Molecular Determinants of the Myristoyl-electrostatic Switch of MARCKS*

(Received for publication, April 9, 1996)

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MARCKS is a protein kinase C (PKC) substrate which binds calcium/calmodulin and actin, and which has been implicated in cell motility, phagocytosis, membrane trafficking, and mitogenesis. MARCKS cycles on and off the membrane via a myristoyl electrostatic switch (McLaughlin, S., and Aderem, A. (1995) Trends Biochem. Sci. 20, 272–276). Here we define the molecular determinants of the myristoyl-electrostatic switch. Mutation of the N-terminal glycine results in a nonmyristoylated form of MARCKS which does not bind membranes and is poorly phosphorylated. This indicates that myristic acid targets MARCKS to the membrane, where it is efficiently phosphorylated by PKC. A chimeric protein in which the N terminus of MARCKS is replaced by a sequence, which is doubly palmitoylated, is phosphorylated by PKC but not released from the membrane. Thus two palmitic acid moieties confer sufficient membrane binding energy to render the second, electrostatic membrane binding site superfluous. Mutation of the PKC phosphorylation sites results in a mutant which does not translocate from the membrane to the cytosol. A mutant in which the intervening sequence between the myristoyl moiety and the basic effector domain is deleted, is not displaced from the membrane by PKC-dependent phosphorylation, fulfilling a theoretical prediction of the model. In addition to the nonspecific membrane binding interactions conferred by the myristoyl-electrostatic switch, indirect immunofluorescence microscopy demonstrates that specific protein-protein interactions also specify the intracellular localization of MARCKS.

EXPERIMENTAL PROCEDURES

Materials—[3H]Myristic acid (0.4–2.2 TBq/mmol), [3H]palmitic acid (1.11–2.22 TBq/mmol), [32P]orthophosphate (320 TBq/mmol), [3H]pro-

*This work was supported by the Paul Ehrlich Foundation for Biomedical Research and National Institutes of Health Grant AI 25032 (to A. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: MARCKS, myristoylated alanine-rich protein kinase C substrate; F-actin, filamentous actin; PAGER, polyacrylamide gel electrophoresis; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.
tein A (370 kDa), and En°hance were obtained from Du Pont NEN. L-[4,5-3H]lysine hydrochloride (3.7 TBq/mmol) was from Amersham. Leupeptin was obtained from Boehringer Mannheim. Hydromount was obtained from National Diagnostics (Manville, NJ). PMA was purchased from LC Laboratories (Woburn, MA). Unless indicated otherwise, all other chemicals were purchased from Sigma.

**Preparation of MARCKS Constructs**—The mutants were generated using cloned native MARCKS cDNA (8) as a template, and a polymerase chain reaction protocol utilizing VENT DNA polymerase (New England Biolabs). Wild type (wt) MARCKS and the various mutants were engineered into the Invitrogen pcDNA I/NEO vector. All polymorphisms were confirmed by restriction digests and DNA sequencing; all mutations are indicated by underlines. The wt construct was generated by engineering the 1.15-kilobase pair HindIII site in codons 158 and 159 so that the desired molecule could be generated in two pieces. The N-terminal half was generated by using coding primer 5'-CGTGCGGATCCCGCAGCTGTTGGTCG-3' and non-coding primer 5'-GGTGAACGATCCTGGTCTTGAGGAAGGGCTG-3'; and the C-terminal half was generated by using coding primer 5'-CGTGCGGATCCCGCAGCTGTTGGTCG-3' and non-coding primer 5'-CGGTCTAGAGGATCCCTCTGGAGCTTACTCGGC-3'. PHOS was generated by engineering a HindIII site in codons 158 and 159 so that the desired molecule could be generated in two pieces. The two polymerase chain reaction products were subjected to HindIII and BamHI digestion then purified, and the mutant was generated in a three fragment ligation. The G43-M mutant combines the first 7 amino acids of GAP-43 (19) with amino acids 6–309 of murine MARCKS (8) using coding primer 5'-GGGAGGATCCCTCTGGAGCTTACTCGGC-3' and 3'-Norm as the non-coding primer. 16–140 was constructed using coding primer 5'-GGGGATCCCTCTGGAGCTTACTCGGC-3' and 3'-Norm as the non-coding primer.

