MARCKS Is a Natively Unfolded Protein with an Inaccessible Actin-binding Site

EVIDENCE FOR LONG-RANGE INTRAMOLECULAR INTERACTIONS

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Myristoylated alanine-rich C kinase substrate (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, despite its unfolded state, the distant N terminus influences binding events at the PSD. We show that the 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 that explain the observed differences between properties of the PSD and MARCKS. Consistent with this hypothesis, chemical modifications 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. Because 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.

Myristoylated alanine-rich C kinase substrate (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 the 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). Although this approach intuitively appears to be logical, given the unfolded state of the native protein, the substitution of the PSD peptide for the intact protein has never been rigorously justified. In fact, there are several reported experiments that imply that the PSD peptide behaves differently from intact MARCKS. The non-phosphorylated PSD peptide is known to have extended structure, to nucleate polymerization, and to cross-link F-actin filaments (5–7), presumably because of two binding sites with a site-specific $K_D$ of ~0.5 μM for F-actin (8). Although the PSD of MARCKS and its homolog 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 μM) and does not cross-link F-actin or induce G-actin polymerization (9). Although 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 104-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 that 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 ($K_D = 3.8$ 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 does non-myristoylated MARCKS (14) unless both binding sites can interact with a single calmodulin molecule simultaneously or myristoylation itself changes the accessibility...
ity of the PSD to bind to calmodulin. Independent binding could change the stoichiometry of the interaction to two calmodulin molecules/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 (14), 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, because of its natively unfolded structure, there is reason to believe that the native protein is equivalent to the recombinant protein.

Many in vivo studies have implicated 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 filament filaments (18, 22). Such data are most readily interpreted as direct effects of MARCKS on actin. One target of MARCKS, phosphatidylinositol 4,5-bisphosphate, has been implicated in controlling the actin cytoskeleton by binding to many other actin-regulating proteins such as neural Wiskott-Aldrich syndrome protein (24), suggesting one possible indirect mechanism of actin control by MARCKS. MARCKS may sequester phosphatidylinositol 4,5-bisphosphate in the plasma membrane by reversible PSD binding (25). However, it should be noted that published estimates of the 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, although there may not be sufficient MARCKS to globally regulate phosphatidylinositol 4,5-bisphosphate metabolism, local pools of phosphatidylinositol 4,5-bisphosphate 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 this study. 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 function of MARCKS.

EXPERIMENTAL PROCEDURES

Materials—Rabbit skeletal muscle actin was prepared from frozen muscle (Pel-Freeze Biologicals, Rogers, AR) in 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 pyrene-actin (actin labeled at Cy5™ with N-(1-pyrene/iodoacetamide)) was prepared with 0.5–0.95 mol of label/mol of protein using the method of Koyama and Mihashi (29). Peptides were synthesized by solid-phase Fmoc-(N-(9-fluorenemethoxycarbonyl) chemistry at the University of Florida (8). The PSD peptide has the sequence KKKKRRPFSKKSKPLG0FSFKKSKK. The modified rhodamine-labeled PSD peptide (Rh-PSD) (8) was N-terminally modified through an amide link with 5(6)-carboxytetramethylrhodamine succinimidyl ester and C-terminally labeled with Oregon Green 488 succinimidyl ester (5-isomer). Calpain I purified from porcine erythrocytes was obtained from Calbiochem. Calmodulin (CaM) isolated from bovine brain was purchased from Sigma. CaM-Sepharose beads were purchased from Amersham Biosciences. Wheat germ CaM (M, 16,800) fluorescently labeled with 2-[(4-maleimidobenzoxy)oxy]ethyl sulfonic acid (MIANS) at Cys²⁷ (MIANS-CaM) was gifted by Dr. J. M. Chalovich (East Carolina University).

