Biochemical and Biophysical Research Communications **276,** 183–188 (2000)
doi:10.1006/bbrc.2000.3430, available online at http://www.idealibrary.com on IDEAL®

Myristoylation Alters Retinoic Acid-Induced Down-Regulation of MARCKS in Immortalized Hippocampal Cells

Le Wang,* David G. Watson,† and Robert H. Lenox*,‡,§,[1]

*Department of Psychiatry, ‡Department of Pharmacology, and §Department of Neuroscience, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104; and †University of Florida College of Pharmacy, Gainesville, Florida

Received August 8, 2000

The *m*yristoylated *a*lanine-*r*ich *C k*inase *s*ubstrate (MARCKS) is a prominent PKC-substrate in the brain, which has been implicated in brain development, cytoskeletal remodeling, calcium/calmodulin signaling, and neuroplasticity. The sequence of the *Macs* gene codes for a protein that has three highly conserved domains including a 5′ myristoylation region and a 25-amino-acid phosphorylation site domain (PSD), which are involved in anchoring MARCKS to the cellular membrane. In this study, we examined the role of the myristoylation signal in the regulation of MARCKS in transfected rat hippocampal cells (H19-7) following retinoic acid (RA) treatment. A mutant MARCKS lacking the myristoylation signal was engineered by substitution of alanine for glycine at position 2 of the *Macs* gene and was found to be exclusively expressed in the cytosol fraction of transfected cells. Exposure of the wild-type MARCKS-transfected cells to RA resulted in an apparent shift of MARCKS from the membrane to the cytosol, while the total protein of wild-type MARCKS was not significantly changed. In contrast, RA-exposed cells transfected with the mutant MARCKS revealed a dramatic reduction of expression of MARCKS protein in both cytosol and total protein fractions. These data suggest that the absence of the myristoyl moiety may not only alter the anchoring of the protein to the membrane but also play a novel role in modulating cellular levels of MARCKS protein in response to RA. © 2000 Academic Press

This work was supported by the National Institutes of Health Grant RO1 MH56247-01 (R.H.L).

Abbreviations used: MARCKS, *m*yristoylated *a*lanine-*r*ich *C k*inase substrate; RA, retinoic acid; H19-7, immortalized hippocampal cells; HN33, fusion of primary hippocampal neurons with the N18TG2 neuroblastoma cells; CMV, cytomegalovirus; G2A, nonmyristoylatable mutant MARCKS.

[1] To whom correspondence should be addressed at Department of Psychiatry, University of Pennsylvania, 549 Clinical Research Building, 415 Curie Boulevard, Philadelphia, PA 19104. Fax: (215) 573-6382. E-mail: rlenox@mail.med.upenn.edu.

Phosphorylation of intracellular substrates by protein kinase C (PKC) results in a wide range of cellular processes including differentiation, mitogenesis, and neurosecretion (see review (1)). The *m*yristoylated *a*lanine-*r*ich *C k*inase substrate (MARCKS) is a prominent protein kinase substrate which binds to calcium/calmodulin and filamentous actin (2), has been shown to be essential for the development of the central nervous system (3–5) and has been implicated in cytoskeletal restructuring (see review (6)) and neuroplasticity in brain (7–9). MARCKS is an acidic rod-shaped protein characterized by three highly conserved domains: a N-terminal myristoylation domain, the MH2 domain of unknown function, and the phosphorylation site domain (PSD) (2, 6). N-terminal myristoylation is one of the major cotranslational modifications of various proteins in signal transduction pathways, and takes place at the amino terminus resulting in attachment of a 14-carbon fatty acid myristate through an amide bond to Gly^2 following cleavage of the initiator methionine (10, 11). Some myristoylated proteins may undergo a signal-induced conformational change that exposes the myristoyl moiety and allows binding of these proteins to membranes (12, 13). This phenomenon has now evolved as the concept of a myristoyl switch. In the case of MARCKS, phosphorylation/dephosphorylation events might be the signals that trigger such a myristoyl switch, causing the protein to be shuttled off and on the membrane (14). Mutation of the N-terminal glycine results in a nonmyristoylatable MARCKS which does not bind to membrane, and is phosphorylated at the same sites as the wild-type (15), albeit less efficiently in intact cells (16). This indicates that myristic acid targets MARCKS to the membrane, where it is efficiently phosphorylated by PKC. *In vivo* nonmyristoylatable MARCKS exhibits most of normal physiological functions possessed

0006-291X/00 $35.00

by myristoylated MARCKS (17); however, the role of myristoylation in the regulation of MARCKS is not well defined.

