1996

MacMARCKS and the Protein Kinase C Signal Transduction Pathway: Role in Neural Secretion and Neural Development

Sandy Chang
MacMARCKS And The Protein Kinase C
Signal Transduction Pathway:
Role In Neural Secretion And Neural Development

Sandy Chang

1996

A thesis submitted to the faculty of The Rockefeller University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
c. Copyright Sandy Chang 1996
DEDICATION

This thesis is dedicated to my father, who instilled in me a love of learning,
and to my mother, who nurtured this love.

Men and women are not content to comfort themselves with tales of gods and
giants, or to confine their thoughts to the daily affairs of life; they also build
telescopes and satellites and accelerators, and sit at their desks for endless hours
working out the meaning of the data they gather. The effort to understand the
universe is one of the very few things that lifts human life above the level of
farce, and gives it some of the grace of tragedy.

Steven Weinberg, 1977, *The First Three Minutes*
ACKNOWLEDGMENTS

Many people helped me complete this thesis, and I am indebted to workers from several labs. I am particularly grateful to my advisor Alan Aderem, who gave me a chance to study a fascinating protein in a stimulating environment. His unflagging support gave me the confidence to pursue unconventional ideas and to make my own mistakes, which have greatly contributed to my development as a scientist. Alan’s contagious enthusiasm and insights into difficult scientific problems continue to inspire me. I look forward to productive interactions with him in the future.

I wish to thank my colleagues in the Aderem lab for their camaraderie and help over the years. Jianmin Chen constructed the MacMARCKS targeting construct and was instrumental in generating the MacMARCKS knockout mice. I will remember the many hours we spent learning and understanding the phenotype of the mutant embryos. Jianmin has been a great friend and collaborator, and his crazy sense of humor has made the lab a fun place to work. Monn Monn Myat helped me analyze the mutant PC12 cells. Although our interactions were often tempestuous, I will always treasure the unique friendship we developed throughout the years. Nurin Veis helped me with the in vitro analysis of the MacMARCKS promoter, and kept my spirits up during difficult times. A special thanks goes to Naomi Morrissette, who devoted heroic efforts in helping me finish my thesis. I am grateful to her for making the past 3 months tolerable. I want to thank Lee-Ann Allen for editing my thesis and for providing valuable technical advises, Jennifer Darnell for help with microglia purification, Paula Yeghiayan for expert technical
assistance, J. C. Ahn for allowing me to complain about the hapless Knicks, and Penelope Anderson for all her help and emotional support during these past four years.

My collaborators Hugh C. Hemmings, Jr. and members of his lab, Anna Adamo and Kamala Murugaiah, helped with synaptosome purifications and $^3$H-NE release assays. Ray Birge performed the PC12 cell transfections. Steve Duncan cut most of the early embryo sections, and James Okano in Bob Darnell’s lab helped cut brain sections. I am indebted to Gord Fishell for his help with the analysis of the MacMARCKS null embryos, and to Pietro De Camilli for providing valuable reagents and advice. I want to thank my thesis committee members Nat Heintz, Bob Darnell and Hugh C. Hemmings, Jr. for their advice and help over the years, and for reading my thesis in such short time.

My dear friends Margie, Chen, and Anna have done their best to get me away from the lab, and their friendship have helped me keep things in perspective. My observing partner Michael O’Gara has kept me sane by taking me out under the stars to enjoy the faint glow of galaxies. Finally, I want to thank my family, especially my mom, for all her support and love during a much overextended graduate career.
# TABLE OF CONTENTS:

<table>
<thead>
<tr>
<th>Section</th>
<th>Page #</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES &amp; TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>1</td>
</tr>
<tr>
<td><strong>CHAPTER ONE: INTRODUCTION</strong></td>
<td>2</td>
</tr>
<tr>
<td>SIGNAL TRANSDUCTION AND THE CYTOSKELETON</td>
<td></td>
</tr>
<tr>
<td>THE CYTOSKELETON AND DEVELOPMENT</td>
<td></td>
</tr>
<tr>
<td>DEVELOPMENT AND SIGNAL TRANSDUCTION</td>
<td></td>
</tr>
<tr>
<td>MARCKS AND MacMARCKS INTEGRATE SIGNAL</td>
<td></td>
</tr>
<tr>
<td>TRANSDUCTION AND THE ACTIN CYTOSKELETON</td>
<td></td>
</tr>
<tr>
<td><strong>CHAPTER TWO: MATERIALS AND METHODS</strong></td>
<td>25</td>
</tr>
<tr>
<td>REAGENTS</td>
<td></td>
</tr>
<tr>
<td>CELL CULTURE</td>
<td></td>
</tr>
<tr>
<td>BIOCHEMICAL AND MORPHOLOGICAL TECHNIQUES</td>
<td></td>
</tr>
<tr>
<td>FUNCTIONAL ASSAYS</td>
<td></td>
</tr>
<tr>
<td>MOLECULAR BIOLOGY TECHNIQUES</td>
<td></td>
</tr>
<tr>
<td>GENERATION OF LACZ TRANSGENIC MICE</td>
<td></td>
</tr>
<tr>
<td>GENERATION OF MacMARCKS NULL MICE</td>
<td></td>
</tr>
<tr>
<td><strong>CHAPTER THREE: CHARACTERIZATION OF MacMARCKS IN SYNAPTOSOMES AND PC12 CELLS</strong></td>
<td>47</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>RESULTS</td>
<td></td>
</tr>
<tr>
<td>DISCUSSION</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER FOUR: A DOMINANT NEGATIVE MacMARCKS MUTATION BLOCKS EVOKED EXOCYTOSIS OF PC12 CELLS

INTRODUCTION
RESULTS
DISCUSSION

CHAPTER FIVE: REGULATION OF THE MacMARCKS GENE IN MACROPHAGES

INTRODUCTION
RESULTS
DISCUSSION

CHAPTER SIX: EXPRESSION OF THE MacMARCKS GENE DURING MOUSE DEVELOPMENT

INTRODUCTION
RESULTS
DISCUSSION

CHAPTER SEVEN: MacMARCKS IS REQUIRED FOR NORMAL BRAIN DEVELOPMENT IN THE MOUSE EMBRYO

INTRODUCTION
RESULTS
DISCUSSION

EXTENDED DISCUSSION

BIBLIOGRAPHY
LIST OF FIGURES AND TABLES:

Figure 1-1. Domain structure and proposed function of MARCKS and MacMARCKS. 14
Figure 3-1. MacMARCKS is expressed and phosphorylated in PC12 cells. 51
Figure 3-2. MacMARCKS is phosphorylated upon depolarization of PC12 cells. 53
Figure 3-3. MacMARCKS and synaptophysin colocalize in NGF-differentiated PC12 cells. 56
Figure 3-4. PKC agonists or depolarization induce the phosphorylation of MacMARCKS. 59
Figure 3-5. Subcellular distribution of MacMARCKS, MARCKS, synaptophysin, and synapsin 1. 61
Figure 4-1. Characterization of wt-HA and S-A-HA MacMARCKS in PC12 cells. 72
Figure 4-2. Actin colocalization and morphological effects of recombinant wt-HA and S-A-HA MacMARCKS in PC12 cells. 76
Figure 4-3. Expression of S-A-HA MacMARCKS in PC12 cells blocks evoked Sytum-Ab staining. 80
Figure 4-4. Quantitation of depolarization-evoked Sytum-Ab labeling and assay for wheat germ agglutinin (WGA) uptake in PC12 cells. 83
Figure 4-5. [3H]-norepinephrine release from S-A-HA PC12 cells is inhibited following depolarization. 86
Figure 4-6. Immunogold labeling of S-A-HA MacMARCKS localizes to the cytoplasmic face of the plasma membrane and to dense core granules. 89
Figure 5-1. Nucleotide sequence of the MacMARCKS 5’ flanking region and mapping of the transcriptional start site. 99
Figure 5-2. Induction of MacMARCKS mRNA by macrophage activating factors. 102
Figure 5-3. Kinetics of MacMARCKS mRNA and protein induction by LPS and the effect of cycloheximide on expression of MacMARCKS mRNA. 105
Figure 5-4. Effect of LPS on MacMARCKS transcription. 108
Figure 5-5. Functional analysis of MacMARCKS-luciferase constructs in RAW 264.7 cells. 111
Figure 5-6. Construction and quantitation of the MacMARCKS-lacZ transgenes. 113
Figure 5-7. LPS stimulation of microglia induces MacMARCKS. 117
Figure 6-1. RNase protection analysis of MacMARCKS expression in tissues. 127
Figure 6-2. MacMARCKS expression patterns during mouse development. 131
Figure 6-3. Expression of β-galactosidase in 4 kb-lacZ transgenic mice during
development. 134
Figure 6-4. LacZ expression patterns in the embryonic retina and the brains
of P₀ and adult transgenic mice. 138
Figure 6-5. Identification of β-galactosidase-expressing cells in the brains
of 4 kb-LacZ transgenic mice. 141
Figure 6-6. Expression of the 1.7 kb-lacZ transgene during mouse development. 145
Figure 7-1. Targeted disruption of MacMARCKS and germline transmission. 158
Figure 7-2. Disruption of MacMARCKS prevents cranial neural tube closure
and results in exencephaly. 164
Figure 7-3. Brain malformations in MacMARCKS null embryos. 169
Figure 7-4. Disruption of the MacMARCKS gene results in anencephaly
and retinal defects. 172
Table 7-1. Genotypes of embryos from MacMARCKS heterozygote matings. 161
ABSTRACT

MacMARCKS is a second member of the MARCKS family of PKC substrates which integrate signal transduction events with changes to the actin cytoskeleton. Work presented here demonstrates diverse roles for MacMARCKS ranging from neural secretion to neural development. Chapters 3 and 4 describe the role of MacMARCKS in neural secretion. MacMARCKS is phosphorylated in a stimulus-dependent manner in rat brain synaptosomes and PC12 cells. It is localized to cell bodies and neurite tips of PC12 cells as well as being present on synaptic vesicles. A dominant-negative mutation in the MacMARCKS effector domain which abolishes PKC-dependent phosphorylation is associated with changes in cellular morphology and blocks agonist evoked exocytosis in PC12 cells. Characterization of the MacMARCKS promoter is presented in Chapter 5. The LPS response elements of the MacMARCKS promoter are localized to a region 113 bp upstream of the transcriptional start site; this region contains multiple SP-1 sites. Regulation of MacMARCKS induction by LPS appears to be by post-transcriptional mechanisms. Chapters 6 and 7 presents evidence that MacMARCKS is involved in mouse neural development. Endogenous MacMARCKS expression is neural specific early during embryonic development but is expressed in other tissues later on. In adult animals, MacMARCKS expression localizes to specific regions of the adult brain. Transgenic mice carrying 4 kb or 1.7 kb of MacMARCKS upstream flanking regions directed neural-specific expression of the β-galactosidase reporter gene in a developmentally regulated manner. Targeted deletion of the MacMARCKS gene in mouse embryos leads a failure of cranial neural tube closure, resulting in exencephaly. MacMARCKS null mice are born anencephalic and die soon after birth. The work presented in this thesis demonstrates that MacMARCKS is essential for processes associated with signal transduction events leading to reorganization of the actin cytoskeleton.
CHAPTER 1: INTRODUCTION

The ability of animal cells to respond to extracellular stimuli is essential for many biological processes. The cortical actin cytoskeleton which underlies the plasma membrane of cells is intimately involved in regulation and execution of many of these responses. The actin cytoskeleton and its associated proteins provide a highly dynamic, organelle-excluding matrix, which is important in such diverse processes as cell motility, phagocytosis, neural secretion, and embryonic development (reviewed by Bretscher, 1991). The signal transduction pathways leading to actin remodeling are only beginning to be elucidated, due to the complexity of events and the large number of actin binding proteins involved in controlling these processes. A host of extracellular signals must be processed to remodel the cortical actin cytoskeleton by modulation of contacts between the actin cytoskeleton and the integrin adhesion receptors, and between integrins and the extracellular matrix (Clark and Brugge, 1995). Actin filament turnover is coordinated with changes in microtubules and intermediate filaments. To elucidate the intracellular signal transduction pathways that control cytoskeletal mediated changes in cell physiology, it is necessary to understand the proteins regulating these events.

In this thesis, the biology of an actin regulating protein, MacMARCKS, is explored. The complexity of the actin cytoskeleton necessitates a multidisciplinary approach utilizing a variety of systems to explore how signal transduction pathways impinge upon the actin cytoskeleton. Protein kinase C (PKC)-dependent phosphorylation of MacMARCKS regulates its interaction with calcium/calmodulin and actin. In this thesis, data are presented that demonstrates a role for MacMARCKS in neural secretion and neural tube closure, events which require dynamic reorganization of the actin cytoskeleton. The introduction briefly presents a general discussion of signal transduction and the
cytoskeleton followed by a consideration of the actin cytoskeleton during neural secretion and embryonic development. Finally, the biology of the MARCKS family of PKC substrates is discussed in detail.

**Signal Transduction And The Actin Cytoskeleton:**

The actin cytoskeleton is an essential component of a host of functions associated with cell shape and motility. The ability of actin to respond differentially to cues associated with these various processes is due to the different signals which stimulate these events and a variety of effectors which coordinate the cytoskeletal response. One way in which the actin cytoskeleton is regulated is through the extracellular matrix (ECM). Integrins are the major family of cell surface receptors that link the actin cytoskeleton on the cytoplasmic face of the plasma membrane to the ECM. They are assembled from a group of 16 α and 8 β transmembrane subunits that heterodimerize to produce more than 20 different receptors (reviewed by Hynes, 1992). These receptors interact with a large variety of ligands that are components the ECM (fibronectin, collagen) or receptors on the cell surface of adjacent cells. ECM ligands cross-link and cluster integrins on the cell surface to activate integrin-mediated responses. This leads to the formation of focal adhesions, regions of the plasma membrane closely adherent to the substratum (Luna and Hitt, 1992; Gumbiner, 1993). Actin binding proteins including α-actinin, talin, vinculin and tensin link actin filaments with integrins in adhesion plaques (Ben-Ze’ev, 1991). These assemblies of structural proteins play important roles in stabilizing cell adhesion and regulating cell motility (Clark and Brugge, 1995). Together these components serve as a framework for the association of signaling proteins which regulate signal transduction pathways leading from cytoskeletal-induced changes to modulations in cell behavior (Clark and Brugge, 1995).

Protein phosphorylation is the earliest event detected following integrin receptor binding. Activation of protein tyrosine kinases, including the focal adhesion kinase (FAK),
is a ubiquitous response to integrin stimulation (Hynes, 1992). After integrin crosslinking, FAK is phosphorylated on tyrosine residues, and its own tyrosine kinase activity is stimulated (Schaller and Parsons, 1994). FAK phosphorylates several other proteins in the adhesion plaque, including paxillin (Clark and Brugge, 1995). Phosphorylation of FAK is modulated by the protein kinase C (PKC) family, a large group of protein-serine/threonine kinases found in mammalian tissues (Nishizuka, 1992). Integrin-mediated activation of PKC precedes cell spreading, and PKC agonists enhance cell spreading and tyrosine phosphorylation of FAK (Vuori and Ruoslahti, 1993). PKC inhibitors prevent cell spreading and eliminate focal adhesion plaques (Vuori and Ruoslahti, 1993). These results suggest that integrin stimulation activates protein-tyrosine kinases and PKC, leading to the formation of focal contacts (Clark and Brugge, 1995).

Considerable evidence suggests that PKC is also involved in neural secretion. Most PKC isozymes are expressed at high levels in neurons (Dekker et al., 1991; Kaczmarek, 1987), and phorbol esters promote release of neurotransmitters (Shapira et al., 1987). Ca\(^{2+}\) and DAG are elevated in active presynaptic terminals (Pozzan et al., 1984). PKC is also implicated in catecholamine secretion from adrenal chromaffin cells. Neuroendocrine cells such as adrenal chromaffin cells have the ability to exocytose neurotransmitters in response to a variety of external stimuli. Activation of PKC by phorbol esters increases evoked catecholamine release in these cells (TerBush et al., 1988; Bittner and Holz, 1990). Nicotinic stimulation of adrenal chromaffin cells induces a rapid (<2 sec) and transient translocation of PKC from the cytosol to the membrane, leading to enhanced Ca\(^{2+}\)-dependent secretion (TerBush et al., 1988; Bittner and Holz, 1990).

Exposure of adrenal chromaffin cells to elevated external K\(^{+}\) or agonists such as nicotine induces membrane depolarization, leading to Ca\(^{2+}\) influx and release of stored neurotransmitters (Williams and McGee, 1982). A meshwork of F-actin underlies the plasma membrane of adrenal chromaffin cells (Lee and Trifaro, 1981; Cheek and
Cortical F-actin impedes exocytosis by acting as a barrier to inhibit the movement of secretory vesicles to the plasma membrane (Koffer et al., 1990; Vitale et al., 1991; Del Castillo et al., 1992). Nicotine or elevated K⁺-induced depolarization causes a rapid disassembly of F-actin at discrete subplasmalemmal zones. Anti-dopamine β-hydroxylase antibodies demonstrate that these regions are sites of active exocytosis (Vitale et al., 1991). Nicotine stimulation also causes the cellular redistribution of scinderin to sites occupied by F-actin. Scinderin is a Ca²⁺-dependent actin severing protein, and depolarization-induced Ca²⁺ influx presumably activates scinderin to shorten actin filaments, allowing exocytic vesicles to fuse with the plasma membrane (Vitale et al., 1991). Scinderin redistribution and actin filament disassembly induced by depolarization precede catecholamine release. The PKC agonist phorbol myristate acetate (PMA) induces scinderin redistribution in a Ca²⁺-independent manner, and this redistribution is completely blocked by PKC inhibitors (Del Castillo et al., 1992). PMA treatment of permeablized mast cells causes a decrease in F-actin underlying the plasma membrane and results in enhanced secretion (Koffer et al., 1990). These data suggest a role for PKC in regulating the cortical actin cytoskeleton during neural secretion.

**Regulation of the Actin Cytoskeleton During Embryonic Development:**

Embryonic morphogenesis is driven by a complex series of coordinated changes in cell shape and size; these changes are in turn based on developmentally regulated alterations in the underlying cytoskeleton. The actin cytoskeleton is an essential part of *Drosophila* development, including oogenesis. It is also essential to chordate development, as illustrated by the complex morphogenetic events associated with neurulation. Both processes are discussed below.
**Drosophila oogenesis:** Oogenesis is one of the best understood developmental systems from the perspective of the actin cytoskeleton. It culminates with the formation of an oocyte surrounded by nurse cells which synthesize most of the ooplasmic components (reviewed by Cooley and Theurkauf, 1994). During this process, nurse cell cytoplasm streams into the oocyte through specialized conduits called ring canals. These structures are composed of F-actin and products of the *hts* and *kelch* genes. The NH$_2$-terminal half of the Hts protein is homologous to the actin binding protein adducin (Yue and Spradling, 1992) and the Kelch protein has six copies of a 50 amino acid motif that is also found in the actin binding protein scruin (Way *et al.*, 1995). Kelch is thought to dimerize to produce a bifunctional actin binding molecule that crosslinks and stabilizes actin filaments in the ring canal (Cooley and Theurkauf, 1994). Proper formation of the ring canals is required for oogenesis, as mRNAs and proteins synthesized by the nurse cells are selectively transported to the oocyte through these structures. Cytochalasin inhibits movement of particles through the ring canals, suggesting that transport involves actin based mechanisms (Bohmann and Biber, 1994). Late in oogenesis, nurse cells undergo major cytoskeletal rearrangements in preparation for final cytoplasmic transfer. Actin filament bundles connect the nuclear membrane with the plasma membrane, and this dynamic process requires the *chickadee, singed* and *quail* genes (Cooley and Theurkauf, 1994). The *chickadee* gene encodes *Drosophila* profilin, a 15 kDa actin binding protein involved in the tyrosine kinase signal transduction pathway (Cooley *et al.*, 1992, Aderem, 1992b). Null alleles of *chickadee* are lethal, suggesting that profilin is essential for cell function (Cooley *et al.*, 1992). The product of the *singed* gene is homologous to sea urchin fascin, which crosslinks actin into hexagonal arrays (Cant *et al.*, 1994; DeRosier et al., 1977). The *quail* gene product is homologous to villin, an actin cross-linking and severing protein (Matsudaira *et al.*, 1983).
In addition to these actin binding proteins, the product of the \textit{armadillo} gene is required for assembly of the cytoplasmic actin bundles in the nurse cells (Peifer \textit{et al.}, 1993). Armadillo is 65\% homologous to \(\beta\)-catenin and plakoglobin, both cadherin-associated proteins (Peifer and Wieschaus, 1990). Cadherins are a molecular superfamily of adhesion proteins which mediate cell-cell interaction, and which associate with \(\beta\)-catenin and plakoglobin. Both cadherins and catenins localize to the cell-cell adherens junctions where they anchor the actin cytoskeleton. This interaction is required for proper formation of cell-cell adherens junctions (Peifer and Wieschaus, 1990). Disruption of \textit{armadillo} function in \textit{Drosophila} oocytes leads to a collapse of the cortical actin cytoskeleton and distortion of cell shape (Peifer, 1995).

\textbf{Wingless signaling pathway:} The armadillo protein in \textit{Drosophila} has dual functions. In addition to being an integral component of cell-cell adherens junctions (discussed above), it is also involved in the wingless signal transduction cascade required for cell fate specification during embryogenesis. \textit{Wingless} (a segment polarity gene) organizes pattern formation by activating signal transduction pathways that direct particular cell fates. The wingless protein is secreted by a subset of cells within each fly segment. Cells in different positions within each segment are exposed to a gradient of wingless. Binding of this protein to putative receptors activates the disheveled protein. Disheveled, in turn, negatively regulates the protein-serine/threonine kinase \textit{zeste-white3} (homologue of mammalian glycogen synthase kinase 3\(\beta\)), thereby de-repressing armadillo (Siegfried \textit{et al.}, 1994). Armadillo accumulates in cells which receive the highest levels of wingless (Peifer \textit{et al.}, 1994; Van Leeuwen \textit{et al.}, 1994). The wingless signal stabilizes armadillo by suppressing its phosphorylation by \textit{zeste-white3}. Phosphorylated armadillo is degraded, whereas unphosphorylated armadillo accumulates in the cytoplasm and activates as yet unidentified effectors of wingless signaling. These effectors must ultimately impinge upon the nucleus,
since the *engrailed* gene (which encodes a transcription factor) is activated by the wingless signal transduction pathway (Dinardo *et al*., 1988). Recent evidence suggests that at elevated expression levels, plakoglobin (an armadillo homologue) is detected in the nucleus and most likely interacts with nuclear targets (Funayama *et al*., 1995).