Preparation of MARCKS and Myristoylated MARCKS—Marine full-length MARCKS DNA (GenBank™/EBI accession number M60747) was inserted into the plMW172 vector (30) and transformed into Escherichia coli BL21(DE3) competent cells. Cells (300 ml) and further grown overnight in LB medium and used to inoculate 2 liters of culture. After 3 h, protein expression was further induced by the addition of isopropyl β-D-thiogalactopyranoside (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 EDTA, 5 mM β-mercaptoethanol, 2 mM EDTA, 100 µM phenylmethylsulfonyl fluoride, and 0.6 mM diisopropylphosphosphate (pH 7.9). Resuspended cultures were heated to 85 °C for 10 min, 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 blotting using goat anti-MARCKS polyclonal antibody raised against a C-terminal synthetic peptide (Serotec Inc., Raleigh, NC)) were combined and concentrated on a hydroxylapatite column and further purified by gel filtration on Sephacryl 300 HR. Concentration was determined by UV absorption at 258 nm (ε₂₅₈ = 1100 at 258 nm) or by amino acid analysis. MARCKS typically eluted as a monomer at 40–70 µM and was stored at −80 °C in the column buffer, which contained 5 mM Tris-HCl, 5 mM β-mercaptoethanol, and 50 mM KCl (pH 7.9) (MARCKS buffer).

Preparation of myristoylated MARCKS, E. coli strain BL21 was transformed with both the pBB131NMT plasmid (a gift from Dr. J. I. Gordon, Washington University), which contains the gene for yeast N-myristoyltransferase (31), and the pMW172-MARCKS plasmid described above and selected in the presence of 50 µg/ml kanamycin and ampicillin. A frozen stock of transformed colonies was used to inoculate 200 ml of LB medium containing 50 µg/ml kanamycin and ampicillin. The overnight culture (2 ml) was grown to log phase: 400 mM isopropl β-D-thiogalactopyranoside was added to induce protein expression; and the culture was grown for an additional 3 h. Myristoylated MARCKS was purified according to the protocol for MARCKS described above. Myristoylated MARCKS runs at ~83 kDa on SDS-polyacrylamide gel (a slightly higher apparent molecular mass than that of MARCKS). It was characterized by mass spectrometry with a peak at 29,574 Da compared with 29,664 Da for non-myristoylated MARCKS, and these correspond to the predicted masses based on the sequence. 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 × g for 15 min through a 20% sucrose cushion either 1 or 12 h after dilution. Pellets and supernatants were analyzed by SDS-PAGE or 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. Mg²⁺- F-actin was prepared by activating F-actin (5 µM) with Mg²⁺-G-actin by the addition of 0.125 mM EDTA and 0.05 mM MgCl₂ for 10 min at room temperature and then polymerizing by the addition of MgCl₂ to a 2.0 mM final concentration. MARCKS (0–10 µM) was added to Mg²⁺-actin (0–40 µM) in a total volume of 100 µl and equilibrated for varying times (20 min to 4 h). F-actin was pelleted at 140,000 × 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. Supernatants 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, Inc., 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 centrifugal forces. Proteins or peptides were added to Mg²⁺-F-actin (7 μM final concentration) to a final volume of 90 μL. After a 10-min incubation, samples were centrifuged at 8000 × g for 20 min to pellet actin filament aggregates and any associated proteins. Supernatants (30 μL) were removed and loaded onto 10% SDS-polyacrylamide gel. The presence of bundling or cross-linking is indicated by the depletion of actin from the supernatant. The effects of MARCKS on the time course of actin filament polymerization were measured by the fluorescence change associated with the polymerization of pyrenyl-actin (28). Ca²⁺-G-actin (3 μM, 4% pyrenyl-actin) was converted to Mg²⁺-actin as described above, and polymerization was initiated by adjustment to 50 mM KCl and 2 mM MgCl₂. (Experiments without KCl are specifically indicated below.) Seeded polymerization assays employed cross-linked oligomeric F-actin seeds (32) and 4% pyrenyl-labeled Mg²⁺-actin monomer (0.5 μM). The assay was shown to be linear in response to seed concentration and actin monomer concentration with variations in 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 that could be fit with a line without systematic deviation. The time course of actin filament depolymerization was assayed by dilution of 10% pyrenyl-labeled F-actin (10 μM) polymerized with 2 mM MgCl₂ to 0.1 μM in the depolymerizing buffer (MES and 400 mM NaCl) for 10 min at room temperature. Freshly prepared EDC (200 mM) was added to give varying final EDC concentrations (0, 4, 12, and 20 mM). Reactions were incubated at room temperature for 1 h, stopped by the addition of β-mercaptoethanol (100 mM), and dialyzed into 5 mM Tris-HCl and 5 mM β-mercaptoethanol (pH 7.9). Aliquots of treated MARCKS from each reaction mixture were subjected to 12% SDS-PAGE as described above. The shift in electrophoretic mobility, as unmodified MARCKS runs anomalously as an ~80-kDa protein because of its unusual pl, and modification of the acidic residues shifts it toward 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 (2.5 μg) in 0.5 mM Tris-HCl, 100 mM NaCl, and 2 mM CaCl₂ (pH 7.9) for varying lengths of time. The reaction was stopped by addition of 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 polyvinylidene difluoride membrane and stained with 0.02% Coomassie Blue R-250 in 40% methanol and 5% acetic acid for 30 s, followed by destaining (40% methanol and 5% acetic acid) and air-drying. Amino acid sequence was obtained by Edman degradation on a Procise instrument (Model 494 HT, Applied Biosystems).