**Transfection and Culture Conditions**— Constructs were stably transfected into Ltk° cells using calcium-phosphate precipitation (20). Transfection was performed in Dulbecco's modified Eagle's medium supplemented with 1% L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (all from JRHBiosciences, Lenexa, KS), 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), and 400 μg/ml G418 (Life Technologies, Inc., Grand Island, NY), in an atmosphere of 5% CO2 in air. Individual G418-resistant colonies were harvested by localized trypsinization and cloned by limiting dilution. Clones expressing equivalent amounts of wild-type or mutant MARCKS were identified by Western blotting. Where indicated, PMA was used at 200 nM.

**Western Blotting**—Monolayers were scraped into 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, 15 mM EDTA, 50 mM potassium fluoride, 50 mM NaH2PO4, 10 mM sodium pyrophosphate, and 1% Nonidet P-40) supplemented with the protease inhibitors indicated below. Protein was determined using the Coomassie Plus kit (Pierce). Cell lysates were resolved by 7.5% SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Blots were blocked for 1 h at room temperature in milk buffer: 25 mM Tris-HCl, pH 8.0, 0.02% NaN3, and 150 mM NaCl (TBS) supplemented with 5% nonfat dry milk. Blots were then incubated with a 1:200 dilution of rabbit anti-murine MARCKS antiserum (21) in milk buffer for 1 h, washed sequentially with milk buffer, and Western blot buffer (TBS supplemented with 0.1% Triton X-100), and then incubated for an additional hour in horseradish peroxidase-conjugated anti-rabbit IgG (Amersham). Bands were visualized using enhanced chemiluminescence (Amersham).

**Two-dimensional Thin Layer Phosphopeptide Mapping**—This was performed essentially as described (21). Protein Kinase C Phosphorylation of Myristoylated and Nonmyristoylated MARCKS—Ltk° cells stably expressing either wt- or Myr° MARCKS were radiolabeled with [3H]myristic acid and stimulated with 200 nM PMA for the indicated times. The incubations were terminated by washing twice with ice-cold PBS, and the cells were lysed with 0.1% Nonidet P-40 supplemented with the protease inhibitors indicated below. Nuclei were pelleted at 400 × g for 5 min and MARCKS was immunoprecipitated from the postnuclear supernatants using the RIPA method. Bovine immunoprecipitates were separated by 8% SDS-PAGE, and bands were visualized by autoradiography.

**Lysate Labeling**—Cell lysates were prepared from 35-mm dishes of cells labeled with [3H]myristic acid, or [3H]myristate, or [3H]palmitic acid as described (22). Cells were scraped into 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, 15 mM EDTA, 50 mM potassium fluoride, 50 mM NaH2PO4, 10 mM sodium pyrophosphate, and 1% Nonidet P-40) supplemented with the protease inhibitors listed above. Nuclei were pelleted at 400 × g for 5 min and MARCKS was immunoprecipitated from the postnuclear supernatants using the RIPA method.

### RESULTS

Rationale for Mutant Construction—Comparison of known MARCKS sequences revealed two highly conserved regions: the N-terminal half which contains a myristoylation consensus sequence, and a basic effector domain which contains the PKC phosphorylation sites and a calmodulin- and actin-binding site (1, 2) (Fig. 1). To determine the role of myristoylation, the N-terminal glycine, which serves as the myristoyl acceptor, was mutated to alanine (Myr°, Fig. 1). Further investigation of the role of fatty acid acylation in the subcellular targeting of MARCKS, we constructed a chimera in which the myristoylation domain of MARCKS was replaced by the palmitoylation sequence of a related PKC substrate, GAP-43 (19) (G43-M, Fig. 1). To examine the role of phosphorylation, serines 152, 156,
that the G43-M mutant labels only with [3H]palmitic acid. The position of the mutant MARCKS are designated on top. The immunoprecipitates of the stable transfectants labeled with 32Pi and peritoneal macrophages (21). Two-dimensional thermolytic phosphopeptide mapping was performed (Fig. 2A). Phosphopeptide 1, representing Ser-152 and -163 (both are cleaved to FS), has twice the intensity of phosphopeptide 2 (cleaved to FKKKS), which contains Ser-156 (21). The pattern is identical to MARCKS isolated from murine peritoneal macrophages (21). Two-dimensional thermolytic phosphopeptide mapping of all the mutants, except Phos-*, revealed an identical pattern (data not shown).