The members of the wingless signal transduction pathway are conserved in all multicellular animals examined to date. The armadillo homologues, β-catenin and plakoglobin, and the zeste-white3 homologue, glycogen synthase kinase 3 (GSK3), all are involved in signaling of the *wingless* homologue *wnt-1* during *Xenopus* development (Peifer, 1995). Catenins and plakoglobin are present in the *Xenopus* egg (Fouquet *et al*., 1992), and injection of β-catenin or plakoglobin leads to axis duplication in the *Xenopus* embryo (Karnovsky *et al*., 1995; Funayama *et al*., 1995). Overexpression of cadherins can suppress this axis duplicating activity (Heasman *et al*., 1994). These observations suggest that β-catenin and plakoglobin transduce signals during embryonic development, and that the cadherin-cytoskeleton complex can sequester and suppress these cytosolic signals. GSK3 phosphorylates serines in the NH2-terminal domain of β-catenin, targeting it for ubiquitination and proteosomal degradation (Cowin and Burke, in press). In the mouse, the *wnt-1* gene is essential for murine brain patterning and development (McMahon and Bradley, 1992). αN-catenin and *E-cadherin* are expressed in the embryonic forebrain and midbrain, regions that parallel the expression of *wnt-1* (Shimamura *et al*., 1994). In *wnt-1* null mice, *E-cadherin* expression is upregulated, while αN-catenin expression is suppressed (Shimamura *et al*., 1994). These results suggest that *wnt-1* is involved in the regulation of *E-cadherin* and αN-catenin expression in specific domains of the embryonic murine brain.

**Mouse neurulation:** Regulation of the actin cytoskeleton is also pivotal to chordate development. Neurulation, the formation of a neural tube that eventually gives rise to the
brain and spinal cord, is driven by a complex series of cell shape changes coordinated by the actin cytoskeleton. During neurulation, contraction of the actin cytoskeleton transforms neuroepithelial cell shape from columnar to pyramidal, a process referred to as cell wedging. This morphogenic alteration drives the neural plate to fold into the neural tube (for review see Copp et al., 1990; Schoenwolf and Smith, 1990). Circumferential microfilament bands are present in the apices of neuroepithelial cells, and they appear increasingly dense during bending of the neural plate (Morriss-Kay et al., 1994). As the neural folds begin to elevate, actin is localized along the basal aspects of the neuroepithelial cells. When neuroepithelial cells are undergoing active cell wedging, actin redistributes to the apical region of the cells (Sadler et al., 1981). Actin binding proteins such as myosin and fodrin are also present in the apices of neuroepithelial cells (Sadler et al., 1986). It is proposed that the microfilaments contract gradually in a purse-string like fashion, constricting the apices of these cells and thereby transforming them from columns to wedges that aid in the elevation of the neural folds (Schoenwolf and Smith, 1990).

**PKC and the actin cytoskeleton during development:** The PKC-dependent signal transduction pathway regulates the actin cytoskeleton during development. Treatment of *Xenopus* oocytes and eggs with PMA triggers early events of embryonic development, including cortical granule exocytosis, cortical contraction, and cleavage furrow formation, all actin-dependent processes (Bement and Capco, 1989). In the *Xenopus* embryo, neural induction is characterized by a rapid translocation of PKC from the cytosol to the membrane of ectodermal cells (Otte et al., 1988), and PMA stimulates this translocation and induces neural induction.

PKC isozymes are differentially expressed throughout the brain during embryonic development (Sposi et al., 1989). For example, while α-PKC is universally distributed in all tissues, β-PKC isozymes are enriched in the developing brain as early as E9.5, and γ-
PKC is found exclusively in the brain (reviewed by Dekker et al., 1991). Immunoelectron microscopy demonstrates that both β and γ-PKC are localized to the axonal termini of cerebellar Purkinje cells, and phosphorylation studies reveal that active PKC isozymes are present in synaptic termini together with their physiologically important substrates (Dunkley and Robinson, 1986; Wang et al., 1988). PKC isozymes tightly associate with the growth cone presynaptic membranes and mediate neurotransmitter release (Dekker et al., 1991).

Several PKC substrates, including GAP-43 and the MARCKS proteins, have a role in neural development. Axonal growth cones are highly dynamic structures which interact with various soluble or substrate-associated guidance factors, resulting in membrane addition, controlled polymerization and stabilization of cytoskeletal elements in filopodia and lamellipodia (Forscher and Smith, 1988). GAP-43 is highly concentrated in the growth cone membrane and its level of expression, and state of phosphorylation, increases during axonal growth and regeneration (reviewed by Strittmatter and Fishman, 1991; Skene et al., 1986; Dent and Meiri, 1992). In growth cones, the majority of GAP-43 is associated with the cortical actin cytoskeleton (Allsopp and Moss, 1989; Moss et al., 1990).

GAP-43 is thought to be required for axonal extension, since antisense GAP-43 oligonucleotides decrease neurite extension from cultured dorsal root ganglion (DRG) neurons (Aigner and Caroni, 1993). Overexpression of GAP-43 leads to increased neurite extension from PC12 cells (Yankner et al., 1990), although PC12 cells depleted of GAP-43 are still capable of extending neurites (Baetge and Hammang, 1991). When expressed in COS and L6 cells, GAP-43 promotes cell spreading and the transient formation of large filopodia (Zuber et al., 1989; Widmer and Caroni, 1993). Therefore, GAP-43 may modulate neuronal extension, and it can also affect morphological behavior in non-neuronal cells.
GAP-43 interacts with several members of signal transduction pathways (Strittmatter and Fishman, 1991). The effector domain of GAP-43 binds calmodulin with high affinity in the absence of Ca\(^{2+}\); this interaction is abolished by Ca\(^{2+}\) and by PKC-dependent phosphorylation (Alexander et al., 1987). Expression of a mutant GAP-43 protein in which the PKC phosphorylation site was changed to an alanine results in reduced L6 cell spreading and small filopodia formation (Widmer and Caroni, 1993). The in vivo role for GAP-43 has been explored by generating mice lacking GAP-43 by targeted gene disruption. GAP-43 null mice have greatly decreased survival rates; only 10% survive beyond 3 weeks (Strittmatter et al., 1995). The central nervous system (CNS) of these mice appears grossly normal. Cultured dorsal root ganglia from homozygous null mice extend neurites and growth cones which are indistinguishable from wild-type controls. These results indicate that GAP-43 is not absolutely required for either growth cone formation or neurite extension. Examinations of the optic chiasm indicate that retinal ganglion fibers from GAP-43 null mice are tangled and twisted in random directions and do not cross the midline at the chiasm to enter the contralateral optic tract (Strittmatter et al., 1995). The failure of retinal axons to reach their appropriate targets is consistent with the suggestion that GAP-43 plays a role in signal transduction mechanisms of the growth cone.

The MARCKS Family of PKC Substrates Integrate Signal Transduction Pathway With The Actin Cytoskeleton:

The myristoylated alanine-rich C-kinase substrate, MARCKS, has been used as a marker of PKC activation in vivo for at least a decade (Aderem, 1992; Blackshear, 1993). It represents the prototype of a family of PKC substrates which translate extracellular signals into alterations in the actin cytoskeleton. Since this dissertation focuses on MacMARCKS, a second member of the MARCKS family, the biology of these proteins will be discussed in detail.
**MARCKS protein structure:** MARCKS cDNAs have been cloned from several species and the primary structure of the protein is highly conserved. The predicted size of the protein ranges from 31 kDa (bovine) to 29 kDa (mouse), but MARCKS migrates with an anomalously high Mr on SDS-PAGE, ranging from Mr 87 k Da (bovine) to Mr 68 k Da (mouse). This is due to the rod-shaped structure of this protein (Albert et al., 1987; Manenti et al., 1992); electron microscopy reveals its dimensions to be approximately 4.4 x 36 nm (Hartwig et al., 1992). MARCKS is heat-stable and highly acidic, with a pI of 4.1. Although it lacks significant regions of hydrophobicity, MARCKS associates with the membrane in most cells (see below).

MARCKS contains three major domains (Fig. 1-1a). The N-terminal amino acids, which contain the consensus sequence for N-terminal myristoylation (Towler et al., 1988), is conserved in all species examined. The second conserved domain of 20 amino acids, termed the MARCKS homology 2 (MH2) domain, surrounds the site of intron splicing. Although the function of this domain is still not known, a core sequence of 7 amino acids within this domain is identical to the cytoplasmic tail of the insulin-like growth factor II/mannose 6-phosphate receptor (Aderem, 1992; Blackshear, 1993). The third conserved region is the 25 amino acid effector domain which contains the PKC phosphorylation sites, and which also binds calcium/calmodulin, F-actin and acidic phospholipids (Graff et al., 1989; 1990; Hartwig et al, 1992; Kim et al, 1994b).

**Myristoylation and Membrane Binding:** Myristoylation refers to the attachment of a 14 carbon saturated fatty acid (myristate) to the NH₂-terminus of substrate proteins like MARCKS (Towler et al., 1988; Aderem et al., 1988; James and Olson, 1989). The enzyme N-myristoyltransferase cotranslationally transfers a myristoyl moiety from the cofactor myristoyl-coenzyme A to the NH₂-terminal glycine residue of MARCKS through
Fig. 1-1. Domain structure and proposed function of MARCKS and MacMARCKS.

a. The domain structure of MacMARCKS and MARCKS. Squiggley line represents the myristic acid moiety and the dark box represents the N-terminal myristoylation consensus sequence. The MH2 and effector domains are boxed. PKC phosphorylation sites in the Effector Domain are starred.

b. Model indicating a mechanism by which the MARCKS family of PKC substrates might regulate actin-membrane interaction. At rest, MARCKS/MacMARCKS (M) associate electrostatically with the plasma membrane through a negatively charged effector domain and myristic acid moiety. In this non-phosphorylated form, MARCKS/MacMARCKS cross-link actin (dark lines) into a rigid meshwork at the plasma membrane. An agonist receptor activates PKC through a cascade of G proteins (G) and phospholipase C (PLC). PKC phosphorylates MARCKS/MacMARCKS, displacing them from the membrane. Phosphorylated MARCKS/MacMARCKS remain in the cytoplasm and cannot cross-link actin. When MARCKS/MacMARCKS are dephosphorylated by phosphatases 1 and 2a, they reassocciate with the plasma membrane and cross-link actin.

An increase in intracellular Ca\(^{2+}\) promotes the binding of calmodulin (Cal) to MARCKS/MacMARCKS, inhibiting their actin crosslinking ability. A decrease in intracellular Ca\(^{2+}\) shifts the equilibrium to the resting state, leading to calmodulin dissociation from MARCKS/MacMARCKS, allowing actin cross-linking at the plasma membrane. Since Ca\(^{2+}\) levels are known to oscillate following cellular stimulation, MARCKS/MacMARCKS would mediate cycles of Ca\(^{2+}\)-dependent actin cross-linking activity at the membrane.
Calcium Channel

Phosphatase 1 and 2a

Phosphatase

Other actin-binding proteins

MIP/IR. MIPNhu

MacMARCKS ~~~~~~ MH2 ~~~~~~ Effector Domain

MARCKS ~~~~~~ MH2 ~~~~~~ Effector Domain

Phosphorylation
Calmodulin Binding
Actin Binding

KRFKKSFKLSGFSFKK

KiNFPKKFLKLGSFLKR

b

Agonist Receptor

PKC

ATP

DAG

IP3

PLC

G

Cal

↑Ca2+

↑Ca2+

↑Ca2+

Pi

-Pi
amide bond formation. Myristoylation is believed to be a constitutive process resulting in a stably modified protein; however, there is evidence that a pool of non-myrystoylated MARCKS exists in synaptosomes, possibly as a result of demyristoylation activity (Manenti et al., 1993; 1994). Photolabeling experiments show that myristate functions to promote MARCKS binding to the membrane by inserting into the hydrophobic interior of the lipid bilayer (Vergeres et al., 1995). Mutation of the amino terminal glycine to alanine abolishes both myristoylation and MARCKS association with the plasma membrane, resulting in its distribution in the cytosol (Graff et al., 1989; Swierczynski and Blackshear, 1995; Seykora et al., in press). However, myristoylation alone is not sufficient to bind MARCKS to membranes for several reasons. First, myristoylated proteins are found in all subcellular compartments including the cytosol (James and Olson, 1989; Blenis and Resh, 1992; Manenti et al., 1993; Allen and Aderem, 1995b). Second, the Gibbs free energy of binding of myristoylated peptides to membrane vesicles is 8 kcal, which is not sufficient to firmly anchor proteins to membranes (Peitzsch and McLaughlin, 1993; Buser et al., 1995). Third, MARCKS mutants lacking the NH₂-terminal glycine residue bind phosphatidylserine (PS) in vitro with an affinity equal to the wild-type protein, indicating that N-terminal myristoylation is not necessary for MARCKS binding to PS (Nakaoka et al., 1995). Thus, other factors contribute to the association of MARCKS with the plasma membrane.

Results from several groups provide compelling evidence that the basic effector domain of MARCKS participates in membrane binding. The effector domain contains a string of 13 basic residues. Peptides that correspond to this region electrostatically interact with acidic phospholipids such as phosphatidylserine (PS; Taniguchi and Manenti, 1993; Kim et al., 1994a, 1994b; Nakaoka et al., 1995; Yang and Glaser, 1995). PKC phosphorylation of these peptides decreases their affinity for the membranes by 1000-fold causing a rapid dissociation of peptides from the membrane (Kim et al., 1994a).
Phosphorylation of the MARCKS protein reduces its binding to phospholipid vesicles by 20 to 500-fold, depending on the nature of the phospholipid vesicles (Nakaoka et al., 1993; Taniguchi and Manenti, 1993; Kim et al., 1994b; Nakaoka et al., 1995). PKC-dependent phosphorylation of the effector domain introduces three negatively charged phosphate groups, reducing its net charge from +13 to +7. This decreases its association with acidic phospholipids. However, electrostatic interactions alone are not sufficient to firmly bind MARCKS to the membrane; a synergy of both electrostatic interactions from the effector domain and hydrophobic interactions from the myristate moiety are required to stably bind MARCKS to the plasma membrane. This mechanism of phosphorylation-regulated translocation of MARCKS from the membrane to the cytosol is known as the electrostatic switch (Kim et al., 1994s; McLaughlin and Aderem, 1995).

The above in vitro results parallel observations of living cells. PKC-dependent phosphorylation of the effector domain results in the translocation of MARCKS from the plasma membrane to the cytosol of nerve terminals (Wang et al., 1989), neutrophils (Thelen et al., 1991), macrophages (Aderem et al., 1988; Rosen et al., 1990) and fibroblasts (Allen and Aderem, 1995b). This is a reversible process; dephosphorylation of MARCKS leads to its reassociation with the plasma membrane (Thelen et al., 1991). Mutational analysis indicates that this regulated cycle of membrane attachment/detachment is important for MARCKS function; mutations which disrupt the electrostatic switch inhibits cell spreading and directed cell motility in fibroblasts (Myat et al., 1994).

**PKC-dependent phosphorylation:** MARCKS phosphorylation is enhanced by agents that activate PKC, including phorbol esters, synthetic diacylglycerols, mitogens, growth factors, and serum (reviewed by Aderem, 1992; Blackshear, 1993). Electrospray mass spectroscopic analysis indicates that the cytosolic form of MARCKS can be phosphorylated by PKC in vitro to a stochiometry of 3 mol/mol, as compared to 4 mol/mol for the
membrane-bound form. This suggests that one of the PKC phosphorylation sites is already phosphorylated in cytosolic MARCKS (Manenti et al., 1992). The first, second, and fourth serine residues of the MARCKS effector domain (KKKRFS\textsuperscript{152}FKKS\textsuperscript{156}FKLS\textsuperscript{160}GFS\textsuperscript{163} FKK) are in a consensus sequence for PKC phosphorylation. Microsequencing of the individual sites indicates that Ser\textsuperscript{152}, Ser\textsuperscript{156}, and Ser\textsuperscript{163} are phosphorylated in vivo (Amess et al., 1992) although the third serine can still bind PKC with high affinity in vitro (Graff et al., 1991). All PKC phosphorylation sites are found within the effector domain; when these sites are mutated into alanines, PKC-dependent phosphorylation of MARCKS is not observed (Allen and Aderem, 1995b; Seykora et al., submitted). Maximal phosphorylation of MARCKS does not depend upon the presence of myristic acid; non-myristoylated MARCKS is phosphorylated as well as the wild-type protein, though with slower kinetics (Graff et al., 1989; Seykora et al., submitted).

MARCKS is a good substrate for conventional PKC\textalpha{}, novel PKC\textbeta{}, and novel PKC\textepsilon{}, and binds these isozymes with very high affinity (Fujise et al., 1994). Recent evidence suggests that MARCKS is not exclusively phosphorylated by PKC. Of 7 phosphorylated serines from MARCKS purified from bovine brain, only one was within the effector domain (Ser\textsuperscript{152}, Taniguchi et al., 1994). All 6 novel phosphorylated serine residues are followed immediately by a proline, suggesting that MARCKS is also an in vivo substrate for proline-directed protein kinases such as MAP kinase and cdc2 kinase (Taniguchi et al., 1994). This is borne out by the observation that MARCKS is a major substrate for MAP kinase, cdc2 kinase and tau protein kinase II in the brain (Yamamoto et al., 1995; Kusubata et al., unpublished data). Moreover, the sites phosphorylated by cdc2 kinase and tau protein kinase II (TPKII) lie outside the effector domain (Yamamoto et al., 1995). Proline-directed protein kinases are important in signal transduction (reviewed by Hall and Vulliet, 1991; Spencer et al., 1992); MARCKS may mediate interactions between the PKC and MAP kinase dependent pathways.
MARCKS function is also regulated by phosphatases. Okadaic acid, a potent inhibitor of serine/threonine phosphatases 1, 2A, and 2B prevents the dephosphorylation of MARCKS and its reassociation with the plasma membrane in neutrophils (Thelen et al., 1991). Okadaic acid inhibits dephosphorylation of one or two (but not all three) of the serine phosphorylation sites in the effector domain (Amess et al., 1992). PKC-phosphorylated MARCKS or a PKC-phosphorylated effector domain peptide are dephosphorylated by protein phosphatases1 and 2A in cell extracts (Clarke et al., 1993; Yamamoto et al., 1995). In addition, cdc2 kinase or TPKII kinase-phosphorylated MARCKS is dephosphorylated by protein phosphatase 2A (Yamamoto et al., 1995).

**Calmodulin Binding:** Ca$^{2+}$-calmodulin inhibits MARCKS phosphorylation in rat brain synaptosomes by approximately 80% (Wu et al., 1982; Albert et al., 1984). This inhibition is due to calmodulin binding to the effector domain of MARCKS. Both MARCKS and an effector domain peptide bind calmodulin with high affinity (Graff et al., 1989). MARCKS binds calmodulin in the presence of Ca$^{2+}$, in contrast to GAP-43, which binds calmodulin only in the absence of Ca$^{2+}$ (Graff et al., 1989; Alexander et al., 1987). PKC phosphorylation of the effector domain peptide decreases its binding to calmodulin by 200-fold and results in a rapid release of bound calmodulin (McIlroy et al., 1991). Calmodulin binding to recombinant MARCKS inhibits its association with large unilamellar phospholipid vesicles by 20-fold (Kim et al., 1994). Furthermore, non-myristoylated MARCKS binds calmodulin poorly, indicating that the presence of the myristic acid moiety is necessary for calmodulin binding (Manenti et al., 1993). Microinjection of a MARCKS effector domain peptide into Paramecium leads to formation of a complex between the peptide and calmodulin. This complex is disrupted by PMA activation of PKC (Hinrichsen and Blackshear, 1993). Overexpression of MARCKS in rat1 cells reduces the free concentration of calmodulin in the cell (Herget and Rozengurt, 1994). These findings
suggest that MARCKS might have a role in regulating calmodulin availability inside the cell.

Actin Binding: In murine macrophages, MARCKS has a punctate distribution and is enriched at the substrate-adherent surface of pseudopodia and filopodia (Rosen et al., 1990). MARCKS colocalizes with vinculin and talin in these punctate structures (podosomes) which are the transient contacts made by motile cells with the substratum (Rosen et al., 1990). PKC activation of macrophages results in the disappearance of MARCKS from podosomes in conjunction with major cytoskeletal rearrangements leading to cell spreading and loss of filopodia (Rosen et al., 1990). During phagocytosis, MARCKS colocalizes with F-actin, talin and myosin I on the cytoplasmic face of the forming phagosome, suggesting that it regulates actin structure at the membrane (Allen and Aderem, 1995a). Treatment of neutrophils with the chemotactic peptide f-Met-Leu-Phe leads to a rapid, but transient, phosphorylation of MARCKS by PKC and its displacement from the plasma membrane (Thelen et al., 1991). Taken together, these data suggest that MARCKS may modulate the actin cytoskeleton during macrophage and neutrophil locomotion.

Immunoelectron microscopy demonstrates that MARCKS localizes to points where actin filaments interact with the cytoplasmic surface of the plasma membrane (Aderem et al., unpublished data). De-phosphorylated MARCKS binds to and cross-links filamentous but not globular actin in vitro. This cross-linking activity is disrupted by binding MARCKS to calmodulin (Hartwig et al., 1992). Phosphorylated MARCKS binds actin with 50% lower affinity than dephosphorylated MARCKS, and does not cross-link F-actin. The involvement of the effector domain in binding actin is confirmed using an effector domain peptide: dephosphorylated peptide cross-linked F-actin into bundles, whereas peptides
bound to calmodulin or phosphorylated by PKC did not (Fig. 1-1b; Hartwig et al., 1992). For MARCKS to cross-link actin, it must either dimerize, or another region of the protein must bind actin. The highly conserved MH2 domain might be involved in MARCKS dimerization or actin-crosslinking.