**RESULTS**

*Full-length Recombinant MARCKS Fails to Interact with Actin—*MARCKS did 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. Despite a different interpretation, these results are qualitatively similar to those reported by Hartwig et al. (5), in whose study pelleting assays were performed by Western blotting, and the molar ratio of bound MARCKS to actin was ~0.01–0.02 at saturation by MARCKS. Also, Wohlschlager et al. (7) reported that only ~5% of the total amount of MARCKS-related protein was associated with F-actin upon pelleting at 100,000 × g. The results reported here by us and elsewhere by others are consistent either with very low affinity (K_d > 100 μM) 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.
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The PSD peptide on actin. Incubation with MARCKS (8
actin filaments alone (7
non-pelleting contaminant that is most probably a digestion fragment and was not depleted from the supernatant. The extra band in the supernatants of these actin/MARCKS mixtures, including a control of the presence of the PSD peptide, Mg2+
how MARCKS can induce aggregation of actin filaments (8). In
viously associated with the PSD. We previously identified two peptide alone showing a 1:1 stoichiometry with F-actin sub-
are not readily made consistent with binding data for the PSD correct orientation for binding. However, these explanations aligned so that only a few of the F-actin protomers have the created by two adjacent actin filaments in a filament bundle simultaneously accessed, or a MARCKS-binding site could be buried within F-actin so that only a few sites can be plain the low stoichiometry of binding. A MARCKS-binding site could be
was 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 binding of the PSD to CaM. First, the Rh-PSD was bound to CaM-Sepharose beads, and the amount bound was determined from the observed decrease in supernatant fluorescence after pelleting the Rh-PSD bound to CaM-Sepharose beads (Fig. 2A). The experiment was done at two different concentrations of the Rh-PSD peptide (0.6 and 0.06 μM), one of which was substantially higher than the Kd, 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 the Rh-PSD and CaM are equal, assuming a stoichiometry of 1:1. 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 Kd of 10 nM for binding of the Rh-PSD to CaM. Second, CaM (in solution, not bead-bound) was equilibrated with the Rh-PSD peptide, and a binding curve was obtained based on the change in fluorescence that was observed upon binding of the Rh-PSD to CaM (Fig. 2B). At saturation by CaM, the maximum 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 the Rh-PSD can be globally fit to a Kd of 9 nM. As long as the stoichiometry of the interaction between the 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 the PSD with CaM provide direct support for both of these claims (12). When the unlabeled PSD peptide was added to a complex of the Rh-PSD peptide and CaM, the PSD competed directly with the Rh-PSD for binding to CaM; so at high concentrations of the PSD, the fluorescence intensity of the Rh-PSD mixed with CaM was about the same as that of the Rh-PSD alone (Fig. 2B, inset). The theoretical fit to these competitive binding data shows that the rhodamine label on the PSD does not significantly influence binding of the PSD to CaM.