Our laboratory has shown that MARCKS is regulated in an immortalized hippocampal cell line (HN33) by retinoic acid (RA), phorbol ester (PMA), and mood stabilizers (lithium and valproate) used for the treatment of bipolar disorder (18–20). Lithium- and valproate-induced down-regulation of MARCKS expression is thought to utilize PKC-mediated pathways similar to that observed following phorbol ester exposure (21). The expression of endogenous MARCKS in HN33 cells was regulated differently following treatment with either PMA or RA (18). While PMA induced a down-regulation of MARCKS in both cytosol and membrane fractions, RA reduced MARCKS preferentially in the membrane fraction of the cells, with only a small reduction noted in the cytosol fraction (18). These findings suggested that the RA-induced reduction of membrane-associated endogenous MARCKS may be primarily related to the dissociation of MARCKS from the membrane through a myristoyl switch. In order to gain further insight into the role of myristoylation in the regulation of MARCKS following RA exposure, we have studied the expression of recombinant wild-type MARCKS as well as mutant MARCKS lacking the myristoylation signal in a transfected rat hippocampal cell line (H19-7) that was immortalized by retroviral transduction of temperature-sensitive Simian Virus 40 large tumor antigen (22) and does not express endogenous MARCKS at 33°C (23). In spite of the similarity in function between myristoylated and nonmyristoylated MARCKS (17), we observed a myristoylation-dependent RA-induced down-regulation of MARCKS. Our data suggest that N-terminal myristoylation may play a novel role in modulating cellular levels of MARCKS protein in response to RA exposure.

MATERIALS AND METHODS

Cell culture. The rat hippocampal H19-7 are clonal cells derived from embryonic hippocampal cells and immortalized by retroviral transduction of temperature-sensitive Simian Virus 40 large tumor antigen (22). H19-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL, Grand Island, NY) at 33°C, and used for transfection assays. The mouse immortalized hippocampal cell line HN33 was derived from the fusion of primary mouse hippocampus neurons of the postnatal day 21 mice with the N18TG2 neuroblastoma cell line (24). HN33 cells were grown in DMEM containing 5% FBS at 37°C, and used for detection of endogenous MARCKS.

DNA construct and site-directed mutagenesis. A full-length murine MARCKS cDNA (a gift of Dr. A. Aderem, Rockefeller University, NY) (25) was inserted into pcDNA3.1/Zeo (Invitrogen, Carlsbad, CA), a mammalian expression vector driven by cytomegalovirus (CMV) promoter. The wild-type MARCKS construct is called pcDMum. Site-directed mutagenesis of pcDMum was accomplished by a PCR method, using a pair of primers in which the upstream primer (GAA GAA GGT ACC *ATG* G**C**T GCC CAG) has a single base change in the second codon of *Macs* gene (G**G**T/gly → G**C**T/ala) and the downstream primer is identical to the 3′-end of MARCKS cDNA sequence before stop codon. The resulting construct encodes a nonmyristoylatable MARCKS called pcDMum/G2A. The sequences of all the DNA constructs were confirmed by DNA sequencing.

Cell transfection. 1 μg of the purified MARCKS plasmid DNA was mixed with 6 μl of lipofectAMINE reagent (Gibco-BRL, Grand Island, NY) in 600 μl DMEM without FBS, and added to a 60-mm dish seeded with approximately 1.5×10^6 H19-7 cells the day before. The transfected cells were grown in DMEM and split into five new dishes on the next day. After 48 h, 250 μg/ml zeocin (Invitrogen, Carlsbad, CA) was added. Zeocin-resistant clones were then selected and tested for the expression of recombinant MARCKS by Western blotting as described below.