**Biological Functions:** MARCKS is widely expressed in a variety of tissues and cell types (Albert et al., 1986; Lobach et al., 1993). It is postulated to have a role in translating extracellular signals into alterations in actin plasticity and changes in actin-membrane interactions (Aderem, 1992). The concentration of MARCKS is highest in the brain, where it represents approximately 0.2% of cellular protein (Graff et al., 1989). It is localized to presynaptic nerve terminals (Ouimet et al., 1990) and its phosphorylation in synaptosomes is stimulated by depolarization-induced Ca$^{2+}$ influx (Wu et al., 1982; Wang et al., 1989; Coffey et al., 1994). Other agonists, such as acetylcholine (Haycock et al., 1988) and glutamate (Scholz and Palfrey, 1991; Tan et al., 1994), also induce transient MARCKS phosphorylation in neurons and neuroendocrine cells. Phosphorylation of MARCKS in synaptosomes results in its rapid translocation from the membrane to the cytosol, suggesting a role in secretion or membrane recycling (Wang et al., 1989). By analogy with the synapsins (Greengard et al., 1993), MARCKS might link synaptic vesicles to cortical actin. MARCKS could reversibly tether synaptic vesicles to the actin-based cytoskeleton of the nerve terminal, and this linkage would be disrupted when the effector domain of MARCKS is phosphorylated by PKC. Alternatively, MARCKS might regulate the dynamics of actin filament assembly at the presynaptic terminal in a PKC- and Ca$^{2+}$-calmodulin-dependent manner, thereby affecting access of synaptic vesicles to the plasma membrane.

Studies in macrophages and neutrophils suggest that MARCKS is involved in phagocytosis and cell motility. During phagocytosis, MARCKS colocalizes with PKCα,
talin and F-actin to the phagocytic cups of nascent phagosomes (Allen and Aderem, 1995a). Phagocytosis is accompanied by a sustained phosphorylation of MARCKS, suggesting that MARCKS is not cross-linking actin at the site of particle ingestion (Allen and Aderem, 1995a). Stimulation of phagocytic cells with bacterial lipopolysaccharide (LPS) or f-Met-Leu-Phe results in increased cell motility and leads to PKC activation and an elevation of cytosolic Ca\(^{2+}\) concentration. These two signals regulate MARCKS interaction with both the actin cytoskeleton and the plasma membrane. Both phosphorylation and calmodulin binding could modulate the ability of MARCKS to serve as a dynamic, PKC- and calmodulin-regulated crossbridge between the actin cytoskeleton and the substrate-adherent plasma membrane during cell motility (Hartwig et al., 1992).

Targeted disruption of the murine MARCKS gene reveals a role for MARCKS during embryonic development. MARCKS null mice display a wide range of disorders, including exencephaly (severe brain malformation with an absence of the overlying skull or skin), umbilical herniation (abdominal contents protrude through abdomen), and runting (Stumpo et al., 1995). The observed malformations occur in only a subpopulation of MARCKS null mice, indicating that there is incomplete penetrance in the observed phenotypes. MARCKS null brains are severely malformed, with a reduction in brain size, disrupted commissures, and increased ventricular volume (Stumpo et al., 1995). These results suggest that MARCKS expression during mouse embryogenesis is necessary for normal development of the CNS.

**The MacMARCKS protein:** A protein related to MARCKS was cloned from an embryonic mouse cerebellum library (Umekage and Kato, 1991) and from a LPS-induced rabbit alveolar macrophage library (Li and Aderem, 1992). Nucleotide sequence analysis indicates that the predicted protein is very similar to MARCKS (Umekage and Kato, 1991; Blackshear *et al.*, 1991; Li and Aderem, 1992). It is constitutively expressed at high levels
in many organs, including the CNS, spleen and testis (Umekage and Kato, 1991; Lobach et al., 1993). Its expression in macrophages is highly induced after LPS stimulation (Li and Aderem, 1992). The gene for this protein is located on mouse chromosome 4, while the MARCKS gene is located on mouse chromosome 10 (Lobach et al., 1993; Blackshear et al., 1992). This protein is called either MacMARCKS (because it is highly enriched in LPS-stimulated macrophages; Li and Aderem, 1992) or MARCKS related protein (MRP; Blackshear, 1993). MacMARCKS represents the second member of the MARCKS family of PKC-substrates. It has an amino acid composition and domain structures which are very similar to that of MARCKS (Fig. 1-1a), but it is significantly smaller. Despite its predicted molecular mass of 20 kd, MacMARCKS migrates anomalously on SDS-PAGE as a 42-50 kDa protein. MacMARCKS is heat-stable, and has an acidic pI of 4 (Blackshear et al., 1991; Li and Aderem, 1992). The MacMARCKS myristoyl moiety is embedded in the lipid bilayer of membranes (Vergeres et al., 1995). Like MARCKS, the ability of MacMARCKS to bind membranes is dependent upon the electrostatic interaction of its basic effector domain with acidic phospholipids.

The MacMARCKS effector domain contains 24 amino acids which include 10 lysines and 2 arginine residues. This domain is 89% identical to the equivalent region in the MARCKS protein. In contrast to the MARCKS effector domain, the MacMARCKS effector domain (KKKKKFS\textsuperscript{93}FKPFKLSGLS\textsuperscript{103}FKR) is phosphorylated on only the first and third serine residues (Li and Aderem, 1992; Verghese et al., 1994); the second serine residue of the MARCKS effector domain is replaced with a proline residue in the MacMARCKS effector domain. Despite substitution of a proline residue for the second serine, the MacMARCKS effector domain peptide is still a high affinity substrate for PKC and is phosphorylated with significant positive cooperativity (Blackshear et al., 1992).

MacMARCKS binds calmodulin in a Ca\textsuperscript{2+}-dependent manner, and this binding is regulated by PKC-dependent phosphorylation (Li and Aderem, 1992; Vergeres et al.,
A MacMARCKS effector domain peptide bound calmodulin half-maximally at 7.4 ± 0.53 nM compared with 8.2 ± 0.6 nM for the MARCKS peptide, and both peptides complexed with calmodulin with a similar K_d of <3 nM (Blackshear et al., 1992). The time course of dissociation of the MacMARCKS peptide from calmodulin after PKC phosphorylation is identical to the time course for PKC disruption of the MARCKS peptide-calmodulin complex (Blackshear et al., 1992). Preliminary results also indicate that the MacMARCKS effector domain binds and cross-links actin (Aderem, unpublished data). These results suggest that the proline substitution within the MacMARCKS effector domain does not affect calmodulin or actin binding. This hypothesis is supported by the observation that MARCKS exists as a random coil in solution and does not adopt the α-helical conformation previously thought to be required for calmodulin binding (Manenti et al., 1992). MacMARCKS most likely adopts a similar random coil in solution. The affinity of MacMARCKS for acidic lipid vesicles is 30 fold less than MARCKS, and the strong cooperative effect between the effector domain of MARCKS and its myristoyl moiety is not observed for MacMARCKS (Vergeres et al., 1995). This suggests that additional protein(s) are required for the binding of MacMARCKS to intracellular membranes.

The myristoylation domain of MacMARCKS is separated from its effector domain by the MH2 domain; 16 out of 21 residues within this domain are identical between MacMARCKS and MARCKS, with the rest being conservative substitutions (Li and Aderem, 1992). This domain is homologous to a region of the mannose-6-phosphate/insulin-like growth factor II receptor involved in internalization (Aderem, 1992; Blackshear, 1993). As discussed above, this domain may be involved in actin cross-linking or protein dimerization (Aderem et al., 1992).

MacMARCKS is a good in vitro substrate for a mixture of PKC isozymes (Verghese et al., 1994) and is also phosphorylated by cdc2 kinase and tau protein kinase II
(Yamamoto et al., 1995). MacMARCKS phosphorylation is rapidly and transiently increased following glutamate stimulation of hippocampal pyramidal neurons (Scholz and Palfrey, 1991). Phosphorylation is also stimulated by PMA in murine neuroblastoma cells (Blackshear et al., 1992) and in macrophages (Li and Aderem, 1992). In the J774 macrophage cell line, endogenous MacMARCKS concentrates around nascent phagosomes (Zhu et al., 1995). Expression of a mutant MacMARCKS protein lacking the effector domain profoundly inhibits phagocytosis by J774 cells, while endocytosis was unaffected (Zhu et al., 1995).

The work presented in this thesis examines the role of MacMARCKS in neural secretion and neural development. Characterization of MacMARCKS in PC12 cells and rat brain synaptosomes is described in Chapter 3. Chapter 4 describes a dominant-negative MacMARCKS mutant which inhibits agonist-dependent secretion from PC12 cells. The characterization of the MacMARCKS promoter and its regulation by LPS is presented in Chapter 5. Chapter 6 examines endogenous MacMARCKS expression patterns, as well as the expression patterns of MacMARCKS promoter-lacZ constructs, during mouse development. Finally, Chapter 7 presents evidence that MacMARCKS is necessary for normal mouse neural development.
CHAPTER TWO:
MATERIALS AND METHODS

I. REAGENTS

Materials:

$[^3]H$]Myristic acid (0.4-2.2 TBq/mmolt), $[^32]P$]orthophosphate (320 TBq/mmolt), $[^{125}]I$protein A (370 kBq/ug), $[^3]H$]Norepinephrine (555 GBq/mmolt) and Enhance were obtained from New England Nuclear. PMA was purchased from LC Services, Corp (Woburn, MA) and Re595 LPS from Salmonella minnesota from List Biological Labs, Inc. Leupeptin and Nerve Growth Factor (NGF) 2.5S (from rat submaxillary gland, grade II) were obtained from Boehringer-Mannheim Biochemicals. Lipofectamine was purchased from Gibco BRL, and opsonized zymozan was purchased from Molecular Probes. Unless otherwise noted, all other chemicals were obtained from Sigma.

Antibodies:

Polyclonal antiserum against murine MacMARCKS was prepared by injecting rabbits with a purified GST-MacMARCKS fusion protein (J. Liet al., manuscript in preparation). Antibodies were affinity-purified from sera using strips of polyvinylidene difluoride membrane containing recombinant MacMARCKS. The affinity-purified MARCKS polyclonal antibody was prepared as described (Allen and Aderem, 1995; Rosen et al., 1990). Mouse monoclonal antibody against synaptophysin (Jahn et al., 1985) and anti-synapsin 1 antiserum were kind gifts from P. Greengard, A. J. Czernik and A. C. Nairn (The Rockefeller University). The rabbit polyclonal antibody directed against the lumenal amino terminus of synaptotagmin I (anti-Syt1um-Ab) was the kind gift from Drs. O. Mundigl and P. De Camilli (Yale University). The neuron-specific rabbit polyclonal
antibody NOVA was the kind gift from R. Buckanovich and Dr. R. Darnell (The Rockefeller University). The rabbit polyclonal antibody directed against bovine Glial Fibrillary Acidic Protein (GFAP) was the kind gift from Dr. M. B. Hattan (The Rockefeller University). The rat monoclonal antibody F4/80 directed against a macrophage/microglia-specific epitope (Hume and Gordon, 1987) was the kind gift from Dr. R. Steinman (The Rockefeller University). Commercial antibodies include both the mouse monoclonal and the rabbit polyclonal anti-HA antibodies (Berkeley), polyclonal anti-β-galactosidase antibody (3′-5′), monoclonal anti-β-galactosidase antibody (Johns Hopkins), Texas red-conjugated goat anti-rabbit IgG and Texas red-conjugated goat anti-mouse IgG (Tago), FITC-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rat IgG (Jackson), and non-specific rabbit IgG (Sigma).

II. CELL CULTURE

PC12 cell culture:

PC12 cells (obtained from the American Type Culture Collection, Bethesda, MD) were cultured in the absence of NGF as described previously (Greene and Tischler, 1976). They were maintained in RPMI medium (Gibco, Grand Island, NY) supplemented with 5% fetal calf serum and 10% horse serum (JRH Biosciences, Lenexa, KS) in an atmosphere of 5% CO₂/95% air (vol/vol) at 37°C. Two days before an experiment, cells were subcultured on rat tail collagen-coated plastic dishes (60 mm) at a density of 10⁶ cells/ml. Where indicated, NGF at 100 ng/ml was added 24 h prior to the experiment.

Transfection of PC12 Cells:

5 x 10⁵ PC12 cells were plated onto 100 mm collagen-coated dishes one day prior to transfection. Each plate was incubated with a lipofectin-DNA mixture (20-50 µg DNA
per plate) for 18 hrs. (Hempstead et al., 1994). After 48 hrs. the cells were placed in medium containing 400 µg/ml G418 (Gibco BRL). Following 5 weeks of selection surviving colonies were individually subcloned and expanded into 24 well plates.

**RAW Cell Culture:**

The RAW 264 macrophage-like cell line was obtained from ATCC and maintained in bacteriological dishes in RPMI 1640 medium containing 5% fetal calf serum supplemented with 2 mM L-glutamine (Stacey et al., 1995). Cells were passaged three times per week. One day before an experiment, RAW 264 cells were plated onto 60 mm tissue culture dishes (Corning) at a density of $1 \times 10^6$ cells/ml.

**Isolation of Murine Peritoneal Macrophages:**

Female ICR mice (Charles River) were sacrificed with CO₂. 4 ml of ice-cold sterile PBS was injected into the peritoneum, and the dislodged cells were transferred into a sterile tube. Another 4 ml of PBS was used to remove residual macrophages. The cells were pelleted at 1000 x g for 15 min. at 4°C, resuspended in α-MEM plus 10% FCS, and plated at approximately $1 \times 10^6$ cells/ml. Cells were allowed to adhere for two hours; nonadherent cells were removed by washing with PBS. Macrophages were incubated in fresh α-MEM plus 10% FCS overnight prior to the start of an experiment.

**Isolation and Culture of Microglial Cells:**

Brains from 1-3 day old neonatal mice were removed into ice-cold PBS. Cortices were isolated and stripped of meninges under the dissecting microscope, finely minced and incubated in PBS containing 0.5% trypsin, 0.025% DNase I (Worthington Biochemicals) for 15 min. at 25°C. After incubation, the cells were pelleted, resuspended in 2 ml α-MEM
plus 10% FCS, and disrupted by trituration with a drawn pipette. Cells were then plated at 10\(^6\) cells/ml in T-75 flasks. After one week of culture microglial cells were dislodged by shaking the flask at 180 rpm in an orbital shaker at 37°C. The supernatant containing microglia was isolated, while contaminating astrocytes remained in the flasks. Purified microglia were maintained in media containing 20% astrocyte conditioned medium.

Isolated microglia were routinely assayed for purity by several different criteria: immunocytochemistry with F4/80 antibody and anti-GFAP antibody; nonspecific esterase staining using a commercial kit (Sigma); DiI-Ac-LDL staining (Biomedical Technologies, Inc.) and phagocytosis of opsonized zymozan particles. By these methods, the isolated microglial population was >95% pure.

II. BIOCHEMICAL AND MORPHOLOGICAL TECHNIQUES

Myristoylation and phosphorylation of MacMARCKS:

PC12 or RAW 264 cells were labeled overnight with \(^{3}\text{H}\)myristic acid (40 mCi/ml), and lysed in ice-cold lysis buffer containing 10 mM Tris-HCl, pH 7.5, 15 mM EDTA, 1% (w/v) Nonidet P-40, 50 mM NaH\(_2\)PO\(_4\), 50 mM KF, 10 mM sodium pyrophosphate, supplemented with the following protease inhibitors: 0.09 TIU/ml aprotinin, 1 mM PMSF, 1 mM diisopropyl fluorophosphate, and 0.5 mg/ml leupeptin (Allen and Aderem, 1995). After cell lysis, MacMARCKS was immunoprecipitated as described below. Protein phosphorylation in intact cells was performed by radiolabeling cells with 250 mCi/ml \(^{32}\text{P}\)orthophosphate either in PO\(_4\)-free RPMI or in Hepes-buffered saline as described (Aderem \textit{et al.}, 1988). Where indicated, cells were treated with 200 nM PMA for 30 min. at 37°C. PC12 cells were depolarized with depolarization buffer (85 mM NaCl, 10 mM Hepes, 5.5 mM D-glucose, 60 mM KCl, 1 mM MgSO\(_4\), pH 7.4) either in the presence or absence of 1.8 mM CaCl\(_2\) for 1 min. at room temperature. After
stimulation, the cells were scraped into lysis buffer and the lysates subjected to immunoprecipitation.

Purified microglia were labeled for 3 hrs. with $[^3]H$ myristic acid (40 mCi/ml) either without treatment or in the presence of 100 ng/ml LPS; 200 nM PMA; or 50 ug/ml opsonized zymozan. Cells were washed, lysed as described above and equal amounts of proteins were loaded in all lanes. Proteins were resolved by 10% SDS-PAGE and visualized by fluorography.

**Subcellular fractionation:**

Agonist-treated PC12 cells labeled with either $[^3]H$ myristic acid or $[^32]P$ orthophosphate were scraped into 1 ml homogenization buffer (250 mM sucrose, 20 mM Hepes, pH 7.2, 1 mM EDTA) supplemented with protease inhibitors (see above). Cells were disrupted by nitrogen cavitation (1000 psi, 15 min. at 4°C), nuclei were removed by low speed centrifugation (500 x g, 5 min.), and the particulate and cytosolic fractions were prepared by centrifugation at 400,000 x g for 20 min. at 4°C in a Beckman TLA-100.2 rotor (Allen and Aderem, 1995; Thelen et al., 1991). The cytosolic fraction (supernatant) was removed and the pellet was resuspended in 1 ml of homogenization buffer. Proteins from each fraction were precipitated with TCA (final concentration 10%, w/v) and after solubilization were subjected to immunoprecipitation.

Synaptic fractions were prepared as described (Huttner et al., 1983) with minor modifications (McPherson et al., 1994). To obtain S3 and P3, the S2 fraction was centrifuged at 165,000 x gav for 2 h. The eluate from the controlled-pore glass (CPG) column was pooled into three fractions (CPG1-CPG3). These fractions were kindly prepared by Drs. P.S. McPherson and P. De Camilli (Yale University).
**Immunoprecipitation:**

Cell extracts were precleared with 20% (vol/vol) protein-A Sepharose, and MacMARCKS was immunoprecipitated with MacMARCKS antiserum at a 1:100 dilution (Thelen et al., 1991). TCA-precipitated proteins were immunoprecipitated as previously described (Allen and Aderem, 1995). HA-tagged proteins were immunoprecipitated with either the mouse monoclonal anti-HA antibody (1:200) or the rabbit polyclonal anti-HA antibody (1:400). Following immunoprecipitation, samples were boiled, and analyzed by 10% SDS-PAGE. $^{3}$H-labeled proteins were visualized by fluorography, and $^{32}$P-labeled proteins were visualized by autoradiography.

**Immunoblotting:**

Lysates prepared from PC12 cells, and proteins from synaptic fractions were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Towbin et al., 1979). The nitrocellulose membranes were blocked for 2 h with Tris-buffered saline (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 20 mM sodium azide) containing 5% (w/v) nonfat dry milk and incubated overnight with primary antibody at the indicated dilutions (1:200 for the anti-MacMARCKS and the anti-MARCKS antibodies, 1:5000 dilution for the anti-synaptophysin antibody, and 1:1000 for the anti-synapsin-1 antibody). Immunoblots were then washed three times in Tris-buffered-saline milk, incubated for 5 h with a 1:1000 dilution of $^{125}$I-labeled protein-A in Tris-buffered-saline, and washed 5 times with Tris-buffered-saline. Labeled proteins were visualized by autoradiography.

To identify HA-tagged proteins, heat treated lysates were resolved by SDS-PAGE, transferred onto nitrocellulose, and blocked overnight with PBST (1 mM Na$_2$HPO$_4$, 1 mM NaH$_2$PO$_4$, 150 mM NaCl, 0.01% Triton X-100, pH 7.1) containing 5% (w/v) nonfat dry milk. The blot was incubated for 2 hrs. with a 1:1000 dilution of anti-HA rabbit polyclonal antibody in PBST plus 5% milk, washed 3 times in PBST and incubated for 1 hr with a
1:1000 dilution of HRP-conjugated goat anti-rabbit 2° antibody. The blot was washed 3 times in PBST and subjected to enhanced chemiluminescence detection (Amersham) following manufacturer’s recommendations.

2-Dimensional polyacrylamide gel electrophoresis:

$^{32}$P-Labeled PC12 cell lysates were immunoprecipitated with the anti-MacMARCKS antibody, and subjected to 2-dimensional polyacrylamide gel electrophoresis as described (Li and Aderem, 1992). Radiolabeled MacMARCKS was visualized by autoradiography.

Preparation and labeling of rat brain synaptosomes:

Synaptosomes were purified from rat cerebrocortices as described (Dunkley et al., 1986) with minor modifications (Wang et al., 1989). The final purified synaptosomal pellet was resuspended in phosphate-free incubation buffer (140 mM NaCl, 5 mM KCl, 5 mM NaHCO$_3$, 1 mM MgCl$_2$, 10 mM glucose and 10 mM Hepes, pH 7.4) to 2 mg/ml and radiolabeled with $[^{32}\text{P}]$orthophosphate (1 mCi/ml) for 45 min. (Wang et al., 1988). Extrasynaptosomal $[^{32}\text{P}]$orthophosphate was removed by centrifugation of the synaptosomes at 1000 x g for 5 min. Radiolabeled synaptosomes (100 µg of protein) were incubated in the presence of 300 nM phorbol 12,13-dibutyrate (PDBu) or were depolarized by the addition of KCl to a final concentration of 40 mM (isotonicity being maintained by a corresponding decrease in the concentration of NaCl). Incubations were terminated by the addition of 4 vol of cold lysis buffer. Samples were then immunoprecipitated with the MacMARCKS antibody as described above.
**Immunofluorescence microscopy:**

PC12 cells were plated on poly-L-lysine coated coverslips and differentiated for 5 days in complete RPMI medium containing 100 ng/ml NGF. The cells were fixed in 4% (w/v) paraformaldehyde in 120 mM phosphate buffer containing 4% (w/v) sucrose, and processed for indirect immunofluorescence microscopy as previously described (Cameron et al., 1991; Mundigl et al., 1993). The coverslips were incubated with affinity purified anti-MacMARCKS antibody (1:50 dilution), followed by a 1:1000 dilution of a Texas red-conjugated goat anti-rabbit IgG (Tago, Burlingame, CA). Synaptophysin was visualized using a 1:1000 dilution of anti-synaptophysin antibody in conjunction with a 1:200 dilution of FITC-conjugated goat anti-mouse IgG (Jackson Immunoresearch Labs, Westgrove, PA). HA-tagged proteins were visualized using a 1:400 dilution of rabbit polyclonal anti-HA antibody, or a 1:200 dilution of the mouse monoclonal anti-HA antibody. Stained cells were visualized with a Zeiss Axiophot fluorescence microscope using a 63X Plan-Apochromat objective. Confocal microscopy was performed using the Molecular Dynamics' Image Space software and a Zeiss Axioskop microscope.

