 20      | 10           |
| 30         | 0       | 10           |

MARCKS
Actin

B

F-actin alone
PSD + F-actin
MARCKS + F-actin

MARCKS
actin

C

Fluorescence (cts)

Time (s)

0 100 200 300 400

0 300 600 900 1200 1500 1800

0 200 400

6000 4000 2000 0
Figure 2

A

B

C
MARCKS is a natively unfolded protein with an inaccessible actin-binding site: Evidence for long-range intramolecular interactions
Hazel Tapp, Iman M. Al-Naggar, Elena G. Yarmola, Alexis Harrison, Gerry Shaw, Arthur S. Edison and Michael R. Bubb

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