The third assay was steady-state fluorescence anisotropy of the 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 CaM-Rh-PSD contributed 2.3 times as much to the measured value of fluorescence anisotropy as did the free Rh-PSD, necessitating correction of the data by this weighting factor (Fig. 2C). As observed with the fluorescence intensity measurements, the unlabeled PSD competed with the Rh-PSD; so at high concentrations of the unlabeled PSD, the anisotropy of a solution of

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 the PSD peptide alone showing a 1:1 stoichiometry with F-actin sub-units at saturation by the PSD (8).

MARCKS does not have actin-regulating 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 the PSD peptide, Mg2+-actin filaments pelleted at low centrifugal forces (8000 × g), but the filaments remained in the supernatant despite a large excess of intact MARCKS (Fig. 1B). The results in Fig. 1B, in which the concentration of MARCKS exceeded 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 were 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 Mg2+-actin in 50 mM KCl and 2 mM MgCl2 was similar to the control (Fig. 1C). The PSD peptide showed an ~4-fold increase in the rate of actin filament polymerization over the first 100 s 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 the PSD peptide, again with no effect attributable to MARCKS, but an ~30-fold increase in the rate of polymerization by the PSD peptide (Fig. 1C, inset). Because the effects of KCl on this particular assay in the absence of the 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).
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Sepharose beads were equilibrated with 0.6 M CaCl2, compared with a Kd of 10 nM for the PSD under the same conditions (Fig. 2A). Schleiff et al. (36) have shown that maximum binding of MARCKS to CaM is measured at 0.1 mM CaCl2 and that the affinity of the complex is decreased by 6-fold as the concentration of Ca2+ is increased to 1 mM. Using the increment in 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 Kd of 2.5 μM for binding of MARCKS to MIANS-CaM in 0.6 mM CaCl2, but the measured Kd dropped to 0.1 μM in 0.1 mM CaCl2 (Fig. 3). Titration of MIANS-CaM with CaCl2 alone had an insignificant effect on fluorescence intensity, thus ruling out the possibility that the change in fluorescence upon the addition of MARCKS was caused indirectly by binding of MARCKS to Ca2+ with a secondary decrease in free Ca2+. Although the assays are different, the measured values for Kd are all significantly higher than those obtained for the PSD peptide at either 0.1 or 0.6 mM CaCl2. 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 Kd. Qualitatively, upon binding CaM in B. At saturation with CaM, this increment is a factor of 2.3, so, for example, when the anisotropy change is 2.3/3.3 or 70% of the maximum anisotropy change, then one-half of the Rh-PSD is bound. A global fit to the fluorescence and anisotropy data with the same set of parameters yields Kd = 6 ± 2 nM for binding of the PSD peptide and 9 ± 4 nM for binding of the Rh-PSD peptide to CaM (solid lines). cps, counts/s.