Subcellular fractionation. Cells were treated with 10 μM RA in 70% ethanol or the same concentration of ethanol as controls for 3 days, scraped into a homogenization buffer (20 mM Hepes, pH 7.4, 2 mM EGTA, 1 mM PMSF, 2 mM DTE, and 10 μg/ml aprotinin), and sonicated for 30 sec on ice. The resulting homogenates were subjected to ultracentrifugation at 100,000*g* for 60 min at 4°C. The supernatants were collected as cytosol fractions. The pellets were resuspended in a buffer containing 0.1% Triton X-100 and solubilized for 30 min. Solubilized fractions were then centrifuged at 30,000*g* for 30 min at 4°C and the supernatants containing the solubilized membrane protein were collected as membrane fractions. Total homogenates were generated by treatment with 0.1% Triton for 45 min, and followed by centrifugation at 100,000*g* for 30 min at 4°C. The resulting supernatant was taken as the total protein fraction. The protein concentration was determined by Bradford method (Bio-Rad, Hercules, CA).

Western blot analysis. Proteins from SDS–PAGE were transferred to polyvinylidine fluoride membrane (Millipore Corp., Bedford, MA). The membranes containing immobilized proteins were blocked with 5% skim milk in TS buffer (20 mM Tris, pH 7.5, 0.5 M NaCl) and incubated with a polyclonal rabbit antibody to mouse MARCKS at room temperature. After washes, the membranes were incubated with a goat anti-rabbit IgG HRP conjugated antibody as the secondary antibody in TS buffer. Immunoreactive bands were visualized by a standard ECL procedure and quantitated by densitometry scan (Bio Rad, Model GS-700 imaging densitometer). The data are expressed as % of MARCKS in untreated controls and represent a mean of 3–5 individual experiments.

RESULTS AND DISCUSSION

N-terminal myristoylation is a key event for the association of certain proteins to the membrane leading to the subsequent stable anchorage of the protein (10, 11, 26). For MARCKS, the protein is attached to membrane by hydrophobic interactions via myristoyl group as well as by electrostatic interaction via PSD region (15, 27–30). The purpose of this study is to assess whether the myristoyl moiety plays a role in the regulation of MARCKS using stable transfection of immortalized hippocampal cells with both recombinant wild-type MARCKS and mutant MARCKS lacking the myristoylation signal. Unlike the HN33 cells, H19-7 cells lack endogenous MARCKS expression at 33°C (23). After transfection with recombinant MARCKS cDNA, the transfected H19-7 cells were able to express constitutively either wild-type or mutant MARCKS. Stably transfected H19-7 cells that expressed either wild-type MARCKS or G2A mutant MARCKS were

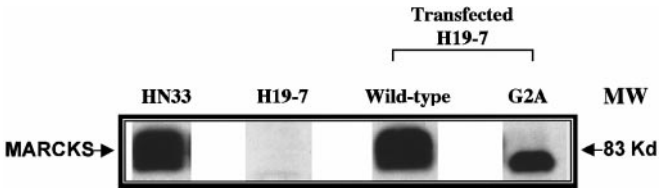

FIG. 1. Expression of wild-type and mutant MARCKS lacking the myristoylation signal in transfected H19-7 cells at 33°C. Panel 1 shows the endogenous MARCKS present in HN33 cells. Panel 2 shows an untransfected H19-7 cell that does not express endogenous MARCKS at 33°C. Panels 3 and 4 present the transfected H19-7 cells that express wild-type MARCKS and mutant MARCKS lacking the myristoylation signal (G2A), respectively.

selected for this study. The SDS–PAGE gels showed that the expressed recombinant wild-type MARCKS co-migrated with the endogenous MARCKS present in HN33 cells, while the mutant MARCKS lacking the myristoylation signal migrated slightly faster than the corresponding myristoylated form (Fig. 1). This increase in SDS–PAGE mobility of G2A mutant is likely due to the lack of myristoyl moiety in the molecule (31). In addition, the expression level of wild-type MARCKS in the stably transfected H19-7 cells appears to be higher than that of G2A mutant MARCKS. This phenomenon may be related to the different number of MARCKS construct copies integrated into the chromosome or maintained as stable episomal plasmids in the transfected cells.

Since the G2A mutation (an amino-terminal substitution of alanine for glycine) results in the synthesis of nonmyristoylatable MARCKS protein (15), little or no G2A mutant protein was thus detected in the membrane fraction of the transfected cells (Fig. 2A), consistent with myristoylation acting as an intracellular membrane-associating signal. Similar to our previous finding that RA exposure resulted in a reduction of membrane associated endogenous MARCKS in HN33 hippocampal cell line (18), we observed a significant RA-induced reduction of recombinant wild-type MARCKS in the membrane fraction of the transfected SV40 immortalized H19-7 hippocampal cells (Fig. 2), which are otherwise devoid of endogenous MARCKS expression. The level of the recombinant wild-type MARCKS in the cytosol fraction remained statistically unchanged although there was a trend in a number of experiments for a modest increase (Fig. 3A), suggestive of a shift of MARCKS from membrane to cytosol. As a consequence, the wild-type MARCKS in total protein fraction was only slightly decreased (Fig. 4), consistent with the relatively small contribution of membrane MARCKS to the total protein fraction similar to that observed in HN33 cells (18). Thus, these data reinforce the suggestion that RA-induced reduction of membrane-associated wild-type MARCKS may be primarily attributable to an altered distribution between membrane and cytosol.