**Electron Microscopy:**

S-A-HA clone 28 PC12 cells were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde for 30 min., dehydrated and embedded in L. R. White resin (EM Science). Sections were collected on nickel grids and incubated with the anti-HA epitope 12CA5 monoclonal antibody at 1:1 dilution. Goat anti-mouse antiserum conjugated to 10 nM gold (Amersham) was used to probe the samples, which were visualized with a JEOL 100CX electron microscope (Peabody, Mass.).
III. FUNCTIONAL ASSAYS

Synaptotagmin Exocytosis Assay:

PC12 cells were differentiated for 5 days in the presence of 100 ng/ml NGF, plated onto poly-L-lysine coated coverslips, and washed twice for 5 min. at 37°C with Krebs-Ringer-HEPES (KRH) buffer (130 mM NaCl, 1.0 mM KCl, 1.0 mM Na2HPO4, 1.2 mM MgSO4, 2.7 mM CaCl2, 20 mM HEPES, and 11 mM glucose; Mundigl et al., 1995). The rabbit polyclonal anti-synaptotagmin lum antibody (Matteoli et al., 1992) was diluted 1:100 in Krebs-Ringer-HEPES buffers containing the following ionic compositions: KRH (128 mM NaCl, 3.0 mM KCl, 1.0 mM Na2HPO4, 1.2 mM MgSO4, 2.7 mM CaCl2, 20 mM HEPES, 11 mM glucose), KRH/55 mM K+ (76 mM NaCl, 55 mM KCl, 1.0 mM Na2HPO4, 1.2 mM MgSO4, 2.7 mM CaCl2, 20 mM HEPES, 11 mM glucose), Ca2+-free KRH (128 mM NaCl, 3.0 mM KCl, 1.0 mM Na2HPO4, 1.2 mM MgSO4, 2.7 mM CaCl2, 20 mM HEPES, 11 mM glucose), and KRH/55 mM K+ / Ca2+-free (76 mM NaCl, 55 mM KCl, 1.0 mM Na2HPO4, 1.2 mM MgSO4, 1.0 mM EGTA, 20 mM HEPES, 11 mM glucose). To control for nonspecific IgG binding to the cell surface, a rabbit IgG was used at a 1:100 dilution into the Krebs-Ringer-HEPES buffers. Cells were incubated with the antibodies for 1 hr at 37°C, immediately chilled on ice, and washed with ice-cold wash KRH. For experiments performed in Ca2+-free media, the last wash was done in Ca2+-free KRH. The cells were then fixed by 4% (w/v) paraformaldehyde in 120 mM phosphate buffer containing 4% (w/v) sucrose. Fixed cells were permeablized and blocked with goat serum dilution buffer (450 mM NaCl, 20 mM NaPO4, 17% goat serum, 0.3% Triton-X-100) and labeled with a 1:200 dilution of mouse anti-HA monoclonal antibody. Synaptotagmin was visualized with a 1:1000 dilution of Texas Red-conjugated goat anti-rabbit IgG, and HA-tagged MacMARCKS was visualized with a 1:400 dilution of FITC-conjugated goat anti-mouse IgG.
3H-Norepinephrine Release Assay:

One day before an experiment, PC12 cells were subcultured at 5 x 10^5 cells per 35 mm collagen-coated dish. The cells were washed twice with KRH buffer and incubated with 2 ml of KRH buffer containing 1 μCi/ml of 3H-norepinephrine (3H-NE) for 1 hr at 37°C. The labeled cells were pipetted off the dishes and loaded into sample assay columns of a superfusion apparatus (Brandel), which traps the cells between porous filter disks while being exposed to exogenously introduced solutions. The cells were washed with Ca^{2+}-free KRH buffer for 30 min. before the start of the experiment. Cells were perfused with fresh Ca^{2+}-free KRH buffer for 5 minutes and 0.5 ml fractions were collected every minute to establish a baseline of spontaneous 3H-NE release. To stimulate regulated 3H-NE release, the cells were perfused for 5 min. with either KRH, Ca^{2+}-free KRH, KRH/55 mM K^+, KRH/55 mM K^+ / Ca^{2+}-free, KRH + 4 μM ionomycin, or Ca^{2+}-free KRH + 4 μM ionomycin. Fractions were collected every minute for 5 minutes. Following agonist stimulation the cells were again perfused with Ca^{2+}-free KRH for a final 5 min., and fractions were again collected every minute. The filter disks were removed and the cells hydrolyzed with 0.5N NaOH to determine the amount of cell-bound 3H-NE. 3.5 ml of Ready-Safe scintillation fluid (BioRad) were added to all fractions and the samples were counted in a LKB scintillation counter. Each experiment was done in triplicate.

Wheat Germ Agglutinin Endocytosis Assay:

Texas red-conjugated wheat germ agglutinin (TR-WGA; Molecular Probes) was diluted in culture medium at 50 μg/ml and applied to NGF-differentiated PC12 cells grown on polylysine coated coverslips at 37°C (Mundigl et al., 1995). After one hour the cells were washed extensively, fixed and the pattern of WGA distribution examined by confocal microscopy.
IV. MOLECULAR BIOLOGY TECHNIQUES

Generation of HA-Tagged MacMARCKS cDNA Constructs:

PCR was used to generate a wild type MacMARCKS cDNA construct containing a hemaglutinin epitope (HA) sequence at the 3' end. The 5' oligo A1 (5'GCGGGATCCATGGGCAGCCAGAGCTCT3') contained a BamHI site (underlined) to facilitate cloning, and the 3' oligo A2(5'GCGGGATCCTCACAAGCTAGCGTAATCTGGAACA TCGTATGGGTACTCATTCTGCTCAGC ACT3') contained both the HA sequence (bold) as well as a BamHI site (underlined) for cloning. Mouse MacMARCKS cDNA (Li and Aderem, 1992) was used as the template. It was denatured for 30 sec at 95° C, annealed at 62° C for 30 sec, and extended at 72° C for 30 sec. This reaction was repeated for 30 cycles. The reaction product was subcloned into pBlueScript and completely sequenced; this construct is named wt-HA MacMARCKS.

To obtain a construct encoding serine to alanine mutations within the MacMARCKS effector domain, two DNA fragments spanning this domain were generated by PCR, using WT-MacMARCKS-HA as the template. The first fragment encoded amino acids 1-100, and was generated with the 5' A1 oligo and 3' B1 oligo (5'AAGCTTGAAAGGCTTCTTGAAACGAGAATTTCT 3'). The second DNA fragment encoded amino acids 99-200 as well as the 11 amino acid HA epitope, and was generated with the 5' B2 oligo (5'AAGCTTAGTGGCCTGGCCTTCAAGAGAAAT3') and the 3' A2 oligo. Letters in bold indicate nucleotide changes that converted serines to alanines at position 93 (5'TCT3' to 5'GCT3') and at position 104 (5'TCC3' to 5'GCC3'). Underlined letters represent nucleotide changes that generate a novel HindIII site while maintaining the correct MacMARCKS amino acid sequence. Both fragments were digested with HindIII and ligated together at this site. The mutated MacMARCKS fragment was
then subcloned into the BamHI and EcoRI sites of pBlueScript and completely sequenced. This construct is named S-A-HA MacMARCKS.

For high levels of expression in PC12 cells, both the wt-HA MacMARCKS and the S-A-HA MacMARCKS constructs were digested with BamHI and EcoRI and subcloned into the pcDNA3 vector (Invitrogen) under the control of the cytomegalovirus promoter. Transfections into PC12 cells were as described above.

Isolation of RNA and RNase Protection:

RAW 264 cells were either untreated or stimulated with the compounds indicated and total RNA was isolated with the guanidinium method (Chomczynski and Sacchi, 1987). To map the RNA initiation site of the MacMARCKS gene, a 620 bp XbaI-Sacl fragment from the MacMARCKS genomic DNA was subcloned into pBlueScript. This construct was linearized at the XbaI site and T3 RNA polymerase (Promega) was used to generate $^{32}$P-labeled run-off RNA transcripts complementary to the MacMARCKS sequence from -413 to +203. The RNA transcripts were gel-purified and allowed to hybridize with 20 µg of total RAW 264 RNA at 50° C overnight. After hybridization, the reaction was digested with a mixture of RNase A and RNase T1 for 30 min. using the RPAII kit (Ambion). The approximately 200 bp protected fragments were resolved in a 6% sequencing gel and detected by autoradiography.

To analyze the levels of MacMARCKS message following agonist stimulation of RAW 264 cells, the S-A MacMARCKS-HA construct (see above) was linearized with HindIII and T3 RNA polymerase was used to generate a 315 bp run-off transcripts complementary to the MacMARCKS coding sequence (+485 to +745). The RNase protection assay was performed as described above, except that 5 µg of total RNA was used per reaction, and a 250 bp β-actin antisense RNA probe was included as an internal
control. Protected fragments (251 bp for MacMARCKS, 210 bp for β-actin) were resolved in a 6% sequencing gel and detected by autoradiography.

**Mapping of MacMARCKS Transcriptional Start Site By Primer Extension:**

Total RNA was isolated from RAW 264 cells that were either untreated or stimulated for 4 hrs. with LPS (100 ng/ml). A 30-base oligonucleotide complementary to the 5' end of the MacMARCKS cDNA (positions +174-203; 5’GAGCTCTGGCTGC CCATGATGGGGTCTGC3’) was 32p end-labeled at the 5' terminus and mixed with 50 μg of total RNA. After hybridization in buffer A (Ambion) at 30°C overnight, the primer was extended with Moloney murine leukemia virus reverse transcriptase (Boehringer-Mannheim Biochemicals) in the presence of 50 μg/ml actinomycin D. The 205 bp radiolabeled products of this reaction were resolved on a 6% polyacrylamide/7 M urea sequencing gel alongside a dideoxy-sequencing ladder generated with the same oligonucleotide and visualized by autoradiography.

**Transfection and Transient Expression of Luciferase Reporter Constructs:**

The 1.7 kb NheI restriction fragment upstream from the MacMARCKS gene was cloned into the pBlueScript vector (Stratagene) to generate a 1.7 kb-pBlue construct. Deletion constructs were generated in two ways. First, the 1.7 kb-pBlue construct was digested with AvrI and the resultant fragment was subcloned into the GeneLight luciferase vector system (Promega). The second method generated nested deletions in 1.7 kb-pBlue by Exonuclease III digestion (Henikoff, 1984), which were subcloned into the same GeneLight vector. The 1.7 kb-pBlue construct was first digested with SacI and NotI and the Erase-a-Base system (Promega) was used to generate an ordered set of deletion clones of various sizes, spanning 1.7 to 0.4 kb (see Fig. 5-4A). All constructs were then sequenced for definitive characterization.
Plasmid DNA was purified by CsCl banding and 10 µg of the constructs were introduced into RAW 264 cells by electroporation as described (Stacey et al., 1993). To control for differences in the amounts of transfected DNA a CMV-CSF-I construct was co-transfected with the MacMARCKS promoter-reporter constructs. Cells from 4 separate electroporations were pooled and distributed equally between 4 60 mm tissue culture dishes, and the medium was changed 2 hrs. later. After 48 hr the transfectants were stimulated for 4 hrs. with medium alone or with LPS at 100 ng/ml. Supernatants were radioimmunoassayed for CSF-1 (Stacey et al., 1995). The cells were washed once with PBS and lysed in buffer containing 1% Triton X-100 (Promega). Aliquots of the lysate were assayed for luciferase activity in a luminometer.

**Nuclear Run-On Analysis:**

Nuclei were isolated from RAW 264 cells as described (Stacey et al., 1995) after the cells were incubated either in control medium or in medium supplemented with cyclohexamide (10 µg/ml). The labeled run-on transcripts were prepared and hybridized with denatured linearized plasmid DNA (10 µg/slot) immobilized on nitrocellulose. Equal amounts (cpm) of radiolabeled RNA were used for each filter. Filters were prehybridized and hybridized in 5 X SSPE, 5 X Denhardt’s solution, 0.5% SDS, 50 µg of denatured herring sperm DNA/ml, and 200 µg of yeast tRNA/ml at 42°C. After hybridization for 36 hrs., the filters were washed twice for 20 min. each with 2 X SSC at 42°C, twice for 20 min. with 1 X SSC-0.1% SDS at 55°C and once for 30 min. with 2 X SSC with 10 µg of RNase A at 37°C. The filters were then rinsed and subjected to autoradiography.
V. GENERATION OF LAC-Z TRANSGENIC MICE

Transgenic DNA Constructs:

Using MacMARCKS cDNA probes specific to 5’ ends a 17 kb genomic clone containing the entire murine MacMARCKS gene was isolated from a 129SV genomic library. By restriction enzyme mapping, the clone encompassed the entire MacMARCKS coding region as well as 8 kb upstream and 4 kb downstream sequences. A 4 kb BamHI-NheI fragment of the MacMARCKS 5’ upstream sequence containing the transcriptional start site plus 30 bp of the untranslated region was subcloned into pBlueScript to take advantage of convenient flanking restriction sites. A lacZ reporter construct was made by subcloning the 4 kb MacMARCKS SacI-SalI fragment from this construct into the EcoRI and XhoI sites of the pNASSb vector (Clontech; Li et al., 1995); this clone was named 4kb-TM. The plasmid was digested with SpeI and Sall, the linearized DNA construct was separated from the vector sequence by agarose gel electrophoresis, and finally purified by elute-tip (S&S). This DNA was reconstituted in 10 mM Tris pH 7.5 and 2 mM EDTA at 4 ug/ml. A second construct, 1.7 kb-TM, was generated by digesting 4kb-TM with PstI and purified as described above.

Generation and Analysis of Transgenic Mice:

Transgenic mice were generated by the Rockefeller transgenic facilities according to the method of Hogan et al., (1994) using B6/CBA hybrid mice as donors. Transgenic mice were identified by analysis of genomic DNA isolated from tail tips by both PCR and Southern blot analysis. To type the transgenic mice, 0.3 cm tail fragments were incubated with lysis buffer (see above) at 55° C overnight and precipitated with an equal volume of 100% ethanol. The genomic DNA was spooled out with a drawn out pipette and dissolved in 1 mM Tris-HCl, pH 7.4 and 0.2 mM EDTA.
For PCR analysis, PCR primers flanking the first 750 bp of the *E. coli* β-galactosidase open reading frame were used: 5’ lacZ oligo (5’GCGCTGTACTGGAGGCTG3’) and the 3’ oligo (5’GCGATACAGCGCGTCGTG3’). DNA was denatured for 1 min. at 95°C, annealed at 62°C for 45 sec, and extended at 72°C for 45 sec. These conditions specifically detected a 750 bp fragment from positive transgenic animals, while little signal was detected in non-transgenic animals.

For Southern blot analysis, the DNA samples were digested with either HindIII or SacI overnight, resolved on a 1% agarose gel and transferred to Nytran nylon membranes. The membranes were prehybridized overnight in prehybridization buffer (50% formamide, 6X SSC, 10X Denhardt’s, 1% SDS, 100 μg/ml salmon sperm DNA) and hybridized in the same solution with either the first 750 bp of the coding sequence of the lacZ gene, or with a 0.6 kb Xba-SacI fragment from the MacMARCKS gene to estimate copy numbers.

Positive founders were bred with wild type CD1 mice to generate F1 mice. One positive F1 mouse from each line was sacrificed for histological analysis, and the rest were either interbred to generate homozygous transgenic mice or bred with CD1 mice to generate embryos.

β-galactosidase enzyme staining of embryos and tissue sections:

Embryos prior to E18.5 were dissected out of their dissidua and yolk sac, fixed with 0.2% glutaraldehyde, and washed in wash buffer (0.1 M NaPO₄, 0.02% NP-40, 0.01% deoxycholate, 2 mM MgCl₂). Embryos were stained in 1 mg/ml X-gal, 20 mM K₃Fe(CN)₆, 20 mM K₄Fe(CN₆) in wash buffer at 37°C overnight in the dark. To stain E18.5 embryos, the mother was perfused transcardially with 4% paraformaldehyde containing 2 mM MgCl₂. The embryos were then dissected out, individually tailed to isolate genomic DNA, and fixed in 0.4% paraformaldehyde containing 2 mM MgCl₂. After PCR analysis, transgenic embryos were infiltrated with 30% sucrose as a cryoprotectant.
and frozen in OCT (Miles Inc.). 20 μ sagittal sections obtained on a Microm cryostat were stained as described above, or with antibodies to β-galactosidase (see below).

To obtain organs from adult transgenic mice for staining, transcardial perfusions were performed prior to organ dissection, as described above. Small organs such as spleen and testis were stained whole, while the brain was either sectioned with a razor blade to obtain 1-2 mm coarse sections, or frozen in OCT and sectioned at 20 μ. Brain sections were post-fixed on ice with 0.4% paraformaldehyde in PBS containing 2 mM MgCl₂, rinsed with ice cold wash buffer, and stained for β-galactosidase reactivity as described above.

**Immunohistochemical detection of β-galactosidase:**

Brain sections were prepared as described above and blocked in goat serum dilution buffer (GSDB; 450 mM NaCl, 20 mM NaPO₄, 17% goat serum, 0.3% Triton-X-100) for 2 hours. All antibodies were diluted in GSDB. For double-labeling with GFAP (Dako), the samples were incubated with a 1:1000 dilution of GFAP and a 1:100 dilution of affinity-purified JIE7 monoclonal mouse anti-β-galactosidase antibody (Developmental Studies Hybridoma Bank) for 1 hour. For double labeling with NOVA (Buckanovich *et al.*, 1995), a 1:100 dilution of NOVA was added along with JIE1, and for double-labeling with F480 a 1:1 dilution of the mouse monoclonal anti-F480 antibody was added together with the β-galactosidase antibody. Both fluorescein anti-mouse and fluorescein anti-rat secondary antibodies were used at 1:400, while Texas Red anti-rabbit secondary antibody was used at 1:1000.

To co-localize cells that stained positive with X-gal to a cell-type specific antibody marker, brain sections were first stained for β-galactosidase enzymatic activity, followed by immunohistochemistry with antibodies against NOVA, GFAP and F-480.
VI. GENERATION OF MacMARCKS NULL MICE

Construction of MacMARCKS Targeting Vector:

The targeting construct was generated in the pPNT vector containing the neomycin (neo) and herpes simplex virus thymidine kinase cassettes for positive and negative selection, respectively (Tybulewicz et al., 1991). The expression of both genes is driven by the mouse phosphoglycerate kinase-1 promoter and PGK-1 polyadenylation sequences are located at their 3' ends. To facilitate cloning, the pPNT vector was modified by the addition of a SalI site in between the unique NotI and XhoI sites, the EcoRI site was replaced with a XbaI site, and the XbaI site was replaced with an EcoRI site (see Fig. 7-1A).

A 17 kb genomic clone containing the entire murine MacMARCKS gene was isolated from a 129SV genomic library using MacMARCKS cDNA probes. By restriction enzyme mapping, the clone encompassed the entire MacMARCKS coding region as well as 8 kb upstream and 4 kb downstream sequences. A 7.5 kb Sac I fragment containing the 5' upstream sequences including part of exon 1 was inserted into the SacI site of the pTZ-neo vector to utilize that vector's 5' flanking NotI and 3' flanking SalI restriction sites. This construct was subsequently digested with NotI and SalI and the 7.5 kb NotI-SalI fragment was subcloned into the corresponding sites upstream of the pgk-neo gene. The 1.3 kb EcoRI-Xba I fragment containing 3' flanking sequences was inserted into corresponding sites in the pPNT vector 3' of the pgk-neo gene and upstream of the pgk-tk gene. To aid in the identification of the mutant allele, a HindIII site was engineered into the SalI site of the modified pPNT vector upstream of the neo gene.
Transfection and Culture of ES Cells:

50 ug of the MacMARCKS targeting vector was linearized by NotI digestion and electroporated into 1.0 x 10⁷ J1 (Li et al., 1992) or E14 (Hooper et al., 1987) embryonic stem (ES) cells at 400V and 250 uF. ES cells were cultured on neo-resistant mouse embryonic fibroblasts in the presence of LIF (500U/ml) (Robertson, 1987). Positive selection was carried out with 250 ug/ml G418, and negative selection was carried out with 2 uM FIAU (O’Classen Pharmaceuticals). 10 days post transfection, G418 and FIAU resistant colonies were picked and expanded into 96 well plates. After 6 days, the colonies were expanded to 2 sets of 24 well plates. 2 days later one set of plates were frozen as stock and 5 days later the other set was used for Southern blot and PCR analysis (see below).

Generation of Chimeric Mice:

To identify positive ES cells, ES cells were incubated in lysis buffer (20 mM EDTA, 1% Sarkosyl, 0.2 M Tris-HCl, pH 8.0, 0.4 M NaCl, 8 M urea) with 0.8 mg/ml proteinase K overnight at 55 C to extract genomic DNAs for Southern blot and PCR analysis. After digestion, the DNA was ethanol precipitated and redissolved in 500 µl 1mM Tris-HCl, pH 8.0, 0.1 mM EDTA. 1 µl of the DNA was subjected to PCR, using the primers mm1 (5'GAGGAGTCTGACTACCTGAGAAGTC3') derived from the 3' end of the 1.3 kb EcoRI-XbaI fragment, and mm2: (5'GCCAAGTTCTAATTCCATCAGAAGC3') derived from the 3' end of the pgk neo gene. DNA was denatured at 94°C for 30 sec, annealed at 65°C for 30 sec and extended at 72°C for 1.5 min. The cycle was repeated 39 times. DNAs positive from the PCR screen were digested with HindIII, electrophoresed, transferred onto nitrocellulose and hybridized at high stringency to a MacMARCKS cDNA to confirm the sites of recombination.
Fifteen to twenty ES cells were microinjected into C57Bl/6J blastocysts by the Rockefeller Transgenic Facility. Injected blastocysts were transferred into the uterine horn of pseudo-pregnant (C57Bl/6J x CBA/J) F1 recipients (Bradley, 1987). Chimeric mice were backcrossed to 129/sv or C57Bl/6J and germline transmission was determined by the presence of the agouti coat color in a black background. Heterozygous offspring were identified by PCR analysis. Each agouti mouse was analysed with two reactions: one to detect the presence of the neo gene (730 bp product), using the primers JM 43 (5’CTCTTCCTCATCTCCGGGC CTT3’) derived from the 3’ end of the pGK promoter, and N2 (5’AATATCACG GGTAGCCAACGCTATG3’) derived from the 5’ end of the neo gene. The other PCR reaction was designed to detect the MacMARCKS gene (200 bp product) using primers IN1 (5’TGAACGTGAGAGGAGGAGGAGGAGGAGGAGG3’) derived from the 5’ end of the MacMARCKS intron, and IN2 (5’AATATCACGGGTAG CCAACGCTA TG3’), derived from the 3’ end of the intron.