**FIG. 2.** Binding of the PSD peptide to calmodulin. A, CaM-Sepharose suspended beads (0–2 μM) were equilibrated with the Rh-PSD peptide at 0.6 μM (●) or 0.06 μM (○); 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 excitation at 522 nm and emission at 575 nm. Both sets of data were fit simultaneously to give a Kd of 10 nM for binding of the 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 (○). CaM-Sepharose beads were equilibrated with 0.6 μM MARCKS in the same buffer as described for the PSD; the beads were pelleted; and the amount of free MARCKS remaining in the supernatant was measured by quantification of Coomasie Blue staining after SDS-PAGE. For MARCKS, the solid line is the expected result for Kd = 1.3 μM. B and C, binding of the Rh-PSD to CaM in solution (not bead-bound) was studied using fluorescence intensity and fluorescence anisotropy, respectively, in samples at Rh-PSD concentrations of 20 nM (●) and 67 nM (○) with varying amounts of CaM. The buffer included 50 mM KCl, 0.6 mM CaCl2, and 10 mM Tris-HCl (pH 7.9). Insets, shown is the competition between the PSD and 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 in fluorescence intensity observed upon binding CaM in B. At saturation with CaM, this increment is a factor of 2.3, so, for example, when the anisotropy change is 2.3/3.3 or 70% of the maximum anisotropy change, then one-half of the Rh-PSD is bound. A global fit to the fluorescence and anisotropy data with the same set of parameters yields Kd = 6 ± 2 nM for binding of the Rh-PSD peptide and 9 ± 4 nM for binding of the Rh-PSD peptide to CaM (solid lines). cps, counts/s.

**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 (○) or myristoylated MARCKS (●). At 0.6 mM CaCl2, Kd values of 2.5 and 0.9 μM were calculated for binding of MARCKS and myristoylated MARCKS to CaM, respectively (solid lines). Inset, at 0.1 mM CaCl2, the data were fit to Kd = 0.1 μM for MARCKS (○) and Kd < 9 nM for myristoylated MARCKS (●). cps, counts/s.
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However, this assay uniquely provides a straightforward comparison of binding of MARCKS relative to the 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 the PSD and MIANS-CaM did not alter the fluorescence intensity of MIANS-CaM. These results independently confirm that the interaction of the PSD with CaM is attenuated by the presence of the N and C termini despite 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 for MIANS-CaM by a factor of 3 in 0.6 mM CaCl₂ and by a factor of >10 in 0.1 mM CaCl₂ (Fig. 3). A simple explanation for the attenuation of binding by full-length MARCKS relative to the 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 the PSD and 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 intramolecularly 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 the PSD and a site on an oppositely charged terminus would be expected to have complicated equilibrium kinetics. The consequences of localization of a binding site for the 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 intramolecular binding events measured experimentally using a synthetic PSD peptide would occur in competition with intermolecular binding, so the intramolecular equilibrium kinetics may also be expected to be complicated. Using the Rh-PSD peptide, we observed saturatable binding by MARCKS based on the changes in fluorescence amplitude and anisotropy that were consistent with association of the Rh-PSD and MARCKS (Fig. 4A). The unlabeled PSD was able to displace the Rh-PSD from MARCKS as determined by a return of the amplitude of fluorescence intensity and the anisotropy to levels measured for the free Rh-PSD peptide (Fig. 4A, inset). Qualitatively, the decline in anisotropy as a function of the unlabeled PSD is steep, suggesting that the 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 the Rh-PSD peptide and samples in which variable amounts of unlabeled PSD peptide were added to a fixed mixture of MARCKS and the 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 $\leq 40$ nM, and the apparent (effective) concentration of the intrinsic PSD was estimated as 0.3–0.8 $\mu$M. The fitting algorithm for the anisotropy data included a correction for the larger fluorescence contribution of the free Rh-PSD relative to the bound Rh-PSD. The effective local concentration of the intramolecular PSD depends on the distance between the PSD and its binding site on MARCKS and on steric factors that reflect limitations in relative orientations and conformations of the MARCKS protein. Based only on geometric considerations and no steric constraints, the theoretical maximum effective concentration is $\approx 50$ $\mu$M (this assumes a 20-nm radius). Although the data fit excellently based on the assumption of site-specific binding, we could 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, an $\sim 24$-mer with low polydispersity (32), to displace the rhodamine-labeled PSD peptide (44 nM) from MARCKS (0.25 $\mu$M) 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 polylysines interacts with a single site 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 ($\leq 14$ 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 presumably compensated for in vivo by the high effective local concentration of the intramolecular PSD.