In contrast, Fig. 4 shows that the mutant MARCKS lacking the myristoylation signal was significantly reduced in the total protein fractions of the transfected H19-7 cells (34.5 ± 5% of MARCKS in untreated controls) as compared to the wild-type MARCKS (96 ± 8% of MARCKS in untreated controls). These data suggest that the presence of the myristoyl moiety may be playing a role in protecting MARCKS protein from the RA-induced degradation. There are at least two possibilities: (i) cytosolic MARCKS may be more accessible to cellular proteolytic enzymes than its membrane-MARCKS; and/or (ii) G2A mutation (or the absence of the myristoyl moiety) may induce a conformational change in the N-terminus of the MARCKS protein which increases the vulnerability of MARCKS to cellular proteolytic enzymes. In the first case, both wild-type and G2A mutant MARCKS in cytosol fractions should have been reduced to a similar extent after RA treatment. However, the G2A mutant MARCKS underwent a dramatic reduction in both the cytosol and total protein fractions, while there was little evidence for a change in the wild-type MARCKS in either cell fraction (Figs. 3 and 4). These findings suggest that the altered protein conformation induced by the absence of myristoyl moiety, rather than the accessibility of the protein in cytosol fraction to proteolytic apparatus,

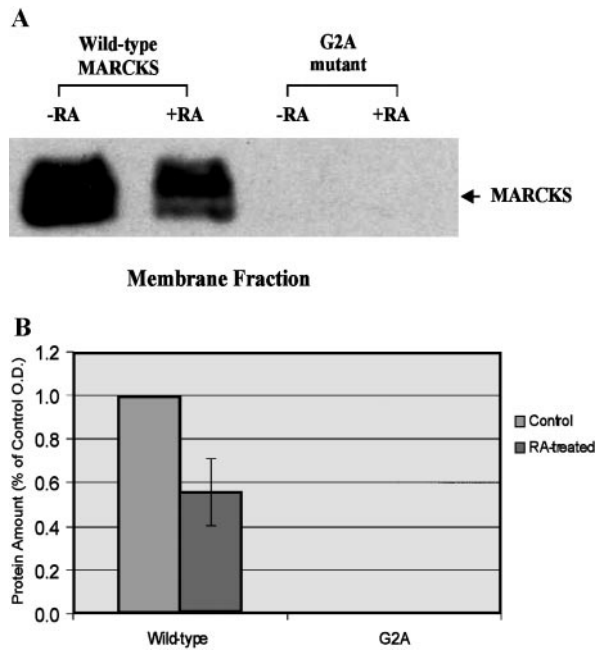

FIG. 2. Absence of G2A mutant MARCKS in the membrane fraction of the transfected H19-7 cells. (A) A representative Western blot of wild-type and G2A mutant MARCKS in membrane fractions of the transfected H19-7 cells exposed to 10 μM RA for 3 days. Note that G2A mutant MARCKS was rarely detected in the membrane fractions. (B) Quantitation of membrane-associated MARCKS immunoreactive bands by densitometry scan. Data represent mean ± SEM from three independent experiments, and are expressed as % of MARCKS in untreated controls ($P < 0.05$ using Student's t test).