Genotypic Analysis of Chimeric and Mutant Mice:

MacMARCKS embryos obtained from heterozygote matings were genotyped by PCR. Noon of the day of the plug was considered as E0.5. For embryos older than E8.5, the yolk sac was used to generated DNA after solublization in lysis buffer overnight at 55°C. 1 µl of the DNA was used in each PCR reaction, and the reaction conditions were the same as those for analysis of the ES cells. In some of the analyses primers specific for the testis-determining gene Sry (5’ oligo: 5’GAGAGCATG GAGGGCCAT3’; 3’ oligo: 5’CCACCTCTCTGTGACACT3’) were used to unambiguously identify male embryos.

Histology:

E8.5 to E12.5 embryos were dissected out of their decidua and yolk sac in PBS under a Nikon Stemi 6000 dissecting microscope. Dissected embryos were fixed with 4%
paraformaldehyde in PBS, washed, and subjected to a glycerol series (20%, 50%, 80%) before being photographed under dark-field luminescence. To analyze E18.5 embryos, the pregnant female mouse was first perfused transcardially with 4% paraformaldehyde, and the embryos were then dissected out and photographed in PBS. The brains from mutant and heterozygous animals were then dissected for further analysis.

For wax sections, embryos and E18.5 brains were dehydrated in methanol and transferred to 100% xylene. Cleared samples were transferred to a mixture of 50% xylene/50% paraplast at 60°C for 0.5 to 4 hrs., depending on the size of the embryo. The samples were then incubated in 100% paraplast at 60°C for 3 hrs. After mounting, embryos embedded in Paraplast were sectioned in a Microm microtome and 10-15 μm sections were collected. Serial 5 mm sections were collected for the E18.5 brains. Embryo sections were dewaxed in xylene, rehydrated and subjected either to in situ hybridization or mounted in Permount for observation. Brain sections were similarly dewaxed, rehydrated, counterstained with eosin and hematoxylin (Sigma) following standard procedures, dried and mounted with Permount (Sigma). Sections were photographed under Normarski optics with a Zeiss Axiophot microscope.

Whole-mount in situ hybridization:

A *MacMARCKS*- pBlueScript plasmid containing 1.5 kb full length cDNA was used to prepare antisense RNA probes (Li and Aderem, 1992). *Emx-2* and *En-2* containing plasmids were as previously described (Simeone et al., 1992; Davis et al., 1988). Single stranded riboprobes were synthesized from linearized plasmids in the presence of digoxygenin-UTP and a trace of $^{32}$P-UTP to measure incorporation. Whole mount in situ hybridization was performed essentially as described (Hogan et al., 1995). Dissected embryos were fixed in 4% paraformaldehyde for 2 hrs. at 4°C. Hybridization was carried out in 50% formamide buffer at 70°C overnight, followed by antibody
detection with alkaline phosphatase coupled anti-digoxygenin antibodies with purple blue as a substrate (Boehringer Mannheim). The embryos were stained by reaction with NBT and BCIP, washed, and dehydrated through a methanol series before being photographed under dark-field luminescence using a Nikon Stemi 6000 stereomicroscope.

**Section in situ hybridization:**

*MacMARCKS* antisense RNA probes were synthesized from the linearized plasmid in the presence of $^{35}$S-UTP. Sections for in situ hybridization was prepared as described (Duncan et al., 1994). Sections hybridized to the MacMARCKS sense probe showed no signal above background. All slides were photographed under both bright and dark field illumination using a Nikon Stemi 6000 stereomicroscope.

**Immunohistochemistry Of Whole Mount Embryos:**

MacMARCKS antibody staining of whole mount embryos were performed as described (Hogan *et al.*, 1995; Dent *et al.*, 1989). Affinity purified anti-MacMARCKS antibody was used at 1:50 dilution, and the affinity purified peroxidase-conjugated goat anti-rabbit secondary antibody (Jackson) was used at a 1:300 dilution.
CHAPTER THREE

CHARACTERIZATION OF MacMARCKS IN SYNAPTOSOMES
AND PC12 CELLS

INTRODUCTION

The Protein kinase C (PKC) family of enzymes have key roles in intracellular signal transduction for a wide variety of cellular functions, including hormone and neurotransmitter release (reviewed in Robinson, 1992). However, relatively little is known about the downstream events which occur after PKC activation, and it is not understood how the PKC signal is transduced into the interior of the cell. Therefore, it is important to identify and characterize physiological substrates for PKC. PKC phosphorylates many proteins in vitro; however, few substrates have been well characterized in intact cells. One physiologically important PKC substrate is MARCKS, a membrane-associated, actin-binding protein widely distributed in all tissues examined (Aderem, 1992; Blackshear, 1992). The membrane association and actin binding activities of MARCKS are regulated both by Ca\(^{2+}\)/calmodulin and by PKC-mediated phosphorylation (Thelen et al., 1991; Hartwig et al., 1992). MARCKS is implicated in a variety of actin-cytoskeleton-related cellular processes including motility (Rosen et al., 1990), secretion (Sawai et al., 1993) and phagocytosis (Allen and Aderem, 1995).

In the brain MARCKS represents approximately 0.2% of cellular proteins (Graff et al., 1989). Phosphorylation of the MARCKS protein is stimulated by depolarization or phorbol ester stimulation of intact synaptosomes (Dunkley et al., 1986) or brain slices (Rodnight and Leal, 1990). Stimulation of phosphorylation by either depolarization or phorbol esters results in the translocation of MARCKS from the synaptosomal membrane...
fraction to the cytosol (Wang et al., 1989), indicating that PKC-catalyzed phosphorylation of MARCKS occurs on the membrane, and regulates its translocation to the cytosol.

MacMARCKS is a second member of MARCKS family of protein kinase C substrates (Aderem, 1992, Blackshear, 1992). MacMARCKS cDNA was initially cloned by screening a mouse cerebellar cDNA library for transcripts preferentially expressed in the cerebellum (Kato, 1990; Umekage and Kato, 1991). In situ hybridization experiments indicate that MacMARCKS is expressed throughout the brain, with enrichment in specific regions, including the olfactory cortex and dentate gyrus (Umekage and Kato, 1991). MacMARCKS cDNAs have also been cloned from mouse macrophages (Li and Aderem, 1992) and rat kidney epithelial cells (Hyatt et al., 1994). Northern blot analysis indicates that although MacMARCKS is expressed in a wide variety of mouse tissues, elevated levels of expression are seen in the cerebellum, cerebrum, testis and uterus (Lobach et al., 1993). MacMARCKS is also highly expressed in bacterial lipopolysacharide (LPS)-stimulated macrophages (Li and Aderem, 1992), where it is associated with endocytic compartments and the plasma membrane (Li et al., unpublished observations).

This chapter describes the characterization of MacMARCKS in both PC12 cells and in rat brain synaptosomes. MacMARCKS is phosphorylated in PC12 cells and, like MARCKS (Wang et al., 1989), is phosphorylated in rat brain synaptosomes upon depolarization and phorbol ester treatment. Within PC12 cells MacMARCKS colocalizes with the vesicular marker synaptophysin in distal neuronal processes and in perinuclear endosomes. MacMARCKS also associates with a number of membrane vesicular fractions purified from rat brain, including a synaptic vesicle fraction.
RESULTS

**MacMARCKS is expressed and phosphorylated in PC12 cells:**

A myristoylated protein with an apparent molecular mass of 48 kDa was immunoprecipitated from cell lysates of PC12 cells labeled with \[^3\text{H}\]myristic acid using a rabbit antiserum raised against murine MacMARCKS (Fig. 3-1a). The 48 kDa protein was heat stable, and was the only protein recognized upon immunoblotting with antiserum to MacMARCKS (Fig. 3-1b). The protein was phosphorylated when \[^32\text{P}\]-labeled PC12 cells were stimulated with PMA, suggesting that it is a PKC substrate (Fig. 3-1c). Furthermore, the immunoprecipitated phosphoprotein had an isoelectric point of 4.2 (Fig. 3-1c), identical to that of MacMARCKS (Li and Aderem, 1992). The 48 kDa protein was also phosphorylated in PC12 extracts by purified PKC, and two-dimensional thermolytic phosphopeptide mapping showed a single phosphopeptide that was identical to that generated from purified recombinant murine, or from purified rabbit macrophage MacMARCKS phosphorylated by PKC (Li and Aderem, 1992). These data indicate that the 48 kDa PKC substrate in PC12 cells is MacMARCKS.

**MacMARCKS is phosphorylated in PC12 cells upon phorbol ester stimulation and KCl-induced depolarization:**

MacMARCKS displayed a basal level of phosphorylation in unstimulated PC12 cells (Fig. 3-2a). PMA treatment or KCl-induced depolarization increased MacMARCKS phosphorylation 4-fold (Fig. 3-2a). Depolarization-induced phosphorylation was completely dependent on Ca\(^{2+}\) in the medium (Fig. 3-2a, lane 3). Since the related PKC substrate, MARCKS, is released from membranes upon phosphorylation (Aderem et al., 1988; Thelen et al., 1991), we examined whether phosphorylation similarly displaced MacMARCKS from membranes. In both unstimulated cells, and in cells depolarized with...
**Figure 3-1.** MacMARCKS is expressed and phosphorylated in PC12 cells.

a. PC12 cells were labeled with \(^3\)Hmyristic acid and MacMARCKS was immunoprecipitated from the cell lysate. The lysate and immunoprecipitate were resolved by 10% SDS-PAGE, and myristoylated proteins were visualized by fluorography. Left lane; total cell lysate labeled with \(^3\)Hmyristic acid. Right lane; immunoprecipitated 48 kDa protein.

b. Lysates from PC12 cells were heat treated and centrifuged. The heat stable proteins (supernatant) were resolved by SDS-PAGE, blotted onto nitrocellulose and subjected to Western blotting with the anti-MacMARCKS antibody.

c. \(^3\)P-labeled PC12 cells were treated with 200 nM PMA for 30 min., lysed, and immunoprecipitated with anti-MacMARCKS antibody. The immunoprecipitates were then subjected to two-dimensional polyacrylamide gel electrophoresis.
Figure 3-2. MacMARCKS is phosphorylated upon depolarization of PC12 cells.

a. PC12 cells radiolabeled with $^{32}$P$_i$ were either not treated (lane 1), exposed to PMA (200 nM, 30 min., lane 2), or subjected to depolarization (60 mM KCl for 1 min.) either in the absence (lane 3) or presence (lane 4) of 1.5 mM extracellular Ca$^{2+}$. PC12 cells were solubilized in lysis buffer and immunoprecipitated with anti-MacMARCKS antibody. Immunoprecipitates were resolved by 10% SDS-PAGE and visualized by autoradiography.

b. PC12 cells were prelabeled with $[^3]$H]myristic acid, treated with control buffer (C), 60 mM KCl (K$^+$), or 200 nM PMA (PMA) and fractionated to isolate particulate (P) and cytosol (S). The fractions were immunoprecipitated with anti-MacMARCKS antibody, and MacMARCKS was visualized by fluorography after 10% SDS-PAGE.

c. PC12 cells were radiolabeled with $^{32}$P$_i$, treated and fractionated as described in (b). Immunoprecipitated MacMARCKS was visualized by autoradiography after 10% SDS-PAGE.
elevated KCl, greater than 95% of [\(^3\)H]myristoylated MacMARCKS was associated with the membrane fraction (Fig. 3-2b). Similarly, most of the phosphorylated MacMARCKS was associated with the membrane fraction of control or depolarized PC12 cells (Fig. 3-2c), although the stoichiometry of phosphorylation of the cytosolic protein appears higher than its membrane-bound counterpart (Fig. 3-2b and c). PMA treatment resulted in the release of approximately 20% of [\(^3\)H]myristoylated MacMARCKS from the membrane. The stoichiometry of PMA-induced phosphorylation of the cytosolic form was also greater than that of the membrane bound protein (Fig. 3-2b and c).

**Immunolocalization of MacMARCKS in PC12 cells:**

NGF-treated PC12 cells immunofluorescently labeled with an affinity purified antibody to MacMARCKS revealed that MacMARCKS is localized throughout the cell in a punctate pattern, with prominent staining in neuronal processes and varicosities (Fig. 3-3a and b). Confocal microscopy confirms these results, and further demonstrates the anti-MacMARCKS staining in neuronal processes (Fig. 3-3c). By double-labeled immunofluorescence microscopy, MacMARCKS co-localizes with synaptophysin (Fig. 3-3c and d), a marker of small synaptic vesicles and endosomes in PC12 cells (Cameron *et al.*, 1991; Linstedt *et al.*, 1992). Co-localization of MacMARCKS and synaptophysin was especially evident in distal neuronal processes (Fig. 3-3c and d, arrows), and was also prominent in the perinuclear region. NGF-induced differentiation did not influence the level of expression of MacMARCKS protein, as determined by immunoblotting (data not shown).
**Figure 3-3.** MacMARCKS and synaptophysin co-localize in NGF-differentiated PC12 cells.

a. Differential interference contrast image of NGF-differentiated PC12 cells.
b. MacMARCKS immunoreactivity is present in the tips of neurite processes and in the perinuclear region in a punctate manner.
c, d. Confocal immunofluorescence microscopy demonstrates the overlapping distribution of MacMARCKS (c) and synaptophysin (d) in PC12 cells. Arrows indicate neuronal processes where MacMARCKS and synaptophysin immunoreactivity are abundant.
PKC agonists or depolarization induce the phosphorylation of MacMARCKS in rat brain synaptosomes:

The presence of MacMARCKS in the tips neuronal processes of PC12 cells and its co-localization with synaptophysin suggested that it might be found in isolated nerve terminals. $^{32}$P-Labeled synaptosomes were treated with either the active phorbol ester PDBu or elevated KCl, and phosphorylated MacMARCKS was immunoprecipitated and resolved by SDS-PAGE (Fig. 3-4a). Stimulation of endogenous PKC with PDBu resulted in near maximal phosphorylation of synaptosomal MacMARCKS within 20 sec, and these levels were sustained for at least 2 min. (Fig. 3-4a and b). The initial kinetics and extent of depolarization-dependent phosphorylation of MacMARCKS paralleled that of PDBu-induced phosphorylation, but was more transient (Fig. 3-4a and b).

Subcellular distribution of MacMARCKS:

The distribution of MacMARCKS in subcellular fractions from rat cerebral cortex was examined using a procedure which results in the isolation of a variety of vesicular populations including a highly purified synaptic vesicle fraction (Huttner et al., 1983). As expected, both synapsin 1 and synaptophysin were highly enriched in purified synaptic vesicles (CPG3) and were absent from soluble fractions (S3 and LS2), (Fig. 3-5). MacMARCKS was detected in most membrane fractions, including small vesicles (P3 and LP2), and purified synaptic vesicle fractions (CPG3). MacMARCKS was also present in the soluble fractions (S3 and LS2). MARCKS was detected in most membrane fractions including small vesicles (P3 and LP2), and was also enriched in soluble fractions (S3 and LS2). In contrast to MacMARCKS, MARCKS was not detected in the synaptic vesicle (CPG3) fraction (Fig. 3-5).
Fig. 3-4. PKC agonists or depolarization induce the phosphorylation of MacMARCKS in rat brain synaptosomes.

a. Percoll-purified synaptosomes were radiolabeled with $^{32}$P$_i$, and incubated with control buffer (lane 1), depolarized with 40 mM KCl for 30 sec (lane 2) or treated with 300 nM PDBu for 1 min. (lane 3). The reactions were terminated by the addition of cold lysis buffer, and MacMARCKS was visualized by autoradiography following immunoprecipitation and 10% SDS-PAGE.

b. Time course of PDBu and depolarization-induced MacMARCKS phosphorylation. $^{32}$P$_i$-labeled synaptosomes were exposed to 300 nM PDBu (circles) or 40 mM KCl (squares) for the indicated times, and MacMARCKS phosphorylation was quantitated after immunoprecipitation and 10% SDS-PAGE using a Molecular Dynamics PhosphorImager. The experiments were done in triplicate, and mean and standard deviations are indicated.
Fig. 3-5. Subcellular distribution of MacMARCKS, MARCKS, synaptophysin, and synapsin 1.

Proteins from rat brain subcellular fractions were fractionated by 10% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with polyclonal antibodies against MacMARCKS, MARCKS, synapsin 1 or synaptophysin as indicated. H; Homogenate, P; pellet, S; supernatant, LP; lysed pellet, LS; lysed supernatant, CPG controlled pore glass.
DISCUSSION

Considerable evidence suggests that PKC has a role in neurosecretion (Dekker et al., 1991; Robinson, 1991). First, most conventional isozymes of PKC are expressed at high levels in neurons, and are present in synaptic terminals (Dekker et al., 1991; Kaczmarek, 1987). Second, phorbol esters promote the release of a variety of neurotransmitters (Shapira et al., 1987; Zurgil et al., 1985; Nichols et al., 1987; Malenka et al., 1987). This effect is stereospecific: only phorbol esters which activate PKC induce neurotransmitter release (Kikkawa et al., 1986). Third, PKC inhibitors prevent KCl induced neurotransmitter release (Dekker et al., 1991; Robinson, 1991), although these data should be interpreted with caution because of poor specificity of the inhibitors. Fourth, Ca\(^{2+}\) and diacylglycerol, which activate PKC, are elevated in active presynaptic terminals (Pozzan et al., 1984). Although there is much evidence that PKC is involved in neurotransmitter release, the mechanism by which PKC exerts this affect is unknown. Several phosphoproteins that are believed to be important in signal transduction in the nervous system have been described (Robinson, 1992; Dekker et al., 1991; Hemmings et al., 1989). In particular, two prominent PKC substrates, MARCKS and GAP-43 have been implicated in a variety of neuronal processes (Albert et al., 1986, 1987; Hemmings et al., 1989; Alexander et al., 1987). Most investigations of the role of PKC in neurosecretion have concentrated on these two proteins. This study indicates that a third PKC substrate, MacMARCKS, might also have a role in neurosecretion.

MacMARCKS is present in the distal neuronal processes of PC12 cells, in rat brain synaptosomes, and on small vesicles and synaptic vesicles isolated from rat brain. MacMARCKS is phosphorylated when PC12 cells or rat brain synaptosomes are stimulated with phorbol ester or depolarized with elevated levels of KCl. Depolarization-induced phosphorylation of MacMARCKS is dependent upon external Ca\(^{2+}\). MacMARCKS is phosphorylated by PKC \textit{in vivo}, since phosphopeptide maps generated
from the protein phosphorylated in PC12 cells are identical to those generated from recombinant MacMARCKS phosphorylated \textit{in vitro} by purified PKC. The phosphopeptides correspond to the underlined amino acids (KKKKFS[1]FKKPFKLGS[2]FKR), and are found within the effector domain of MacMARCKS, where both serines [1] and [2] are phosphorylated by PKC (Li and Aderem, 1992). Interestingly, Scholz and Palfrey (1991) have identified a 48 kDa protein in hippocampal pyramidal neurons that is rapidly phosphorylated after glutamate stimulation in a PKC dependent manner. The apparent molecular weight of this protein, the nearly identical time course of phosphorylation by PKC and the pattern of phosphopeptide fingerprints all suggest that this 48 kDa protein is likely to be MacMARCKS.

MARCKS and MacMARCKS are related proteins which share a number of features. Both are elongated, rod-shaped molecules with a similar domain structure. They have myristoylated N-termini, highly conserved MH2 domains, and basic effector domains which contain the PKC phosphorylation sites, and which bind calmodulin in a Ca$^{2+}$-dependent manner (Li and Aderem, 1992, Blackshear \textit{et al.}, 1992). The interaction between MacMARCKS and calmodulin is prevented by MacMARCKS phosphorylation. Similarly, PKC-dependent phosphorylation prevents calmodulin binding to GAP-43 (Alexander \textit{et al.}, 1987; Apel \textit{et al.}, 1990) and MARCKS (Wu \textit{et al}, 1982; Graff \textit{et al.}, 1989). It has been proposed that both GAP-43 and MARCKS act as regulated calmodulin stores (Alexander \textit{et al.}, 1987; Blackshear, 1993), and it is possible that MacMARCKS has a similar function. This is an attractive hypothesis since PKC-dependent phosphorylation has been shown to mobilize calmodulin, an event which activates calmodulin-dependent protein kinases implicated in neurosecretion (MacNicol \textit{et al.}, 1992). However, GAP-43 would be more suitable as a Ca$^{2+}$ sink than MARCKS or MacMARCKS, since it binds calmodulin in a Ca$^{2+}$-independent manner (Benowitz \textit{et al.}, 1987). Since MARCKS, MacMARCKS, and the calmodulin-dependent protein-kinases would compete for
calmodulin, as the concentration of intracellular Ca\textsuperscript{2+} increases calmodulin availability and hence Ca\textsuperscript{2+}/calmodulin-dependent protein kinase activity could be regulated subtly by the phosphorylation of MARCKS and MacMARCKS.

MARCKS binds and crosslinks actin in a phosphorylation-regulated manner (Hartwig et al., 1992), and preliminary data suggest that the effector domain of MacMARCKS also binds actin (Aderem, unpublished observation). Besides being an internal scaffolding for defining cell shape, the actin cytoskeleton is also involved in the guidance of organelles to sites of accumulation within specific compartments. This is especially critical in highly polarized cells such as neurons. Both KCl-induced MacMARCKS phosphorylation, and KCl-induced neurotransmitter release are dependent upon external Ca\textsuperscript{2+}. Our subcellular fractionation data suggest that MacMARCKS associates with synaptic vesicles, while MARCKS does not. The function of MacMARCKS in the presynaptic terminal is not yet clear, but by analogy with the synapsins (Greengard et al., 1993), it may have a role in linking synaptic vesicles to cortical actin. MacMARCKS could reversibly tether synaptic vesicles to the actin-based cytoskeleton of the nerve terminal, and this linkage would be disrupted when the effector domain of MacMARCKS is phosphorylated by PKC. Alternatively, MacMARCKS might regulate the dynamics of actin filament assembly at the presynaptic terminal. Dephosphorylated MARCKS causes F-actin to aggregate by crosslinking (Hartwig et al., 1992), whereas phosphorylated MARCKS does not aggregate nor crosslink actin. MacMARCKS might similarly regulate actin assembly at the nerve terminal in a PKC-dependent manner, thereby affecting access of synaptic vesicles to the plasma membrane.
CHAPTER FOUR

A DOMINANT-NEGATIVE MacMARCKS MUTATION BLOCKS EVOKEY EXOCYTOSIS OF PC12 CELLS

INTRODUCTION

Neurons are cells which are specialized for signal propagation. The data shown in chapter 3 have demonstrated a role for MacMARCKS in depolarization-dependent signal transduction in neurons and neuroendocrine cells. Neurons and neuroendocrine cells have two independently regulated secretory pathways which use separate secretory organelles: large dense-core vesicles and synaptic vesicles. Neuronal communication is mediated by regulated exocytosis of neurotransmitters which are stored in synaptic vesicles, the most abundant organelle in neurons. Synaptic vesicles are very homogeneous in diameter (50 nm), have an electron-translucent core and store and secrete classical nonpeptide neurotransmitters such as glutamate, γ-aminobutyric acid (GABA), glycine and acetylcholine. These substances are responsible for the rapid, point-to-point signaling characteristic of neurons (Thomas-Reetz and De Camilli, 1994; Kelly, 1993).