MARCKS (5 $\mu$M) could be successfully cross-linked to a synthetic rhodamine-labeled PSD peptide (25 $\mu$M) 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 EDC-modified MARCKS exhibited decreased electrophoretic mobility (Fig. 4B, lanes 2 and 3), perhaps because of an intermolecular cross-link consistent with limited self-association, as discussed below in relation to Fig. 4D. The same rhodamine-labeled peptide bound to a band on polyvinylidene difluoride membrane (after SDS-PAGE and transfer) that was at the same position as MARCKS (Fig. 4C). Not only did the peptide bind to purified recombinant protein, but it also demonstrated apparent selectivity for MARCKS, as it recognized a band at the expected position for MARCKS in whole cell extracts from RAW 264.7 macrophages. The position of the murine MARCKS band for both the 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 appeared to recognize only the band that would correspond to post-translationally modified MARCKS, but because the Western blots are non-quantitative (and in this instance, demonstrate some variation even when using the same samples), this may be due either to
Evidence for Long-range Intramolecular Interactions in MARCKS

If the 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 to 100 μM for 1 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 (but not basic) residues and not to induce intra/intermolecular cross-links (35). We used a conservative treatment of EDC at various concentrations for only 1 h. Amino acid analysis showed that, at the highest concentration of EDC (20 mM), only an average of 6 of the 59 acidic residues were modified, whereas averages 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). Although SDS-PAGE showed that the electrophoretic mobility of MARCKS generally increased with neutralization (Fig. 5A) (experimental repeats not shown), the bandwidth also increased, presumably because of heterogeneity in the covalent modifications (Fig. 5A). High speed pelleting of F-actin (24 μM) and EDC-treated MARCKS (16 μM) resulted 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.

The affinity of MARCKS for MIANS-CaM was similarly increased by EDC treatment (Fig. 5B). The data for untreated MARCKS are superimposable in Figs. 3 and 5C, but are shown on different scales. Treatment of MARCKS with EDC caused an increase in the fluorescence increment of MIANS-CaM that was 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-treated MARCKS gave KD = 2.5 μM, whereas 4, 12, and 20 mM EDC-treated MARCKS gave KD = 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 MARCKS treated with 12 mM EDC 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 a prior report has shown that the effects of the PSD on actin vary non-monotonically with PSD concentration (37), so activation of MARCKS to <100% of the PSD activity may be expected to cause either no effect or activity opposite of that seen for the PSD alone, de-

![Image](http://www.jbc.org/)

**Fig. 4.** Intramolecular interactions between the Rh-PSD and MARCKS. A, when MARCKS was added to the Rh-PSD (44 nM) in 50 mM KCl, 0.6 mM CaCl2, and 10 mM Tris-HCl (pH 7.9), the fluorescence of the Rh-PSD (not shown) decreased with increasing MARCKS concentrations, 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, the unla-
pending 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 partial activation of MARCKS might not be expected to produce the full spectrum of PSD-related functions. Despite these arguments, the inhibition of actin depolymerization by EDC-treated MARCKS in 2.0 mM MgCl2 has a very similar dose dependence compared with the effect on polymerization, with only the 20 mM sample having substantial activity (Fig. 5D). In this assay, 2.0 μM MARCKS treated with 20 mM EDC had activity roughly equivalent to that of 0.2 μM PSD alone. A prior study reported that the PSD slows depolymerization and attributed this effect to a lower critical concentration in the presence of the PSD (9). Alternatively, barbed end capping activity might be expected to produce the observed effect.

Modification of Full-length MARCKS by Myristoylation Enhances 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 above, the PSD of MARCKS affects multiple parameters that alter the time course of actin polymerization, including effects on nucleation rates and 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 the control. Thus, it is possible that myristoylation selectively activates 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 rates.