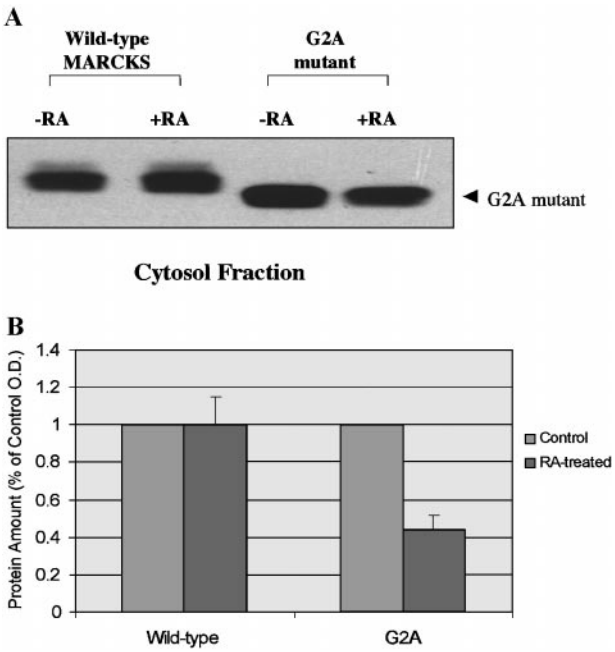

FIG. 3. Effect of RA on the levels of wild-type MARCKS and G2A mutant MARCKS in the cytosol fractions of the transfected H19-7 cells. (A) A representative Western blot of wild-type and G2A MARCKS in cytosol fractions of the transfected H19-7 cells exposed to 10 μM RA for 3 days with a MARCKS-specific antibody. (B) Quantitation of MARCKS immunoreactive bands by densitometry scan. Data represent mean ± SEM from three independent experiments, and are expressed as % of MARCKS in untreated controls ($P < 0.05$ using Student's t test).

may play a critical role in promoting the degradation of G2A mutant protein after RA treatment. Since the two constructs used in this study (pcDMum and pcDMum/G2A) are devoid of both 5′- and 3′-flanking sequences of the *Macs* gene and are driven only by the constitutive CMV promoter which is not repressed by RA (32, 33), transcriptional changes in mRNA are highly unlikely. Furthermore, examination of RA-treated HN33 cells did not indicate any down-regulation of endogenous MARCKS mRNA (data not shown). Hence, the difference observed in recombinant MARCKS protein expression likely resulted from the difference in their posttranslational regulation. While the definitive determination will require further experiments including the measurement of the rates of protein turnover for both wild-type and mutant MARCKS, existing studies have shown that the proteolytic cleavage of MARCKS protein may be associated with targeting of the protein to the lysosomal pathway (34, 35). Based on our data, we suggest that the absence of the myristoyl moiety may alter the conformation of MARCKS protein leading to enhanced nonmyristoylated MARCKS instability in response to RA.

RA is well known to promote differentiation and inhibit growth of many cell types and tumors (see reviews (36 and 37)). For example, RA induces differentiation of acute promyelocytic leukemia (APL) cells and causes partial differentiation of human neuroblastoma cells towards a neuronal phenotype (38, 39). Many of these actions are mediated by transcriptional effects through nuclear RA receptors known as RARs located in the 5′ promotor regions by which RA activates or represses target genes (see review (40)). More recently, RA has also been shown to mediate nontranscriptional regulation of protein expression following completion of polypeptide synthesis. Some RA-induced nongenomic properties include enhancement of retinoylation (41) of proteins in many eukaryotic cell lines. On the other hand, studies have shown enhancement of ubiquitination and proteolysis of proteins such as cyclin D1 in tumor and immortalized human cell lines after RA treatment (42, 43). Myristoylation is essential for proper functioning of a number of proteins. In addition to its role in membrane association, protein myristoylation has also been shown to be involved in protein–protein interaction, which can be further regulated by the interplay between protein phosphorylation, calmodulin binding, and membrane phospholipids (44). In this study, we have demonstrated for the first time that myristoylation of MARCKS may posttranslationally regulate the expression of the MARCKS protein in immortalized hippocampal cells during RA