Synaptic vesicles exist in two pools within the nerve terminal: a reserve pool and a releasable pool (Kelley, 1993). The releasable pool of synaptic vesicles dock along specialized sites of the nerve terminal called the active zone, a region of the presynaptic membrane that is physically connected by extracellular matrix to the postsynaptic region of the target cell. Exocytosis selectively occurs at active zones, and only docked vesicles are exocytosed (Sudhof and Jahn, 1991). Upon neuronal stimulation, an action potential is generated which opens voltage-gated Ca$^{2+}$ channels located in clusters at the active site of the nerve terminal. This results in a local and extremely transient elevation of cytosolic Ca$^{2+}$ concentration near the membrane, which in turn triggers the synaptic vesicles to rapidly (<1
msec) fuse with the plasma membrane and release transmitters. After exocytosis, synaptic vesicle membrane proteins are retrieved by endocytosis and reutilized for the generation of new synaptic vesicles. Newly formed vesicles are reloaded with neurotransmitters from cytoplasmic pools and returned to the nerve terminal for further rounds of exocytosis (Kelley, 1993; Pevsner and Scheller, 1994).

The reserve pool of synaptic vesicles is anchored by the actin-cytoskeleton near the active zone and is mobilized during repeated cycles of neuronal stimulation to dock along the active zone. The synapsin family of phosphoproteins are candidates for anchoring these synaptic vesicles to the actin cytoskeleton. Synapsin I is thought to reversibly tether synaptic vesicles to actin; this linkage is disrupted after Ca\(^{2+}\)-induced phosphorylation of synapsin I by CaM kinase II, allowing vesicles to be mobilized to docking sites (Greengard et al., 1993). Pharmacological evidence also implicates PKC in neurotransmitter release (Shapira et al., 1987). MARCKS, a major PKC substrate in neurons, is phosphorylated upon depolarization-induced Ca\(^{2+}\) influx into the nerve terminal (Wu et al., 1982). PKC-dependent phosphorylation of MARCKS causes it to translocate from the membrane to the cytosol, and dephosphorylation returns it to the membrane (Wang et al., 1989; Aderem et al., 1991). MARCKS binds and crosslinks actin in a phosphorylation-regulated manner (Hartwig et al., 1992). Dephosphorylated MARCKS causes F-actin to aggregate by crosslinking filaments, whereas phosphorylation of MARCKS by PKC disrupts its ability to crosslink actin at the plasma membrane. Increased plasma membrane accessibility due to rearrangement of cortical actin is required to facilitate synaptic vesicle mobilization to the active zone (Vitale et al., 1991), suggesting that cycles of MARCKS crosslinking and release of F-actin may regulate accessibility of vesicles to this region.

In contrast to synaptic vesicles, large dense-core vesicles are 70-200 nm in diameter and have an electron-dense core when visualized by transmission electron microscopy. These vesicles store and secrete peptide neurotransmitters and catecholamines, substances
which have a modulatory effect on their target cells (Thomas-Reetz and De Camilli, 1994). These vesicles are stimulated to undergo exocytosis by a slow rise in intracellular calcium. Exocytosis does not occur at the active zone (Thureson-Klein and Klein, 1990). After exocytosis, the membranes of these vesicles also recycle and are repackaged with neurotransmitters at the Golgi apparatus in the cell body. It is believed that PKC plays a role in the actin cytoskeleton rearrangement associated with vesicle-plasma membrane fusion, although this process is not well understood. In insulin-secreting cells, stimulation of exocytosis results in the redistribution of PKC from the cytosol to the membrane and the phosphorylation of MARCKS (Calle et al., 1992). Phorbol esters, which activate PKC, cause disassembly of the cortical actin cytoskeleton in chromaffin cells and enhance exocytosis (Grant and Aunis, 1990). Due to their lower abundance and heterogenous size, large dense-core vesicles are not as well characterized as synaptic vesicles. They appear to have a distinct set of membrane proteins from those of the synaptic vesicles, although several proteins are present on both membranes (Jahn and Sudhof, 1993).

In recent years there has been an explosion in our knowledge of the protein machinery underlying vesicle targeting, docking and fusion in both regulated and constitutive secretory pathways (for reviews see Bennett and Scheller, 1993; Thomas-Reetz and De Camilli, 1994). In particular, the role of the protein synaptotagmin has received widespread attention. Synaptotagmin is an abundant protein found in both neurons and endocrine cells. It is localized to the membranes of both synaptic vesicles and large dense core-vesicles. Synaptotagmin has a single transmembrane domain with the amino terminus on the intravesicular side (Matthew et al., 1981) and a cytoplasmic domain containing two repeats homologous to the Ca$^{2+}$ and phospholipid binding C2 domain of PKC (Brose et al., 1992). These C2 repeats are highly conserved across species; similar domains in PKC have been implicated in the Ca$^{2+}$-dependent interaction with membrane phospholipids (Nishizuka, 1992). Synaptotagmin binds Ca$^{2+}$ and phosphatidylserine through these C2
repeats, suggesting that it may act as a Ca\textsuperscript{2+}-sensor on the surface of the synaptic vesicle (Brose et al., 1992). Microinjection of anti-synaptotagmin antibodies and soluble C2 synaptotagmin fragments into PC12 cells inhibits K\textsuperscript{+}/ Ca\textsuperscript{2+}-dependent release of catecholamines (Elferink et al., 1993). Injection of peptides from the C2 domain of synaptotagmin into the giant presynaptic terminal of the squid also leads to a significant reduction in evoked transmitter release (Bommert et al., 1993). These results indicate that synaptotagmin is important for regulated exocytosis in neurons and neuroendocrine cells.

The classical way to study synaptic vesicle exocytosis is by postsynaptic recording, but this method cannot be used to study exocytosis in neuroendocrine cell lines because they do not form synapses. However, antibodies directed against the luminal domain of synaptotagmin (Syt\textsubscript{lum}-Abs) can be used in an immunocytochemical assay to detect exposure of the synaptic vesicle membranes to the cell surface (Matteoli et al., 1992). Since the luminal domain of synaptotagmin is exposed to the extracellular medium only during exocytosis, antibodies directed against this domain which become cell-bound reflect the number of exocytotic events irrespective of synaptic vesicle reinternalization. In the original characterization of this assay, hippocampal neuronal processes rapidly and specifically internalize Syt\textsubscript{lum}-Abs, which are retained within the synaptic vesicles during cycles of exo-endocytosis (Matteoli et al., 1992; Kraszewski et al., 1995).

MacMARCKS is highly abundant in the distal neuronal processes of PC12 cells and in rat brain synaptosomes where it is localized on a number of membrane compartments including synaptic vesicles (Chang et al., 1996; see Chapter 3). It is phosphorylated when PC12 cells or rat brain synaptosomes are stimulated with phorbol esters or depolarized with elevated levels of K\textsuperscript{+}. Ca\textsuperscript{2+}-influx is required for MacMARCKS to become phosphorylated by PKC, and phosphorylation translocates a portion of it from the membrane to the cytosol (Chang et al., 1996). The effector domain of MacMARCKS binds Ca\textsuperscript{2+}-calmodulin and actin (Li and Aderem, 1992; Aderem, unpublished)...
observations). These results suggest that MacMARCKS may have a role in regulating synaptic vesicle exocytosis by modulating the actin cytoskeleton.

This chapter describes the role of MacMARCKS in PC12 cells. Two serine to alanine substitutions were made at positions 93 and 104 in the effector domain of the MacMARCKS protein. These two serines are the major sites of PKC-dependent phosphorylation of MacMARCKS in vivo (Verghese et al., 1994). Expression of this mutant protein in PC12 cells induced aberrant filopodial extensions and inhibited depolarization-induced exocytosis, as measured by a double-immunofluorescence assay. Exocytosis in PC12 cells expressing the wild-type protein was unaffected, and these cells did not extend filopodia in the absence of nerve growth factor (NGF). Compared to parental cells and cells expressing the wild-type transgene, elevated K⁺-induced depolarization of PC12 cells expressing S-A MacMARCKS resulted in dramatically reduced [³H]-norepinephrine release. Results of wheat-germ agglutinin uptake experiments indicated that endocytosis was not inhibited in mutant cells. These data suggest that MacMARCKS is involved in regulating exocytosis in PC12 cells.

RESULTS

Characterization of Transfected PC12 Clones Expressing Wild-type and Mutant MacMARCKS:

The effector domain of MacMARCKS binds calmodulin in a phosphorylation-regulated manner: phosphorylation of MacMARCKS abolishes calmodulin binding, while dephosphorylated MacMARCKS binds Ca²⁺-calmodulin with high affinity, preventing subsequent phosphorylation by PKC (Li and Aderem, 1992; Blackshear et al., 1992; Verghese et al., 1994). Like MARCKS, the MacMARCKS effector domain also crosslinks actin in a phosphorylation-dependent manner (Hartwig et al., 1992; Aderem, unpublished...
results). The MacMARCKS effector domain contains three serines, however, only the first and third are PKC phosphorylation sites in vivo (Verghese et al., 1994). Mutations which change these serines into alanines should therefore abolish PKC-dependent phosphorylation (Fig. 4-1a). An influenza virus hemagglutinin (HA) epitope tag was added to the 3' end of the MacMARCKS cDNA to differentiate the transfected product from the endogenous protein (Nolan et al., 1993), and PC12 cells were transfected with cDNAs encoding either wt-HA MacMARCKS or S-A-HA MacMARCKS in the expression vector pCMV-neo. Following G418 selection, the stable strains of wt-HA PC12 and S-A-HA PC12 cells were generated. In each strain, 48 colonies were screened for transgene expression by immunofluorescence; four demonstrated high levels of expression and were chosen for further studies. An anti-HA monoclonal antibody (12CA5) specifically detected HA-epitope tagged MacMARCKS proteins of the correct molecular weight in both wt-HA and S-A-HA cell lines (Fig. 4-1b). HA-tagged protein was not detected in parental cells (Fig. 4-1b).

To determine if the expressed proteins were myristoylated, [3H]-MA-labeled PC12 cell lysates were quantitatively immunoprecipitated with 12CA5. As shown in Fig. 4-1c, transfected PC12 clones displayed a doublet protein band of approximately 48 kDa characteristic of endogenous MacMARCKS (Li and Aderem, 1992; Chang et al., 1996). 12CA5 did not immunoprecipitate this protein in parental PC12 cells (Fig. 4-1c). Western blotting of the same lysates indicated that the transgene product was completely depleted by 12CA5 immunoprecipitation (data not shown). The same lysates were re-immunoprecipitated with a polyclonal anti-MacMARCKS antibody to detect endogenous MacMARCKS protein (Fig. 4-1c). Expression levels of the [3H]-MA labeled transgene product compared with the endogenous [3H]-MA labeled MacMARCKS protein indicate that the ratio of wt-HA to endogenous MacMARCKS in the four clones ranged from
Figure 4-1. Characterization of wt-HA and S-A-HA MacMARCKS in PC12 cells.

a. Diagram of the constructs used to transfect PC12 cells. The black box is the N-terminal myristoylation domain. The first and third serines in the effector domain (ED) of the S-A-HA construct were mutated into alanines. Both constructs were HA-epitope tagged at the 3’ end and subcloned into pCMV-neo.

b. Immunoblot analysis of PC12 clones expressing wt-HA (left) and S-A-HA (right) constructs. The anti-HA monoclonal antibody, 12CA5, detected epitope-tagged protein from lysates of clones expressing wt-HA (clones 2, 12, 16, 19) and clones expressing S-A-HA (clones 2, 15, 23, 28). No signal was detected in nontransfected parental (p) cells. Lysates were normalized for protein content.

c. Immunoprecipitation of [3H]-myristic acid labeled PC12 clones. PC12 clones were labeled with [3H]-myristic acid, lysed, and quantitatively immunoprecipitated with 12CA5 to detect HA-tagged transgene products (HA). These lysates were re-immunoprecipitated with an anti-MacMARCKS antibody to detect endogenous MacMARCKS (MM). The immunoprecipitates were resolved by 10% SDS-PAGE, and myristoylated proteins were visualized by fluorography. Endogenous MacMARCKS was detected in the parental (p) cells.

d. Immunoprecipitation of 32P-labeled PC12 clones. 32P-labeled PC12 clones were lysed and processed as described in c. Immunoprecipitates were resolved by 10% SDS-PAGE, and visualized by autoradiography. Compared to endogenous MacMARCKS, the S-A-HA transgene product was poorly phosphorylated.

e. The S-A mutation abolishes PKC-dependent phosphorylation of MacMARCKS. 32P-labeled Wt-HA clone 19 and S-A-HA clone 28 PC12 cells were either untreated (C) or treated with 100 nM PMA (P) for 45 min. Samples were processed as in (d).

f. The S-A MacMARCKS mutant does not translocate to the cytosol after PKC stimulation. S-A-HA clone 28 PC12 cells labeled with [3H]-myristic acid were either untreated (Con) or stimulated with 100 nM PMA (PMA) for 45 min. The cells were fractionated to isolate membrane (M) and supernatant (S) fractions. The fractions were first immunoprecipitated with 12CA5 (HA), and the lysates re-immunoprecipitated with an anti-MacMARCKS antibody (MM). Samples were visualized by fluorography after 10% SDS-PAGE.
approximately 0.3:1 (clone 12) to 1.3:1 (clone 19). The ratio of S-A-HA to its endogenous counterpart ranged from 0.9:1 (clone 28) to 1.5:1 (clone 15).

PC12 clones labeled with $^{32}$Pi were lysed and immunoprecipitated with 12CA5. Fig. 4-1d illustrates that 12CA5 specifically immunoprecipitated a single phosphorylated protein with an apparent molecular mass of 48 kDa from PC12 clones expressing wt-HA MacMARCKS. Compared to wt-HA MacMARCKS, S-A-HA MacMARCKS was poorly phosphorylated (Fig. 4-1d). To determine the ratio of basal phosphorylation of the transgene products compared to the endogenous MacMARCKS protein, 12CA5-depleted lysates were re-immunoprecipitated with a polyclonal anti-MacMARCKS antibody (Fig. 4-1d). The stoichiometry of phosphorylation of wt-HA MacMARCKS was equal to or higher than endogenous MacMARCKS (approximately 1:1 for clones 2 and 12; 3:1 for clones 16 and 19) and the stoichiometry of phosphorylation for the S-A-HA MacMARCKS was much lower than endogenous MacMARCKS (approximately 0.1-0.3:1 for the clones analyzed). Treatment of S-A-HA PC12 lines with PMA did not increase the level of phosphorylation of the mutant proteins, indicating that PKC-dependent phosphorylation sites have indeed been mutated (Fig. 4-1e). However, this result also indicates that MacMARCKS has other sites of phosphorylation; recent data suggest that the proline-directed protein kinases cdc2 kinase and MAP kinase phosphorylate both MARCKS and MacMARCKS (Taniguchi et al., 1994; Yamamoto et al., 1995). In contrast to the S-A-HA PC12 lines, the level of MacMARCKS phosphorylation in wt-HA PC12 lines increased after PMA treatment (Fig. 4-1e).

The association of MacMARCKS with the plasma membrane is, to some extent, regulated by PKC-dependent phosphorylation; PMA treatment of PC12 cells displaces approximately 20% of phosphorylated MacMARCKS from the membrane to the cytosol (Chang et al., 1996). To determine whether the S-A-HA protein also cycled between the
plasma membrane and the cytosol after PKC activation, untreated and PMA-treated [³H]-
MA-labeled PC12 cells expressing the mutant protein were lysed and immunoprecipitated
with 12CA5. As shown in Fig. 4-1f, PMA treatment did not lead to an increase of S-A-HA
MacMARCKS in the cytosol, indicating that the mutant protein was not displaced from the
membrane by PKC-mediated phosphorylation. In contrast, PMA treatment resulted in the
displacement of a portion of endogenous MacMARCKS from the membrane to the cytosol
(Fig. 4-1f). Taken together, these data suggest that the S-A mutations in the effector
domain eliminated PKC-dependent phosphorylation of MacMARCKS, and that
myristoylation was unaffected by these mutations. Addition of the HA epitope also did not
affect the ability of MacMARCKS to be myristoylated or phosphorylated.

**Effects of Mutant MacMARCKS on PC12 Cell Morphology:**

Non-NGF treated PC12 cells expressing wt-HA MacMARCKS appeared
morphologically similar to parental PC12 cells by DIC microscopy except for slight
flattening of the cell bodies (data not shown). Immunofluorescence confocal microscopy
with affinity-purified 12CA5 antibody revealed that wt-HA MacMARCKS protein was
distributed in a diffuse pattern with some staining at the cell periphery (Fig. 4-2a). Co-
staining with rhodamine-phalloidin showed that F-actin was distributed as a bright cortical
ring with some cytoplasmic staining (Fig. 4-2b). PC12 cells expressing the S-A-HA
transgene appeared larger and flatter than wt-HA PC12 cells and displayed cell surface
irregularities. In these cells, the mutant MacMARCKS protein appeared as a bright cortical
rim around the periphery of the cell, with some staining in the cytoplasm (Fig. 4-2c). 
Numerous processes resembling pseudopodia extended from the cell body in the absence
of NGF treatment, and these processes also stained brightly with the 12CA5 antibody (Fig.
4-2c). These processes were never detected in the parental and wt-HA cells. Rhodamine-
phalloidin labeling of F-actin revealed that its distribution was altered in cells expressing the
Figure 4-2. Actin colocalization and morphological effects of recombinant wt-HA and S-A-HA MacMARCKS in PC12 cells.

a, b. Wt-HA clone 19 PC12 cells were double-labeled with the mAb 12CA5 to visualize the transgene product (a) and Texas Red-phalloidin to visualize F-actin (b). Note the diffuse staining of 12CA5 compared to the cortical staining pattern seen with TR-phalloidin. These cells display normal morphology.

c, d. S-A-HA clone 28 PC12 cells were double-labeled as in a and b. Numerous pseudopodia and processes-like extensions (arrows) are seen in cells expressing the mutant protein. Both the transgene product (c) and F-actin (d) colocalized to these processes and to the cell periphery (arrowheads).

e, f. S-A-HA clone 22 PC12 cells express the mutant protein at approximately 10 times the level observed in clone 28 (c, d) and display an aberrant morphology. These cells were double-labeled as in (a) and (b). Both the mutant MacMARCKS protein and F-actin colocalized to numerous long processes-like extensions (arrows) and filopodia (arrowheads).
mutant MacMARCKS protein (Fig. 4-2d). F-actin was distributed in a cortical rim which coincided almost exactly with the distribution of S-A-HA MacMARCKS protein. The aberrant processes also stained brightly with rhodamine-phalloidin, indicating that mutant MacMARCKS co-localizes with actin in these structures (Fig. 4-2c and d).

PC12 clone 22 expressed S-A MacMARCKS mutant protein approximately 10 times higher than the other transfectants (data not shown) and displayed aberrant cell surface irregularities. These cells were larger than either parental cells or wt-HA PC12 cells, very flat, and contained multiple blebs throughout the plasma membrane (Fig. 4-2e and data not shown). Most strikingly, these cells extended numerous processes in the absence of NGF treatment. These processes appeared highly abnormal: their calibers were larger, and their lengths longer, than neurites of NGF-treated parental cells (Fig 4-2e; compare Fig. 4-4b to 4-4i). In NGF-differentiated PC12 cells, the tip of a neurite contains a single growth cone which contains filopodia and lamellipodia at the distal end (Varnum-Finney and Reichardt, 1994). In clone 22 cells, numerous filopodia decorated the cell periphery as spiny filaments. Rhodamine-phalloidin labeling revealed that these filopodia contained prominent actin filaments (Fig. 4-2f). Double-immunofluorescence confocal microscopy further demonstrated that both the mutant MacMARCKS protein and F-actin co-localized to the cortical filipodia, and both proteins were found throughout the processes including putative growth cones (Fig 4-2e; f).

Expression of Mutant MacMARCKS Blocks Evoked Synaptotagmin Staining:

An immunofluorescence assay was used to analyze the effect of expression of wt-HA and S-A-HA mutant MacMARCKS protein on exocytosis at the single cell level. Antibodies directed against the lumenal domain of synaptotagmin (Sytum-Abs) was used to detect exposure of the synaptic vesicle membranes to the cell surface (Matteoli et al., 1992).
Since the luminal domain of synaptotagmin is exposed to the extracellular medium only during exocytosis, antibodies directed against this domain which become cell-bound reflect the number of exocytotic events irrespective of synaptic vesicle reinternalization. Parental and transfected PC12 cells were stimulated by KCl-induced depolarization for 1 hr at 37°C in the presence of an antibody directed against the luminal domain of synaptotagmin (Syt_{lum}-Ab). After labeling, the cells were washed, fixed, and processed for double immunofluorescence microscopy as previously described (Matteoli et al., 1992; Kraszewski et al., 1995; Mundigl et al., 1995). The mouse 12CA5 primary antibody and FITC-conjugated anti-mouse secondary antibody were used to detect HA-expressing cells. Cell-bound Syt_{lum}-Abs were identified by rhodamine-conjugated secondary antibodies. As shown in Fig. 4-3b and d, both parental and wt-HA MacMARCKS PC12 cells showed intense labeling of varicose structures outlining entire cell bodies, neurites and growth cones with the Syt_{lum}-Ab. In contrast, all PC12 clones expressing the S-A-HA mutant MacMARCKS protein did not label with Syt_{lum}-Abs, demonstrating a pronounced inhibition of the secretory response (Fig. 4-3f and data not shown). In these cells, the level of syt_{lum}-Ab staining was just above background levels in the cell bodies and neurites, and only a few Syt_{lum}-Ab-fluorescent patches were detected. Three observations suggest that the Syt_{lum}-Ab specifically labels synaptic vesicles in these experiments. First, staining of parental PC12 cells with Syt_{lum}-Ab was not detected at 0°C, suggesting that insignificant amounts of synaptotagmin I exist at the cell surface at any given time (data not shown). Second, an increase in Syt_{lum}-Ab uptake was dependent on the presence of extracellular Ca^{2+}, since KCl-induced depolarization in the absence of Ca^{2+} did not result in significant Syt_{lum}-Ab staining (Fig. 4-3h). Third, no staining was observed with a non-specific anti-rabbit antibody (Fig. 4-3l). These results suggest that Syt_{lum}-Ab specifically labeled synaptic vesicles in PC12 cells, and that expression of the S-A mutations in PC12 cells inhibited depolarization-induced exocytosis.
Figure 4-3. Expression of S-A-HA MacMARCKS in PC12 cells blocked evoked Syt\textsubscript{lum}-Ab staining.