Subcellular Localization and Stimulus-dependent Redistribution of MARCKS Mutants—In macrophages, neutrophils, neurons, and primary fibroblasts, the phosphorylation of MARCKS is accompanied by its release from the membrane (12, 14, 15, 22). In unstimulated cells, ~95% of wt MARCKS partitioned with the membrane fraction (Fig. 3). Treatment of the cells with PMA, which stimulates PKC, resulted in the phosphorylation of MARCKS (Fig. 2B), and the translocation of ~50% of the protein from the membrane to the cytosol (Fig. 3). The translocated cytosolic protein was phosphorylated with approximately twice the stoichiometry of the membrane-bound protein (Fig. 3), consistent with our previous observation that MARCKS is displaced from membranes upon phosphorylation of all its PKC phosphorylation sites (14). Experiments with the Phos- mutant confirmed this observation: non-phosphorylatable MARCKS is membrane-bound and does not translocate from membrane to cytosol upon PMA treatment (Fig. 3).

Myristoylation is absolutely required for membrane binding of MARCKS since the Myr* mutant was present only in the cytosol (Fig. 3). In addition, the rate of phosphorylation of the Myr* mutant was substantially lower than that of wt MARCKS (Fig. 4), suggesting that MARCKS must be membrane bound, and in close apposition to activated PKC, for efficient phosphorylation.

The role of fatty acid acylation in targeting MARCKS to the membrane was further probed using the G43-M chimera, which contains two palmitic acid residues instead of one myristate at the N terminus. Interestingly, the palmitoylated chimera associated tightly with the membrane, but in contrast to myristoylated MARCKS, was not released from the membrane upon PMA-induced phosphorylation (Figs. 2 and 2B). The small amount of cytosolic G43-M was only labeled with [3H]lysine, and not with [3H]palmitic acid, suggesting that it had been depalmitoylated (Fig. 3).

The contribution of the conserved N terminus to membrane binding and phosphorylation-dependent translocation was examined using the Δ6–140 deletion mutant. This molecule associated with the membrane in unstimulated cells, and did not translocate to the cytosol upon PMA-induced phosphorylation (Fig. 3).

Immunolocalization of MARCKS Mutants—The subcellular localization of the wild type and mutant MARCKS molecules was studied using indirect immunofluorescence microscopy. In quiescent cells, wild type MARCKS had a diffused distribution throughout the plasma membrane (Fig. 5B). This distribution is similar to the staining of endogenous MARCKS in unstimulated mouse embryo fibroblasts (22), suggesting that the transfected cells are a good model system in which to study this protein. No staining of MARCKS was seen when the primary antibody was omitted (data not shown). As with endogenous MARCKS in mouse embryo fibroblasts (22), activation of PKC is accompanied by a redistribution of MARCKS to perinuclear vesicles (Fig. 5D), which were previously identified as lysosomes (22). This translocation of MARCKS occurs via a soluble intermediate (22). Myr* stained diffusely throughout the cytoplasm in both control and PMA-treated cells (Fig. 5 I and J), confirming the biochemical fractionation data which demonstrated that myristoylation is required for membrane binding (Fig. 3). The nuclear staining seen in Fig. 5 I and J (and Fig. 5, G and H), is an artifact which becomes apparent as a result of increased exposure times required to visualize MARCKS when it is not associated with the plasma membrane. The nuclear staining is also seen in mock transfected (pL/NEO) Ltk* cells which do not express MARCKS (data not shown). MARCKS is undetectable by immunoblotting in nuclei purified from Myr* and mock transfected cells (data not shown). Phos*-stained the plasma membrane diffusely, was particularly concentrated in membrane extensions, and was not displaced upon PMA treatment (Fig. 5, E and F). This finding is also consistent with the
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Fig. 3. Subcellular fractionation of MARCKS and its mutants. In the upper panels stable transfectants labeled with [3H]myristic acid (WT, Phos−, Δ6–140), or [3H]lysine (Myr−, G43-M) were left untreated (Con) or exposed to 200 nM PMA for 30 min prior to fractionation. Cells were broken using nitrogen cavitation, and separated into total membranes (M) and cytosol (C) as described under “Experimental Procedures.” MARCKS was immunoprecipitated from each fraction prior to SDS-PAGE. [3H]MARCKS and mutants were visualized by fluorography of En3Hanced gels. In the lower panels cells were labeled with 32P and then stimulated and fractionated as described above.