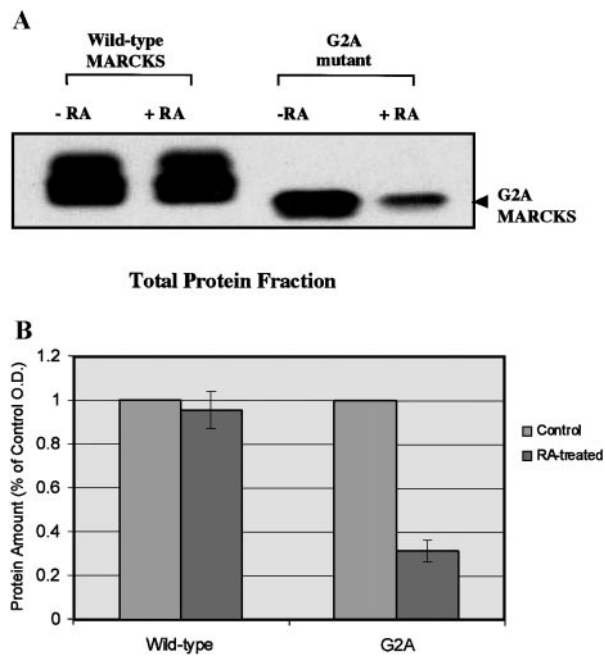

FIG. 4. Retinoic acid induces a marked down-regulation of G2A mutant MARCKS lacking the myristoylation signal in total protein fraction of the transfected H19-7 cells. (A) A representative Western blot of wild-type and G2A MARCKS in total protein fractions of the transfected H19-7 cells exposed to 10 μM RA for 3 days with a MARCKS-specific antibody. (B) Quantitation of MARCKS immunoreactive bands by densitometry scan. Data represent mean ± SEM from three independent experiments, and are expressed as % of MARCKS in untreated controls ($P < 0.001$ using Student's t test).

treatment. This process may be integral to activity-dependent regulation of cytoskeletal restructuring associated with a role for MARCKS in cellular processes such as transformation, differentiation, and neuroplasticity in the brain (5, 7, 8).

ACKNOWLEDGMENTS

We thank Dr. Richard Schnizer for his site-directed mutagenesis work and Ms. Kelly Schraufnagel for her excellent technical assistance. We are grateful to Drs. Michael Bubb and Robert McNamara for helpful comments on the manuscript. These studies were supported in part through NIMH RO1 56247 and a NARSAD Distinguished Investigator Award (R.H.L).