Exocytosis-dependent uptake of Syt\textsubscript{lum}-Ab in living PC12 cells. Double immunofluorescence micrographs of anti-HA 12CA5 mAb (a, c, e, g) and internalized rabbit Syt\textsubscript{lum}-Ab (b, d, f, h) in PC12 cells. Cells were incubated with Syt\textsubscript{lum}-Ab in KRH/55 mM K\textsuperscript{+} for 1 hr at 37°C, washed, fixed and reacted with TR-anti-rabbit Ab to detect internalized synaptotagmin. 12CA5 and FITC-anti-mouse Ab were used to detect the presence of the transgene product. Parental PC12 cells do not stain with 12CA5 (a) but stain heavily with Syt\textsubscript{lum}-Ab, with strong labeling at the neurite tips (b; arrows). In cells transfected with wt-HA MacMARCKS, both the 12CA5 (c) and Syt\textsubscript{lum}-Ab (d) showed intense staining throughout the cell body, neurite (arrowhead) and growth cone (arrow). In S-A-HA PC12 cells, 12CA5 stained the cell body, neurite (arrowhead) and growth cone (arrow), but did not stain with Syt\textsubscript{lum}-Ab (f). Wt-HA PC12 cells incubated in KRH/55 mM K\textsuperscript{+}/Ca\textsuperscript{2+}-free and stained with 12CA5 revealed that the recombinant protein localized to the cell body and growth cone (g, arrow) but these cells do not stain with Syt\textsubscript{lum}-Ab (h), indicating that antibody uptake was dependent on the presence of extracellular Ca\textsuperscript{2+}. Confocal microscopy of parental (i) and wt-HA (j) PC12 cells incubated with Syt\textsubscript{lum}-Ab showed intense staining at the cell periphery, neurites (arrowhead) and growth cones (arrow). In contrast, S-A-HA PC12 cells do not stain with Syt\textsubscript{lum}-Ab (k). Wt-HA PC12 cells stained with a non-specific rabbit antiserum exhibited background staining (l).
Confocal microscopy was used to quantitate the fluorescent patches in PC12 cell lines labeled with Syt\textsubscript{Ium}-Ab. One micron optical sections of parental and wt-HA PC12 cells indicated that multiple Syt\textsubscript{Ium}-Ab-fluorescent patches were localized to hot spots in the neurites and along the cell body of certain optical sections (Fig. 4-3i and j). Finely punctate staining localized to specific hot spots along the cell body and growth cones of neurites. When these sections were quantitated, a sharp peak of Syt\textsubscript{Ium}-Ab-pixel intensity was found concentrated near the bottom of these cells (Fig. 4-4 top). Syt\textsubscript{Ium}-Ab staining is therefore specific to a subset of cellular structures and not due to nonspecific labeling of the cell in general. In contrast, S-A-HA PC12 cells display only a few Syt\textsubscript{Ium}-Ab-fluorescent patches per confocal section (Fig. 4-3k), and these do not show any specific cellular distribution after quantitation (Fig. 4-4 top). Wt-HA PC12 cells stained with a nonspecific antibody showed a similar intensity profile as that seen for cells expressing the S-A mutant, again reaffirming the observation that exocytosis is inhibited in S-A-HA PC12 cells (Fig. 4-4 top).

Receptor-mediated Endocytosis of Wheat-Germ Agglutinin Is Not Affected by Mutant MacMARCKS:

The observed inhibition of Syt\textsubscript{Ium}-Ab surface staining in PC12 cells expressing mutant MacMARCKS protein could be due to either a decrease in vesicle exocytosis or an enhancement in the rate of vesicle endocytosis. It is also possible that general membrane trafficking was disrupted by the mutant MacMARCKS protein. To distinguish between these possibilities, NGF-differentiated PC12 cells were incubated in the presence of TR-conjugated wheat-germ agglutinin (WGA) for 1 hr at 37°C (Matteoli \textit{et al.}, 1992). The uptake of WGA is mediated by adsorptive endocytosis in these cells (Stoeckel \textit{et al.}, 1977). Cells were then fixed and examined by confocal microscopy. As shown in Fig. 4-4 bottom, WGA staining occurred as large punctate patches clustered primarily in the cell
Figure 4-4. Quantitation of depolarization-evoked Syt_{lum}-Ab labeling and assay for wheat germ agglutinin (WGA) uptake in PC12 cells.

**Top.** Quantitation of pixel intensities of Syt_{lum}-Ab labeling of parental PC12 cells (squares), wt-HA PC12 cells (circles), S-A-HA PC12 cells (triangles), and labeling of wt-HA PC12 cells incubated with a non-specific rabbit antiserum (background; crosses). The fluorescent intensity in confocal sections obtained from PC12 cells were quantified by measuring the average pixel intensity per square micron in each section using Image space software version 3.01 (Molecular Dynamics).

**Bottom.** Receptor-mediated endocytosis of WGA is not affected by mutant MacMARCKS. NGF-differentiated PC12 cells were incubated in the presence of TR-conjugated WGA for 1 hr at 37°C. Cells were then fixed and examined by confocal microscopy. WGA staining occurred as large punctate spots clustered primarily in the cell body. S-A-HA mutant cells (e) showed similar amounts of TR-WGA uptake when compared to both wt-HA PC12 cells (b) and parental PC12 cells (a).
Parental PC12 cells
Wt-HA PC12 cells
S-A-HA PC12 cells
background

Pixel Intensity / (micron^2)

Optical Section Number

a)

parental

b)

wt-HA

S-A-HA
body. S-A-HA mutant cells showed similar amounts of TR-WGA uptake when compared to both wt-HA control cells and parental PC12 cells (compare Fig. 4-4 bottom, a-c). This result indicates that the defect in exocytosis seen in S-A-HA mutant cells did not affect endocytotic uptake of WGA.

**3H-Catecholamine Release Is Inhibited In PC12 Cells Expressing Mutant MacMARCKS:**

PC12 cells have the ability to release neurotransmitters in response to a variety of external stimuli. Exposure of the cells to elevated external K⁺, or the Ca²⁺ ionophore ionomycin, induces membrane depolarization, leading to Ca²⁺ influx and exocytotic release of stored neurotransmitters from dense-core granules (Williams and McGee, 1982). K⁺ induced depolarization results in MacMARCKS phosphorylation (Chang et al., 1996). To test whether neurotransmitter release is inhibited in S-A-HA PC12 cells, cells were incubated with [³H]norepinephrine ([³H]NE) for 1 hr to prelabel dense-core granules. Labeled cells were transferred to a superfusion apparatus, and timed fractions were collected before and after agonist stimulation. All three cell lines exhibited similar levels of basal (unstimulated) release, which constituted 0.5-1% of total initial stores of [³H]NE release/min. throughout the course of an experiment (data not shown). This release was independent of external Ca²⁺ (data not shown). Exposure to 55 mM K⁺ led to [³H]NE release from both parental and wt-HA PC12 cells (Fig. 4-5). In wt-HA PC12 cells, [³H]NE release peaked 3 min. after agonist exposure and rapidly declined to basal levels, while in parental cells the time course of [³H]NE release declined to basal levels at a slower rate. [³H]NE release was completely blocked by the omission of Ca²⁺ (data not shown). In contrast, the amount of [³H]NE release from K⁺-depolarized S-A-HA PC12 cells was markedly reduced when compared to both parental and wt-HA levels (Fig. 4-5). S-A-HA
Figure 4-5. $[^3]$H-norepinephrine release from S-A-HA PC12 cells is inhibited following depolarization.

Release of $[^3]$H-norepinephrine ($[^3]$H-NE) after $K^+$-induced depolarization from parental PC12 cells (circles), wt-HA PC12 cells (squares) and S-A-HA PC12 cells (triangles). PC12 cells were plated onto 100 mm collagen-coated dishes at a density of $8 \times 10^6$ cells per dish. Cells were incubated with $^3$H-NE for 1 hr, transferred to a superfusion apparatus and superfused for 30 min. with KRH buffer. At time 0, cells were exposed to KRH containing 55 mM $K^+$ for 5 min. After agonist stimulation, cells were superfused with KRH for an additional 5 min. Fractions were collected every min. and $[^3]$H-NE release was detected by scintillation. Net stimulated release was calculated as the total release during stimulation minus the basal release during that time. Release is expressed as a percentage of total initial stores released during 5 min. of stimulation. Error bars represent means ± S.E.M. for 4 separate experiments.
K⁺-evoked release

3H-NE release (% basal + SEM)

Minutes of superfusion

- parental PC12 cells
- WT HA PC12 cells
- S-A HA PC12 cells

100 110 120 130 140 150 160
PC12 cells exhibited a slow rise of $[^{3}H]NE$ release which peaked 6-7 min. after stimulation (Fig. 4-5). These results indicate that the S-A-HA mutant MacMARCKS protein inhibited depolarization-induced exocytosis of the large dense-core granules in PC12 cells.

**Localization of S-A-HA MacMARCKS by Immunoelectron Microscopy:**

Immunoelectron microscopy on ultrathin cryosections was carried out to more definitively establish the intracellular location of the mutant MacMARCKS protein. Sections of S-A-HA PC12 cells incubated with 12CA5, followed by 10 nm protein A gold, showed specific circumferential labeling of the plasma membrane (Fig. 4-6A-D). In addition, a significant number of gold particles were associated with the cytoplasmic surface of dense core granules (Fig. 4-6D-F). In all experiments, the amount of nonspecific labeling was estimated to be low based on the virtual absence of gold particles overlying the nucleus. The localization of S-A-HA MacMARCKS to dense-core granules strongly supports the observation that the mutant MacMARCKS protein inhibits $[^{3}H]NE$ release from PC12 cells.

**DISCUSSION**

The effector domain of MARCKS has been shown to bind and cross-link F-actin filaments, and this activity is inhibited by PKC-mediated phosphorylation and binding to Ca$^{2+}$-calmodulin (Hartwig et al., 1992). Much evidence exists that MARCKS regulates the structure of actin at the plasma membrane (Aderem, 1992). MacMARCKS contains an almost identical effector domain to MARCKS, and preliminary evidence suggests that MacMARCKS also binds and crosslinks actin (Aderem, unpublished results). Data presented in this chapter indicate that MacMARCKS regulates actin structure in PC12 cells. Expression of a mutant MacMARCKS protein in which single amino acid substitutions abolish PKC phosphorylation dramatically affects cell shape. S-A-HA MacMARCKS
Figure 4-6. Immunogold labeling of S-A-HA MacMARCKS localizes to the cytoplasmic face of the plasma membrane and to dense core granules.

Immunocytochemistry and electron microscopic localization of the S-A-HA MacMARCKS in PC12 cells. Ultrathin cryosections prepared from S-A-HA clone 28 PC12 cells were labeled with 12CA5. S-A-HA MacMARCKS localized to the plasma membrane in a circumferential fashion: (A), enlargement of a region of the cell in (B). Labeling is associated with both the cytoplasmic face of the plasma membrane (C, D) and dense core granules (D, E, F). Arrows in (D) indicate dense core granules and arrowheads label neurofilament bundles. The scale bar in (B) is 1.0 μ, and in (C) and (D) is 500 nm. The magnification of panels (D, E, F) is identical.
expression in PC12 cells induces aberrant cell surface morphology: cells expressing approximately similar levels of mutant protein as endogenous protein display numerous pseudopodial-like processes extending from the cell body. A PC12 clone which grossly overexpresses the mutant MacMARCKS protein extends filopodia and numerous large-caliber processes in the absence of NGF. Double-immunofluorescence microscopy shows that both the mutant MacMARCKS protein and F-actin co-localize in filopodia and throughout the aberrant processes. Immunoelectron microscopy demonstrates that the mutant protein localizes to the plasma membrane and dense-core granules. In contrast, PC12 cells expressing wild-type MacMARCKS protein do not extend processes in the absence of NGF. Taken together, these results suggest that the overexpression of a phosphorylation-defective MacMARCKS protein leads to perturbations of the actin cytoskeleton, leading to formation of abnormal processes. The filopodia-rich morphology and the associated prominent radial arrays of F-actin-containing filaments correlates with the level of expression of S-A-HA MacMARCKS.

The morphology of processes induced by overexpression of the mutant MacMARCKS protein strongly resembles processes induced by overexpression of GAP-43 in L6 and CHO cells (Widmer and Caroni, 1993; Strittmatter et al., 1994). Expression of wild-type GAP-43 in these cells induces formation of numerous long actin-containing filopodia and pseudopodia (Widmer and Caroni, 1993). Both GAP-43 and MacMARCKS are acylated, acidic rod-shaped PKC substrates which bind calmodulin and interact with the plasma membrane and the actin cytoskeleton (Allsopp and Moss, 1989). It is possible that both proteins are regulated by common signal transduction pathways that modulate cell morphology.

Additional evidence suggests that MacMARCKS regulates the dynamic actin cytoskeleton. Phagocytosis represents a specialized form of actin-based motility (Allen and Aderem, 1996). An effector domain deletion (ED) mutant of MacMARCKS, when stably
expressed in a macrophage-like cell line, inhibits the phagocytosis of zymosan particles (Zhu et al., 1995).

MacMARCKS also appears to participate in neural secretion in PC12 cells. It colocalizes with the synaptic vesicle marker synaptophysin in the cell body and distal neuronal processes of PC12 cells (Chang et al. 1996). MacMARCKS is abundant in rat brain synaptosomes where it localizes to a purified synaptic vesicle fraction (Chang et al., 1996; see Chapter 3). As previously described, MacMARCKS is phosphorylated when PC12 cells or rat brain synaptosomes are stimulated with phorbol ester or depolarized with elevated levels of K+. Depolarization-induced phosphorylation of MacMARCKS is dependent upon external Ca$^{2+}$, and phosphorylation results in translocation of a portion MacMARCKS from the membrane to the cytosol. The effector domain of MacMARCKS binds Ca$^{2+}$-calmodulin and actin (Li and Aderem, 1992; Aderem, unpublished observations), suggesting that MacMARCKS may have a role in regulating synaptic vesicle exocytosis by modulating the actin cytoskeleton.

Work from several laboratories has demonstrated that a meshwork of F-actin underlies the plasma membrane of chromaffin cells (Lee and Trifaro, 1981; Cheek and Burgoyne, 1986). Cortical F-actin impedes exocytosis in these cells by acting as a barrier which inhibits the movement of secretory vesicles to the plasma membrane (Koff er et al., 1990; Vitale et al., 1991; Del Castillo et al., 1992). Agonist stimulation of chromaffin cells leads to a disassembly of cortical F-actin networks, suggesting that removal of the actin barrier is required prior to exocytosis (Cheek and Burgoyne, 1986; 1987). Nicotine or elevated K$^{+}$-induced depolarization causes a rapid disassembly of cortical F-actin at discrete subplasmalemmal zones, and anti-dopamine β-hydroxylase antibodies demonstrate that these zones are sites of active exocytosis (Vitale et al., 1991). Cytochalasin D and deoxyribonuclease I, which depolymerize actin filaments, increase Ca$^{2+}$-dependent secretion from permeabilized cells (Lelkes et al., 1986). In contrast, phalloidin, which
stabilizes actin filaments, partially inhibits secretion (Sontag et al., 1988). Depolarization of rat brain synaptosomes by elevated K⁺ results in a decrease in cytoskeletal F-actin and an increase in monomeric G-actin (Bernstein and Bamburg, 1985). Finally, cortical F-actin network disassembly in mast cells is required prior to stimulation-induced exocytosis (Koffer et al., 1990). These results strongly suggest that F-actin disassembly is a prerequisite to exocytosis.

The role of the non-phosphorylatable MacMARCKS mutant in regulated exocytosis was investigated in PC12 cells. These cells are an ideal system to study exocytosis because they contain both synaptic vesicles and dense-core granules which secrete catecholamines in a Ca²⁺-dependent fashion. Both synaptic vesicles and dense core granules of PC12 cells contain synaptotagmin, and the appearance of the luminal domain of synaptotagmin on the cell surface reflects the steady-state distribution of this protein during the dynamic process of exocytosis and endocytosis of vesicle membranes (Matteoli et al., 1992). PC12 cells expressing wild-type MacMARCKS protein displayed intense Syt₄-Ab associated surface labeling, while cells expressing mutant MacMARCKS protein have a marked decrease in Syt₄-Ab associated surface immunofluorescence. The observed inhibition of Syt₄-Ab surface staining in PC12 cells expressing mutant MacMARCKS protein could be due to either a decrease in vesicle exocytosis or an enhancement in the rate of vesicle recycling. Since Syt₄-Ab staining is negligible in the absence of depolarization and labeling is Ca²⁺ dependent, the elimination of Syt₄-Ab associated surface immunofluorescence in S-A-HA PC12 cells is interpreted to reflect inhibition of vesicle exocytosis, rather than enhanced endocytosis. This interpretation is further reinforced by the observation that when compared to parental and wt-HA PC12 cells, cells expressing the mutant MacMARCKS protein released less [³H]NE after depolarization induced by elevated K⁺. In addition, endocytosis as measured by the rate of wheat-germ agglutinin uptake, is the same for both mutant and wild-type MacMARCKS PC12 cells. These data suggest that MacMARCKS is
involved in regulating exocytosis in PC12 cells and in neurons, and that the S-A-HA protein probably functions as a dominant negative mutant \textit{in vivo} to compete with endogenous MacMARCKS protein.

The mechanism by which MacMARCKS participates in secretion is not clear. As discussed above, MacMARCKS may participate in neural secretion by regulating the levels of calmodulin. Alternatively, it may function to allow access of secretory vesicles to the plasma membrane by regulating cortical actin. Evidence supporting this contention is derived by the profound alterations in membrane cytoskeleton of cells expressing nonphosphorylatable MacMARCKS. However, the presence of MacMARCKS on dense core granules suggests that like the synapsins, MacMARCKS may serve to tether the vesicles to the actin cytoskeleton.
CHAPTER FIVE

REGULATION OF THE MacMARCKS GENE IN MACROPHAGES

INTRODUCTION

Previous work, including research presented in Chapters 3 and 4 suggests that MacMARCKS has a significant role in membrane trafficking in neural and neural-endocrine cells. This chapter describes the regulation of LPS-induced MacMARCKS expression in macrophages. Like neural cells, macrophages have a prominent actin cytoskeleton and undergo significant membrane activity associated with phagocytosis, secretion and migration. Macrophages originate in the bone marrow and circulate in the blood as monocytes before they form a resident population in many tissues of the body. Macrophage populations are dynamic; their numbers increase dramatically in response to infection or injury by recruitment from the blood to the site of injury. These newly recruited macrophages, in contrast to resident cells, are activated by γ-interferon and have potent respiratory burst activities (Gordon, 1986). In the induction of specific immunity, activated macrophages exhibit a wide range of activities, including antigen presentation to T cells. This in turn stimulates B cells to produce antibody, and the activation of T cells to destroy tumor cells and intracellular pathogens. Macrophages also phagocytose antibody-opsonized particles and lyse antibody-coated cells. In addition to their roles during the induction and effector phases of specific immunity, macrophages themselves can recognize bacterial lipopolysaccharide (LPS), the major surface component of gram-negative bacteria (Raetz et al., 1991). LPS exerts a profound effect on macrophages by enhancing the release of cytokines, inflammatory mediators such as prostaglandins and leukotrienes, and the induction of a number of immediate-early genes which encode products which may have effects on early stages of macrophage activation (Morrison and Ulevitch, 1978; Ruco and
LPS also induces cytoskeletal rearrangements important for migration and phagocytosis (Shinji et al., 1993).

A variety of compounds can activate macrophages to secrete nitric oxide (NO), a mediator of several tumoricidal and antimicrobial effects (Lorsbach et al., 1993). However, the most effective means of inducing activation is through a combined stimulation with a priming agent and a triggering agent. In activating macrophages for tumor cell killing, IFN-γ functions as a priming agent; it readies the cell to produce NO. When subsequently exposed to a triggering agent such as LPS, primed macrophages rapidly release a burst of NO. The molecular basis for this effect is increased expression of the gene for macrophage nitric-oxide synthase (mac-NOS), which synthesizes NO (Lorsbach et al., 1993). Without IFN-γ priming, macrophages secrete only low amounts of NO. mac-NOS mRNA levels are induced in the absence of IFN-γ priming only at high LPS concentrations (Lorsbach et al., 1993).

Macrophages are also a major source of arachidonic acid metabolites, which are important mediators of inflammation. LPS alone induces macrophages to secrete low levels of arachidonic acid metabolites, but it primes macrophages for greatly increased release of these metabolites when macrophages are subsequently stimulated with a second agonist such as PMA (Aderem et al., 1986). LPS increases the synthesis of both MARCKS and MacMARCKS proteins in macrophages (Aderem et al., 1986; Li and Aderem, 1992) and it primes these cells for enhanced PMA-induced phosphorylation of these proteins (Rosen et al., 1989; Li and Aderem, 1992). MARCKS synthesis in neutrophils is induced by TNF-α, and this cytokine primes neutrophils for increased MARCKS phosphorylation by PMA or the chemotactic peptide f-Nle-Leu-Phe (Thelen et al., 1990).

While MARCKS and MacMARCKS are well characterized at the protein level, little is known about the regulation of their genes or the mechanisms of induction by LPS. Unlike the promoters of most inducible genes, the MARCKS promoter does not have a
TATA box. It contains potential SP-1 and NF-κB binding sites which might mediate responses to LPS and TNF-α (Harlan et al., 1991; Shin et al., 1994). Promoter deletion analysis indicates that a 248 bp fragment of the MARCKS 5’ flanking region was sufficient to drive a reporter gene, but smaller deletions abolished this activity (Harlan et al., 1991). 407 bp of the MacMARCKS promoter has been sequenced, and computer analysis indicates that this region contains a putative TATA box and numerous transcription factor binding sites (Lobach et al., 1993). The homology between the MARCKS and MacMARCKS 5’ flanking regions is only 37%, but splice sites for the single intron occurs within the MH2 domain encoded by both genes (Blackshear, 1993). Both MARCKS and MacMARCKS are expressed in a wide variety of tissues (Chapter 6; Lobach et al., 1993), although MacMARCKS is more highly expressed in reproductive tissues. The MARCKS gene is located on mouse chromosome 10, and the MacMARCKS gene is located on chromosome 4 (Harlan et al., 1991; Lobach et al., 1993).