Fig. 4. Phosphorylation of wild type and nonmyristoylated MARCKS. Ltk− cells stably expressing either wt or Myr− MARCKS were radiolabeled with [32P]orthophosphate and stimulated with 200 nM PMA for the indicated times. The incubations were terminated by the addition of lysis buffer, and MARCKS or Myr− MARCKS were immunoprecipitated as described under “Materials and Methods.” Phosphoproteins were resolved by SDS-PAGE, and phosphorylation levels were quantitated using a PhosphorImager. The data are the average of four independent experiments.

Fractionation data which demonstrate that nonphosphorylatable MARCKS remained associated with the plasma membrane and was not released upon PMA treatment of the cells (Fig. 3), G43-M had a similar distribution to Phos−, and while PMA induced phosphorylation of the chimera (Fig. 2B), it did not significantly alter its intracellular distribution (Fig. 5, K and L). Once again, this reinforces the fractionation data which demonstrated that activation of PKC was not accompanied by significant translocation of G43-M from the membrane to the cytosol (Fig. 3). Δ6–140 demonstrated a reticular staining throughout the cell (Fig. 5, G and H). Since this mutant fractionates quantitatively with the membrane fraction (Fig. 3), it is likely that Δ6–140 is associated with a variety of intracellular membranes. PMA treatment did not influence the subcellular distribution of this molecule (Fig. 5, G and H), although it did increase its phosphorylation (Fig. 2B).

DISCUSSION

MARCKS associates with the plasma membrane of quiescent cells and is released from the membrane to the cytosol upon PKC-mediated phosphorylation (14, 15). Studies with artificial membranes suggest that binding of MARCKS to membranes requires both hydrophobic insertion of its myristoyl chain into the lipid bilayer and electrostatic interaction of its basic domain with acidic lipids (16, 17). PKC-mediated phosphorylation introduces negative charges into the basic effector domain, thereby decreasing the electrostatic interaction, resulting in the release of the protein from the membrane (16, 17). This cycle of membrane binding and release has been termed the myristoyl-electrostatic switch (18). In this report we use a mutational analysis to confirm and extend this model.

The myristoyl-electrostatic switch makes the following predictions, all of which are confirmed by the data presented here. First, myristic acid is necessary but not sufficient for membrane binding since mutation of the N-terminal glycine to alanine prevents myristoylation and abrogates membrane binding (23, 24, 38). Second, phosphorylation introduces phosphate groups into the effector domain which partially neutralizes the electrostatic interaction of MARCKS with the membrane, resulting in its release to the cytosol. Mutation of serines 152, 156, 160, and 163 which are phosphorylated by PKC results in a MARCKS mutant which is neither phosphorylated nor released from the membrane into the cytosol in phorbol ester-stimulated cells. Third, the model predicts that decreasing the distance between the myristic acid moiety and the basic effector domain would increase the membrane binding affinity of the protein (16, 18). This occurs because the overall association constant is the product of the hydrophobic interaction of myristate with the bilayer (Kb) and the electrostatic interaction of the basic effector domain with acidic lipids (Kb), scaled by the distance, r, between them. Thus the overall apparent association constant, K, is:

\[ K = K_d (1 + aK_b r) = K_d aK_b r \]  