REFERENCES

1. Tanaka, C., and Nishizuka, Y. (1994) The protein kinase C family for neuronal signaling. *Annu. Rev. Neurosci.* **17,** 551–567.
2. Blackshear, P. J. (1993) The MARCKS family of cellular protein kinase C substrates. *J. Biol. Chem.* **268,** 1501–1504.
3. Blackshear, P. J., Lai, W. S., Tuttle, J. S., Stumpo, D. J., Kennington, E., Nairn, A. C., and Sulik, K. K. (1996) Developmental expression of MARCKS and protein kinase C in mice in relation to the exencephaly resulting from MARCKS deficiency. *Brain Res. Dev. Brain. Res.* **96,** 62–75.
4. Stumpo, D. J., Bock, C. B., Tuttle, J. S., and Blackshear, P. J. (1995) MARCKS deficiency in mice leads to abnormal brain development and perinatal death. *Proc. Natl. Acad. Sci. USA* **92,** 944–948.
5. McNamara, R. K., and Lenox, R. H. (1998) Distribution of the protein kinase C substrates MARCKS and MRP in the postnatal developing rat brain. *J. Comp. Neurol.* **397,** 337–356.
6. Aderem, A. (1992) *in* Cell, pp. 713–716.
7. McNamara, R. K., Stumpo, D. J., Morel, L. M., Lewis, M. H., Wakeland, E. K., Blackshear, P. J., and Lenox, R. H. (1998) Effect of reduced myristoylated alanine-rich C kinase substrate expression on hippocampal mossy fiber development and spatial learning in mutant mice: Transgenic rescue and interactions with gene background. *Proc. Natl. Acad. Sci. USA* **95,** 14517–14522.
8. McNamara, R. K., Y. J., Streit, W. J., and Lenox, R. H. (2000) Facial motor neuron regeneration induces a unique spatial and temporal pattern of myristoylated alanine-rich C kinase substrate expression. *Neuroscience,* in press.
9. Ramakers, G. M., McNamara, R. K., Lenox, R. H., and De Graan, P. N. (1999) Differential changes in the phosphorylation of the protein kinase C substrates myristoylated alanine-rich C kinase substrate and growth-associated protein-43/B-50 following Schaffer collateral long-term potentiation and long-term depression. *J Neurochem.* **73,** 2175–2183.
10. Mumby, S. M., Heukeroth, R. O., Gordon, J. I., and Gilman, A. G. (1990) G-protein alpha-subunit expression, myristoylation, and membrane association in COS cells. *Proc. Natl. Acad. Sci. USA* **87,** 728–732.
11. Jones, T. L., Simonds, W. F., Merendino, J. J., Jr., Brann, M. R., and Spiegel, A. M. (1990) Myristoylation of an inhibitory GTP-binding protein alpha subunit is essential for its membrane attachment. *Proc. Natl. Acad. Sci. USA* **87,** 568–572.
12. Randazzo, P. A., Terui, T., Sturch, S., Fales, H. M., Ferrige, A. G., and Kahn, R. A. (1995) The myristoylated amino terminus of ADP-ribosylation factor 1 is a phospholipid- and GTP-sensitive switch. *J. Biol. Chem.* **270,** 14809–14815.
13. Zozulya, S., and Stryer, L. (1992) Calcium-myristoyl protein switch. *Proc. Natl. Acad. Sci. USA* **89,** 11569–11573.
14. McLaughlin, S., and Aderem, A. (1995) The myristoyl-electrostatic switch: A modulator of reversible protein- membrane interactions. *Trends Biochem. Sci.* **20,** 272–276.
15. Graff, J. M., Gordon, J. I., and Blackshear, P. J. (1989) Myristoylated and nonmyristoylated forms of a protein are phosphorylated by protein kinase C. *Science* **246,** 503–506.
16. Seykora, J. T., Myat, M. M., Allen, L. A. H., Ravetch, J. V., and Aderem, A. (1996) Molecular determinants of the myristoyl-electrostatic switch of MARCKS. *J. Biol. Chem.* **271,** 18797–18802.
17. Swierczynski, S. L., Siddhanti, S. R., Tuttle, J. S., and Blackshear, P. J. (1996) Nonmyristoylated MARCKS complements some but not all of the developmental defects associated with MARCKS deficiency in mice. *Dev. Biol.* **179,** 135–147.
18. Watson, D. G., Wainer, B. H., and Lenox, R. H. (1994) Phorbol ester- and retinoic acid-induced regulation of the protein kinase C substrate MARCKS in immortalized hippocampal cells. *J. Neurochem.* **63,** 1666–1674.
19. Watson, D. G., and Lenox, R. H. (1996) Chronic lithium-induced down-regulation of MARCKS in immortalized hippocampal cells: Potentiation by muscarinic receptor activation. *J. Neurochem.* **67,** 767–777.
20. Watson, D. G., Watterson, J. M., and Lenox, R. H. (1998) Sodium valproate down-regulates the myristoylated alanine-rich C kinase substrate (MARCKS) in immortalized hippocampal cells: A property of protein kinase C-mediated mood stabilizers. *J. Pharmacol. Exp. Ther.* **285,** 307–316.
21. Manji, H. K., and Lenox, R. H. (1999) Ziskind-Somerfeld Research Award. Protein kinase C signaling in the brain: Molecular transduction of mood stabilization in the treatment of manic-depressive illness. *Biol. Psych.* **46,** 1328–1351.
22. Eves, E. M., Tucker, M. S., Roback, J. D., Downen, M., Rosner, M. R., and Wainer, B. H. (1992) Immortal rat hippocampal cell lines exhibit neuronal and glial lineages and neurotrophin gene expression. *Proc. Natl. Acad. Sci. USA* **89,** 4373–4377.
23. Watson, D. G., Malhotra, J. K., Eves, E. M and Lenox, R. H. (1994) Regulation of the PKC substrate MARCKS in retrovirus transduced hippocampal cells: Effect of lithium and temperature induced differentiation. *Soc.Neurosci. Abstr.* **20,** 1318.
24. Lee, H. J., Hammond, D. N., Large, T. H., Roback, J. D., Sim, J. A., Brown, D. A., Otten, U. H., and Wainer, B. H. (1990) Neuronal properties and trophic activities of immortalized hippocampal cells from embryonic and young adult mice. *J. Neurosci.* **10,** 1779–1787.
25. Seykora, J. T., Ravetch, J. V., and Aderem, A. (1991) Cloning and molecular characterization of the murine macrophage "68- kDa" protein kinase C substrate and its regulation by bacterial lipopolysaccharide. *Proc. Natl. Acad. Sci. USA* **88,** 2505–2509.
26. Wang, Y., Windh, R. T., Chen, C. A., and Manning, D. R. (1999) N-Myristoylation and betagamma play roles beyond anchorage in the palmitoylation of the G protein alpha(o) subunit. *J. Biol. Chem.* **274,** 37435–37442.
27. James, G., and Olson, E. N. (1989) Myristoylation, phosphorylation, and subcellular distribution of the 80- kDa protein kinase C substrate in BC3H1 myocytes. *J. Biol. Chem.* **264,** 20928–20933.
28. George, D. J., and Blackshear, P. J. (1992) Membrane association of the myristoylated alanine-rich C kinase substrate (MARCKS) protein appears to involve myristate-dependent binding in the absence of a myristoyl protein receptor. *J. Biol. Chem.* **267,** 24879–24885.
29. Kim, J., Shishido, T., Jiang, X., Aderem, A., and McLaughlin, S. (1994) Phosphorylation, high ionic strength, and calmodulin re-