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 was cleaved in vitro by calpain, resulting in several bands on SDS-polyacrylamide gel (Fig. 7A). The most prominent band identified with EDC-treated MARCKS (6.0 μM; same symbols as in B) in 2.0 mM MgCl2. Polymerization buffer alone is shown as an additional control (○). D, time course of actin filament depolymerization after dilution of 10% pyrenyl-labeled F-actin to 0.1 μM in the same polymerization buffer with or without EDC-treated MARCKS (2.0 μM; same symbols as in C) or, for comparison, with 0.2 μM (○) or 2 μM (●) PSD. cps, counts/s.
After digestion of MARCKS by calpain for time points up to 1 h, MARCKS increased the rate of actin polymerization, with the extent of the increase correlating with the duration of digestion (Fig. 7B). Compared with 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 increase 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 Ser134) and/or 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-polyacrylamide gel, that it has the absence of significant secondary structure as determined by circular dichroism spectroscopy, that it yields distinctive correct amino acid analysis results, that it has the expected mass as determined 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. Although 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 the 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 more believable with regard to supposedly nonspecific 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 nonspecific binding, the contribution of distant acidic residues may be relatively small compared with the free energy contribution of the multiple electrostatic interactions occurring at the PSD-phospholipid junction. An analysis of the 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 0.1 M KCl, ΔGc < 0.2 kcal/mol for charges separated by distances of >15 Å and that, in 0.1 M KCl, ΔGc < 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 12–24 Å are likely to have no more impact on

by Western blotting using the goat anti-MARCKS antibody recognizing the C terminus 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 Ser134, so the MARCKS cleavage site has the sequence SSTSS–SPKAEDGAA (where – represents a 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 Cy (42). Cleavage at this site leaves only 3 acidic residues N-terminal to the PSD.

**Fig. 7.** Calpain digestion of MARCKS increases the rate of actin filament polymerization. A, SDS-PAGE of calpain-digested MARCKS revealed several bands after Coomassie Blue staining. In a separate experiment in which MARCKS was digested with calpain for 30 min (Coomassie Blue stain shown in the C lane), the most prominent band appearing after calpain digestion on a Western blot using anti-MARCKS antibody directed against the C terminus (W lane) has an apparent electrophoretic mobility of 40 kDa (labeled with the asterisk) and was N-terminally sequenced, confirming that it corresponds to a C-terminal MARCKS peptide that begins 17 residues before the PSD. B, calpain digestion of MARCKS enhanced the rate of growth of actin filaments comparable with the enhancement seen with the PSD. The digested MARCKS is from the same samples (after various times of digestion) as those subjected to SDS-PAGE in A. Polymerization of Mg<sup>2+</sup>-actin (3 μM, 4% pyrenyl-actin) was initiated in 2.0 mM MgCl<sub>2</sub>. The data points represent 0.8 mM CaCl<sub>2</sub> and 40 mM NaCl with calpain alone (●), with calpain-digested MARCKS (5 μM) after 15 (○), 30 (▲), 45 (●), and 60 (□) min of digestion; or with calpain-digested PSD (2 μM) for 30 min (★).
binding to phospholipids than do the 5 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-fold observed when comparing MARCKS relative to the PSD peptide (11). In contrast, models of PSD-phospholipid structure show coulombic interactions occurring at a range of 3–5 Å (45, 46), comparable with the length of a protein-protein salt bridge. Although 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 present results is that the acidic termini of MARCKS interact, either specifically or nonspecifically, with the PSD so as to shield the PSD from its ligands. The free energy difference between ligand binding to MARCKS and the 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 of 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 the PSD peptide to MARCKS that includes the observation that the PSD competes directly with the Rh-PSD peptide to bind to MARCKS. The salt dependence of nearly every equilibrium reaction studied here does not help to distinguish between the site-specific and non-site-specific binding. Although 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+}-dependent, 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 work 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 is limited to the effects on CaM binding reported here and by others (14) and the observation that myristoylated MARCKS-related protein incorporates significantly more phosphatase than non-myristoylated MARCKS-related protein upon in vitro phosphorylation by the catalytic subunit of protein kinase C (51). Here, we have shown that myristoylation alters the 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 noncovalent ligands (17). Although 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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