(Eq. 1)

where \( a = 4 \text{nm}^{-1} \) is a constant that depends only on the area/ phospholipid (25). Therefore, the insertion of myristate into the bilayer confines the basic effector domain to a hemisphere of radius r, which greatly increases the probability that this basic domain will associate electrostatically with acidic lipids (18). Thus decreasing the distance, r, between the myristoyl moiety and the basic domain will increase the affinity of the protein for the membrane. This prediction is supported by the observation that Δ6–140, a mutant in which the intervening sequence between the myristoyl moiety and the basic effector domain is deleted, is not displaced from the membrane by PKC dependent phosphorylation. There is also a second reason why Δ6–140 associates with membranes more tightly than its wild-type counterpart; amino acids 6–140 are highly acidic and this would have the tendency to both repel the protein from the membrane and neutralize some of the positive charges of the effector domain. This view is supported by the observation that the partition coefficient of the basic domain of the intact pro-
tein onto vesicles containing 20% acidic lipid is about 10,000-fold lower than that obtained with a synthetic peptide derived from the effector domain of MARCKS (16, 26). The final prediction follows from the observation that myristic acid provides barely enough energy to anchor the protein to the bilayer (16, 27–29), allowing for the reversible interaction of the myristoylated protein with the membrane. This appears to be confirmed by experiments demonstrating that the G43-M chimera, which bears two palmitic acids at its N terminus in place of one myristic acid moiety, is not released from the membrane upon phosphorylation. The small amount of cytosolic G43-M appears to have been depalmitoylated since we could not detect any radiolabeled palmitate associated with it. This implies that the membrane binding energy contributed by two palmitic acid moieties is sufficient to anchor the molecule to the membrane without the participation of the basic effector domain. This hypothesis is further supported by the observation that spontaneous desorption of doubly acylated peptides from artificial membranes is much slower than that of their singly acylated counterparts (30).

Why is membrane binding important for the function of MARCKS? MARCKS is phosphorylated by PKC, a kinase which is known to be active at the membrane (31). Thus, membrane binding would place MARCKS in close apposition to activated PKC, thereby facilitating the efficient phosphorylation of the substrate. This appears to be the case. First, MARCKS colocalizes with PKCa on phagosomes of macrophages, as well as in the transient adhesion zones known as podosomes (12, 13). Second, nonmyristoylated, cytosolic, MARCKS is phosphorylated much more slowly than its myristoylated, membrane-bound, counterpart. Since myristoylated and nonmyristoylated MARCKS are similarly phosphorylated in vitro, it appears that MARCKS is more efficiently phosphorylated when membrane bound.

Our data suggest that wt MARCKS must be phosphorylated to high stoichiometry in order to be released to the cytosol. This is consistent with the observation that the related protein, MacMARCKS, which has one less phosphorylation site within its effector domain than MARCKS, is not released from the membrane upon phosphorylation (39). It also explains the apparent anomaly in which phagocytosis in macrophages is accompanied by the phosphorylation of MARCKS and its recruitment to the phagosomal membrane (13). If MARCKS was partially phosphorylated under these conditions, it would be expected to associate with membranes.

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3 J. Ahn and A. Aderem, manuscript in preparation.
In addition to the nonspecific hydrophobic and electrostatic interactions with lipids, there is good evidence that the binding of MARCKS to biological membranes involves specific protein-protein interactions. For example, MARCKS has a punctate distribution in macrophages, and the structures containing MARCKS resemble podosomes, the transient complexes formed at the substrate adherent membrane during locomotion (12). During phagocytosis, MARCKS is concentrated under the forming phagosome, where it colocalizes with PKC 

formed at the substrate adherent membrane during locomotion (12). During phagocytosis, MARCKS is concentrated under the forming phagosome, where it colocalizes with PKC. MARCKS resemble podosomes, the transient complexes distribution in macrophages, and the structures containing protein interactions. For example, MARCKS has a punctate interaction with lipids, there is good evidence that the binding of calcium the myristoyl moiety is occluded within a hydrophobic groove (36, 37). Calcium induces a conformational change in receptor which results in the extrusion of the myristoyl moiety from the hydrophobic pocket and releases it to participate in membrane binding. Thus, an array of myristoyl switch mechanisms appear to facilitate signal transduction by mediating reversible membrane binding.

Acknowledgments—We are grateful to Drs. S. McLaughlin and J. Chen for helpful comments on the manuscript.

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