verse the binding of MARCKS to phospholipid vesicles. *J. Biol. Chem.* **269,** 28214–28219.

30. Vergeres, G., Manenti, S., Weber, T., and Sturzinger, C. (1995) The myristoyl moiety of myristoylated alanine-rich C kinase substrate (MARCKS) and MARCKS-related protein is embedded in the membrane [published erratum appears in *J. Biol. Chem.* **270**(47), 28494, 1995]. *J. Biol. Chem.* **270,** 19879–19887.
31. Swierczynski, S. L., and Blackshear, P. J. (1995) Membrane association of the myristoylated alanine-rich C kinase substrate (MARCKS) protein. Mutational analysis provides evidence for complex interactions. *J. Biol. Chem.* **270,** 13436–13445.
32. Ghazal, P., DeMattei, C., Giulietti, E., Kliewer, S. A., Umesono, K., and Evans, R. M. (1992) Retinoic acid receptors initiate induction of the cytomegalovirus enhancer in embryonal cells. *Proc. Natl. Acad. Sci. USA* **89,** 7630–7634.
33. Angulo, A., Suto, C., Heyman, R. A., and Ghazal, P. (1996) Characterization of the sequences of the human cytomegalovirus enhancer that mediate differential regulation by natural and synthetic retinoids. *Mol. Endocrinol.* **10,** 781–793.
34. Spizz, G., and Blackshear, P. J. (1997) Identification and characterization of cathepsin B as the cellular MARCKS cleaving enzyme. *J. Biol. Chem.* **272,** 23833–23842.
35. Allen, L. A., and Aderem, A. (1995) Protein kinase C regulates MARCKS cycling between the plasma membrane and lysosomes in fibroblasts. *EMBO J.* **14,** 1109–1120.
36. Love, J. M., and Gudas, L. J. (1994) Vitamin A, differentiation and cancer. *Curr. Opin. Cell. Biol.* **6,** 825–831.
37. Evans, T. R., and Kaye, S. B. (1999) Retinoids: Present role and future potential. *Br. J. Cancer* **80,** 1–8.
38. Cheung, W. M., and Ip, N. Y. (1998) Interaction of neurotrophins and retinoic acid in neuronal differentiation. *Mol. Cells* **8,** 496–502.
39. Preis, P. N., Saya, H., Nadasdi, L., Hochhaus, G., Levin, V., and Sadee, W. (1988) Neuronal cell differentiation of human neuroblastoma cells by retinoic acid plus herbimycin A. *Cancer Res.* **48,** 6530–6534.
40. Minucci, S., and Ozato, K. (1996) Retinoid receptors in transcriptional regulation. *Curr. Opin. Genet. Dev.* **6,** 567–574.
41. Takahashi, N., and Breitman, T. R. (1990) Retinoylation of HL-60 proteins. Comparison to labeling by palmitic and myristic acids. *J. Biol. Chem.* **265,** 19158–19162.
42. Langenfeld, J., Kiyokawa, H., Sekula, D., Boyle, J., and Dmitrovsky, E. (1997) Posttranslational regulation of cyclin D1 by retinoic acid: A chemoprevention mechanism. *Proc. Natl. Acad. Sci. USA* **94,** 12070–12074.
43. Spinella, M. J., Freemantle, S. J., Sekula, D., Chang, J. H., Christie, A. J., and Dmitrovsky, E. (1999) Retinoic acid promotes ubiquitination and proteolysis of cyclin D1 during induced tumor cell differentiation. *J. Biol. Chem.* **274,** 22013–22018.
44. Taniguchi, H. (1999) Protein myristoylation in protein–lipid and protein–protein interactions. *Biophys. Chem.* **82,** 129–137.