This chapter describes the isolation and characterization of a MacMARCKS genomic DNA clone. A 1.8 kb fragment from the 5’ flanking region of the MacMARCKS gene was cloned and sequenced, and the mRNA initiation sites were identified by primer extension and RNase protection. Induction of MacMARCKS mRNA levels by LPS was determined to occur via post-transcriptional mechanisms. 4 kb of the MacMARCKS 5’ flanking region was sufficient to direct lacZ expression in microglia (resident brain macrophages) of transgenic mice but not in their peritoneal macrophages, suggesting differential regulation of the MacMARCKS gene in these cell types.
RESULTS

Sequences of the 5' Flanking Region and Mapping of the MacMARCKS Transcriptional Start Site:

A 17 kb genomic DNA clone containing the entire murine MacMARCKS gene was isolated from a 129sv genomic library using MacMARCKS 5' cDNA probes. By restriction enzyme mapping and Southern analysis, this clone encompassed the entire MacMARCKS coding region as well as 8 kb of upstream sequences (data not shown). A 4 kb BamHI-NheI fragment of the MacMARCKS 5' upstream sequence was cloned into the pNASSβ vector (Clontech) for the generation of transgenic mice (see below). A 1.8 kb NheI fragment from this clone was subcloned into pBlueScript and completely sequenced (Fig. 5-1a).

The location of the transcriptional start site was identified by both RNase protection (Fig. 5-1b) and primer extension (Fig. 5-1c) using total RNA from both untreated and LPS stimulated peritoneal macrophages. Both techniques detected a doublet of approximately 200 bp. Comparison of the primer extended products with the corresponding dideoxy-sequencing ladder generated with the same oligonucleotide allowed localization of the primer extension product to the +1 G residue (Fig. 5-1c). The appearance of a doublet indicated that the second G residue in the +2 position also serves as the initiating site. Several minor bands were detected in the primer extension reactions; these are most likely incompletely extended products through the highly GC region just 3' of the initiation site (Fig. 5-1c). The transcriptional start site is 40 bp downstream of a putative TATA box, and 187 bp 5' of the ATG codon. Computer analysis of the sequenced region revealed numerous potential transcription factor consensus sequence elements that might be important both for developmental regulation of MacMARCKS and for its responses to extrinsic cues (Fig. 5-1a). Immediately upstream of the transcriptional start site is a SP-1
Figure 5-1. Nucleotide sequence of the MacMARCKS 5' flanking region and mapping of the transcriptional start site.

a. Sequence of the 1.8 kb MacMARCKS 5' flanking region. The transcriptional start site is indicated by an arrow, the putative TATA box is boxed, and the initiator ATG is underlined. p1.8-luc, p6-luc and p7'-10-luc refer to the 5' end of deletion constructs fused to the luciferase gene used to define LPS response elements. The NheI site used for subcloning is underlined. Consensus sequence elements are underlined and identified above the element. AP-1 or 2, activator protein 1 or 2; C/EBP, CCAAT enhancer binding protein; CREB, cAMP response element binder; GCN4; glucocorticoid nuclear factor 4; GCRE, glucocorticoid response element; NFκB, nuclear factor-κB; RARE, retinoic acid response element; SP-1, stimulatory protein 1.

b. Mapping of the RNA initiation site by RNase protection analysis (RPA). Antisense RNA probes complementary to murine MacMARCKS sequence from -413 to +203 were used for RPA. The RNA transcripts were hybridized to 20 μg of total RNA isolated from control (lane 1) and LPS-treated (lane 2) RAW 264.7 cells. After hybridization, the reaction was digested with a mixture of RNase A and RNase T1. The approximately 200 bp protected doublet fragments were resolved in a 6% sequencing gel and detected by autoradiography.

c. Primer extension mapping of the RNA initiation site. 50 μg of total RNA isolated from control (lane 2) or LPS-treated (lane 3) RAW 264.7 cells were hybridized to a 30 bp 32P end-labeled oligonucleotide complementary to the 5' end of the MacMARCKS cDNA (positions +174-203) and extended with Moloney murine leukemia virus reverse transcriptase. The radiolabeled products of this reaction were resolved on a 6% polyacrylamide/7 M urea sequencing gel alongside a dideoxy-sequencing ladder generated with the same oligonucleotide and visualized by autoradiography. Yeast tRNA was included as a negative control (lane 1). Arrows indicate doublet primer extended products. The primer extension products of 205 and 206 bp correspond to the initiation site determined by RNase protection.
site (Mitchell and Tjian, 1989), one of five such elements that are located at +107, -27, -65, -107 and -119. Three PEA-3 sites (Wasylyk et al., 1990) are located at positions -956, -1152 and -1626. Two retinoic acid receptor half-sites (Schule et al., 1990) are located at -186 and -1646 and two Ap-1 sites (Shin et al., 1994) are located at -374 and -301. A NFκB site (Xie et al., 1993) is located at -119. Finally, two ets/c-EBP sites (Stacey et al., 1995) are located at positions -208 and -365.

**Induction of MacMARCKS mRNA by Macrophage-Activating Factors:**

Macrophages can be stimulated by many different factors, including LPS and PMA (Shin et al., 1994; Stacey et al., 1995). Stimulation of RAW 264.7 cells with LPS induces MacMARCKS protein synthesis (Aderem et al., 1986; Li and Aderem, 1992). To determine whether LPS is capable of enhancing expression of the MacMARCKS gene, total RNA from peritoneal macrophages treated with LPS for 4 hrs. was analysed by RNase Protection Assay (RPA). As shown in Fig. 5-2a, top panel, cells stimulated with medium alone expressed MacMARCKS at very low levels. Addition of LPS induced MacMARCKS message in a dose-dependent manner. As little as 0.1 ng/ml of LPS induced MacMARCKS mRNA, and 1 ng/ml was sufficient to confer near-maximal stimulation after 4 hrs. Parallel experiments performed with RAW 264.7 cells showed similar results (data not shown). An antisense actin probe was used as an internal loading control to normalize expression levels in all experiments. Immunoprecipitation of 3H-myristic acid labeled peritoneal macrophage lysates with an anti-MacMARCKS antibody indicated that MacMARCKS protein levels also increased in a dose-dependent manner after addition of LPS (Fig. 5-2a, bottom panel).

PMA stimulates MacMARCKS phosphorylation by activating PKC (Aderem et al., 1988), and can greatly augment the release of arachidonic acid metabolites from LPS-primed macrophages (Aderem et al., 1986). PMA also increases mRNA levels of a number
Figure 5-2. Induction of MacMARCKS mRNA by macrophage activating factors:

(Top). Peritoneal macrophages were stimulated with various concentrations of LPS for 4 hrs, and total RNA was isolated and analysed by RNase protection. 5 μg of total RNA was hybridized to a 315 bp run-off transcript complementary to the MacMARCKS coding sequence. A 250 bp β-actin antisense RNA probe was included as an internal control. Protected fragments were resolved in a 6% sequencing gel and detected by autoradiography.  
(Bottom). Peritoneal macrophages were labeled with $^3$H]myristic acid and stimulated with LPS as above. MacMARCKS was immunoprecipitated from the cell lysate with an anti-MacMARCKS polyclonal antibody and resolved by 10% SDS-PAGE. Myristoylated proteins were visualized by fluorography.

b. Effects of various macrophage-activating agents on MacMARCKS mRNA accumulation in RAW 264.7 cells. Peritoneal macrophages were stimulated with medium alone (cont), 100 ng/ml LPS (LPS), 100 ng/ml PMA (PMA), a combination of both LPS and PMA (LPS+PMA), 1000 u/ml TNF-α (TNF) or 25 U/ml IL-4 (IL-4) for 4 hrs. Total RNA was isolated and subjected to RNase protection as described above. Protected fragments were resolved in a 6% sequencing gel and detected by autoradiography.
of macrophage-specific genes (Stacey et al., 1995). To determine whether PMA could induce MacMARCKS mRNA, peritoneal macrophages were incubated with 100 ng/ml of PMA for 4 hrs either alone or in the presence of 100 ng/ml LPS. PMA alone did not induce MacMARCKS transcripts, and addition of PMA had no effect on LPS induction of MacMARCKS message (Fig. 5-2). PMA stimulation also had no effect on MacMARCKS induction in RAW 264.7 cells (data not shown). In addition to LPS and PMA, treatment of peritoneal macrophages with recombinant murine TNF-α or recombinant murine IL-4 both failed to induce MacMARCKS mRNA (Fig. 5-2b). Recombinant γ-IF also failed to induce MacMARCKS expression, in agreement with previous results (data not shown; Aderem et al., 1988).

**Time Course of MacMARCKS mRNA Induction by LPS:**

To determine the kinetics of induction of MacMARCKS transcripts, peritoneal macrophages were stimulated with 100 ng/ml LPS for various times (from 5 min. to 4 hrs), and total RNAs were prepared and analyzed by RPA. Induction of MacMARCKS message occurred rapidly, with an increase detectable as early as 5 min. after LPS addition (Fig. 5-3a top). Expression levels peaked after 2 hrs of LPS stimulation and remained at maximal levels even after 4 hrs. After 6 hrs of stimulation, the message level decreased slightly (data not shown). MacMARCKS protein levels showed similar induction kinetics, with an increase in protein levels detectable after 20 minutes of LPS stimulation (Fig. 5-3a bottom). RAW 264.7 cells stimulated with LPS showed the same time course of MacMARCKS mRNA induction as the peritoneal macrophages (data not shown).

**Superinduction of MacMARCKS mRNA by LPS and Cycloheximide:**

Expression of several LPS-inducible genes occurs independently of de novo protein synthesis (Farber, 1992; Shin et al., 1994). To determine whether induction of
**Figure 5-3.** Kinetics of MacMARCKS mRNA and protein induction by LPS and the effect of cycloheximide on expression of MacMARCKS mRNA.

**a.** Kinetics of induction of MacMARCKS mRNA and protein by LPS.  
*(Top)*. Peritoneal macrophages were stimulated with 100 ng/ml LPS and mRNA was isolated at the indicated times after stimulation. Levels of MacMARCKS mRNA were then determined by RNase protection. Protected fragments were resolved in a 6% sequencing gel and detected by autoradiography.  
*(Bottom)*. Peritoneal macrophages were labeled with $[^{3}H]$myristic acid and stimulated with LPS as above. MacMARCKS was immunoprecipitated from the cell lysate, resolved by 10% SDS-PAGE and visualized by fluorography.

**b.** Effect of cycloheximide on expression of MacMARCKS mRNA. RAW 264.7 cells were incubated with medium alone (cont-CHX), medium containing 100 ng/ml LPS alone (LPS - CHX), medium containing 5 ug/ml cycloheximide (cont+CHX) or medium with a combination of both agents (LPS+CHX). Total RNA was isolated after 4 hrs and subjected to RNase protection. Protected fragments were resolved in a 6% sequencing gel and detected by autoradiography.
MacMARCKS mRNA required protein synthesis, both peritoneal macrophages and RAW 264.7 cells were stimulated for 4 hrs with LPS in the presence or absence of the protein synthesis inhibitor cycloheximide, and RNAs were analysed by RPA. As shown in Fig. 5-3b, cycloheximide alone induced MacMARCKS mRNA about 6.5 fold over untreated cells, while LPS alone showed a 10 fold induction of MacMARCKS message. Inclusion of LPS with cycloheximide superinduced MacMARCKS mRNA 20 fold over control levels. MacMARCKS mRNA was induced within 10 min. after cycloheximide addition (data not shown). This rapid induction of MacMARCKS mRNA levels by LPS and cycloheximide suggests that MacMARCKS gene regulation falls within the class of rapid-response or “immediate early” genes (Lau and Nathans, 1987).

Effects of LPS on the Rate of MacMARCKS Transcription:

Several mechanisms could account for the increased amounts of MacMARCKS mRNA in macrophages stimulated with LPS. Increased mRNA levels could be due to either enhanced rate of transcription of the MacMARCKS gene, or to posttranscriptional changes, including increased MacMARCKS mRNA stability. To determine whether the rapid increase in MacMARCKS mRNA following LPS stimulation was due to transcriptional activation, nuclear run-on analysis was performed on RAW 264.7 cells. The MacMARCKS gene was constitutively transcribed at high levels in untreated cells, and LPS stimulation increased the rate of transcription at most two-fold over basal levels even after 12 hrs of LPS treatment (Fig. 5-4). This result suggests that the induction of MacMARCKS mRNA by LPS in macrophages occurs at the post-transcriptional level. The transcription rates for a number of macrophage marker genes were also examined. The rates of transcription of plasminogen-activator inhibitor 2 (PAI-2), urokinase plasminogen activator (uPA), and interleukin 1-β (IL1-β) genes were all induced by LPS (Fig. 5-4). However, both TNF-α and lysozyme showed very high basal levels of transcription, and
Figure 5-4. Effect of LPS on MacMARCKS transcription.

LPS stimulation of MacMARCKS transcription is not due to transcriptional activation. RAW 264.7 cells were incubated in medium alone (control) or in medium containing 100 ng/ml LPS. Nuclear run-on transcripts were prepared from isolated nuclei after the indicated times of LPS treatment. Denatured, linearized plasmid DNA fragments in PBlueScript vector were applied to a nitrocellulose membrane and hybridized to $^{32}$P-labeled run-on transcripts. uPA, urokinase plasminogen activator; iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor-α; IL1-β, interleukin 1-β.
like MacMARCKS, were not appreciably induced by LPS over the time course examined (Fig. 5-4).

**Functional Analysis of Luciferase Reporter Constructs in RAW 264.7 Cells:**

A series of deletion constructs were made containing fragments of the MacMARCKS 5' regulatory region inserted upstream of the luciferase reporter gene pGL-basic (Fig. 5-5). These constructs were transiently transfected into RAW 264.7 cells by electroporation (Stacey *et al.*, 1993). When stimulated with LPS, RAW 264.7 cells that were transfected with a construct containing 4 kb of the 5' MacMARCKS flanking region displayed a 10-fold increase in luciferase activity over cells not treated with LPS (Fig. 5-5). A similar increase in LPS-dependent luciferase activity was seen in all cells expressing deletion constructs. Even p7'-10 luc, the construct containing the shortest 5' regulatory region (-113 to +24), gave a 16-fold increase in luciferase activity after LPS stimulation (Fig. 5-5). This upstream region contains 3 potential SP-1 binding sites (Fig. 5-1a), and these SP-1 response elements have been shown to mediate LPS response (Hume *et al.*, in press).

**Generation of MacMARCKS-lacZ Transgenic Mice:**

To identify cis-regulatory elements that define MacMARCKS expression *in vivo*, transgenic mice carrying MacMARCKS promoter-lacZ constructs were generated. A 4 kb BamHI-NheI fragment from the 5' upstream region containing the MacMARCKS transcriptional start site and 30 bp of the untranslated region was subcloned into the pNASSβ vector (Clontech; Fig. 5-6a). The pNASSβ vector contains a SV40 splice donor/acceptor site, followed by the *E. coli* lacZ gene and a SV40 polyadenylation signal. This vector was previously used to produce promoter-lacZ transgenic mice (Li *et al.*, 109).
Figure 5-5. Functional analysis of MacMARCKS-luciferase constructs in RAW 264.7 cells.

Luciferase activity in transfected RAW 264.7 macrophages. Each regulatory region-luciferase construct (see Fig. 5-1a) was co-transfected into RAW 264.7 cells along with a human growth hormone expressing plasmid phGH. Transfected cells were stimulated with medium alone or medium containing 100 ng/ml LPS for 4 hrs. After assaying for both luciferase activity and hGH, the luciferase relative light units were normalized by dividing them by cpm from the hGHH radioimmunoassay, and the data were reported as relative luciferase units per ug protein. LPS stimulation does not affect hGH production (data not shown).
Relative Luciferase Unit/ug protein

<table>
<thead>
<tr>
<th></th>
<th>medium</th>
<th>LPS</th>
<th>fold induction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2000</td>
<td>20000</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>15000</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>7000</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>8000</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>220</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Restriction enzymes (Sac, Nhe, AvrII, EcoRI, SmaI, Nhe) are marked on the diagram. The luciferase construct regions range from -4010 to -113. The fold induction values indicate the relative increase in luciferase activity upon LPS treatment compared to medium control.
Figure 5-6. Construction and quantitation of the MacMARCKS-lacZ transgenes.

a. Restriction map of the endogenous MacMARCKS gene and MacMARCKS-lacZ transgenes. The relevant restriction sites are: B, BamHI; E, EcoRI; H, HindIII; N, NheI; P, PstI; RV, EcoRV; S, SacI; X, XbaI. A 620 bp XbaI-SacI fragment was used as a probe to detect a 3.6 kb HindIII-Sacl fragment from the endogenous gene and a 5.8 kb HindIII-Sacl fragment from the 4 kb-TM transgene. The same probe detects a 2.9 kb EcoRI fragment from the endogenous gene and a 2 kb EcoRI-EcoRV fragment from the 1.8 kb-TM transgene. The grey box in the transgenic construct diagrams represents the SV-40 splice site, and the black box represents the SV-40 poly-A site of the pNASSβ vector (Clonetech). Arrows denote the transcriptional start sites.

b. Determination of the copy number of the 4 kb-TM transgenes in lines 7 and 13. 20 μg of genomic DNA isolated from the tails of founder mice was digested with HindIII and SacI, electrophoresed, and blotted onto nitrocellulose. The filter was hybridized to a 32P-labeled 620 bp probe (a), washed, and autoradiographed. The intensities of the 5.8 kb transgene (TG) band and the 3.6 kb endogenous (Endo) band were determined by densitometry (LKB). The ratio of the TG/Endo intensities yielded the copy number of the transgenes (approximately 5 copies for line 7, 15 copies for line 13).

c. Determination of the copy number of the 1.8 kb-TM transgenes in lines 28, 29 and 30. 20 μg of genomic DNA was digested with EcoRI and EcoRV and processed as above. Approximately 8 copies of the transgene are present in line 28, 14 copies in line 29, and 15 copies in line 30.
Endogenous MacMARCKS gene

Transgenes

b  4 kb-lacZ lines

7  13

5.8 kb  TG

3.6 kb  Endo

C  1.8 kb-lacZ lines

28  29  30

2.9 kb

2.0 kb  Endo  TG
This construct was named 4 kb-TM and was first transfected into PC12 cells to test for expression of β-galactosidase in PC12 cells. After establishing that 4 kb-TM properly directed lacZ expression in the cell bodies and axons of PC12 cells (data not shown), it was injected into fertilized eggs of B6/CBA mice to generate founders. Founders were detected by PCR, using primers specific to the lacZ gene (data not shown). Southern blot analysis of founder mice tail DNAs indicated that the transgene integrated into the genomes of 8 out of 44 mice. Embryos from two independent lines, TG-7 and TG-13, which exhibited similar β-galactosidase expression patterns were studied in detail (Fig. 5-6b). Compared to the endogenous MacMARCKS gene, 4 kb TG-7 and 4 kb TG-13 contain approximately 5, and 15 copies of the transgene, respectively. The level of transgene expression was not dependent upon copy number, since embryos from both lines incubated in X-gal stained with the similar intensity (data not shown).

Deletion analysis was carried out to further define the location of cis-regulatory elements within the 5' flanking region. Digestion of the 4 kb-TM construct with PstI generated the 1.8 kb-TM construct (Fig. 5-6c), which was used to generate three founder lines (1.8 kb T-28, 29 and 30) which carried different copy numbers of the transgene. TG-28 contains approximately 8 copies, TG-29 14 copies, and TG-30 15 copies of the transgene. Since all three lines showed similar intensities upon X-gal staining for lacZ activity, only results for line TG-29 are presented.

**Expression of the 4 kb-LacZ Transgene in Microglia:**

Peritoneal macrophages isolated from transgenic mice carrying the 4 kb-lacZ construct were either untreated, or stimulated with 100 ng/ml LPS for 4 hrs. The macrophages were fixed and subjected to indirect immunofluorescence using both monoclonal and polyclonal antibodies specific for β-galactosidase. LacZ expression was not observed in macrophages from either transgenic line, suggesting that additional
regulatory elements not present in the 4 kb 5' flanking sequence are needed to confer expression in peritoneal macrophages. However, immunofluorescence microscopy with the pan F4-80 macrophage monoclonal antibody and a polyclonal anti-β-galactosidase antibody double-labeled a population of cells in the brain of transgenic mice which display morphologies characteristic of microglia (see Chapter 6, Fig. 6-5e, f). This indicates that the 4 kb flanking region is capable of driving lacZ expression in a specialized macrophage population in the brain.

**LPS Induced MacMARCKS in Microglia:**

To characterize MacMARCKS in a purified microglial population, subarachnoid membranes were isolated from newborn mice, dissociated in media, and cultured on feeder cells. Purified microglia labeled with [3H]-myristic acid were either untreated, or stimulated with either zymosan, PMA or LPS. Following agonist stimulation, the cells were lysed and immunoprecipitated with an anti-MacMARCKS antibody. As shown in Fig. 5-7, MacMARCKS protein in microglia was specifically induced by treatment with LPS. The level of induction was similar to that seen for peritoneal macrophages (compare Fig. 5-7 to Fig. 5-2a, bottom). In contrast, the level of the MARCKS protein in microglia appears to be unaffected by agonist stimulation (Fig. 5-7).

**DISCUSSION**

Macrophages are activated by a wide variety of substances. The direct response of macrophages to LPS from gram-negative bacteria represents a form of natural immunity to foreign microorganisms. LPS exerts a profound effect on the immune system by enhancing the release of various cytokines and arachidonic acid metabolites from macrophages (Morrison and Ulevitch, 1978). It also activates multiple immediate-early genes that are thought to exert significant effects on the early stages of macrophage activation (Shin et al., 115
**Figure 5-7.** LPS stimulation of microglia induces MacMARCKS.

Purified microglia were labeled for 3 hrs with [3H]myristic acid (40 μCi/ml) either without treatment or in the presence of 50 μg/ml opsonized zymozan, 200 nM PMA or 100 ng/ml LPS. Cells were then washed, lysed in lysis buffer and equal amounts of proteins were loaded in all lanes. Proteins were resolved by 10% SDS-PAGE and visualized by fluorography.
The image shows a gel blot analysis with molecular weight markers (kDa) at 97, 66, and 45. The gel is stained to visualize protein bands labeled as MARCKS and MacMARCKS.

**Lanes:**
- **Control**
- **Zymosan**
- **PMA**
- **LPS**

The MARCKS and MacMARCKS bands are clearly visible under the respective conditions.
Macrophages possess dynamic and highly plastic cytoskeletons necessary for migration into sites of inflammation and efficient phagocytosis of microorganisms. LPS enhances phagocytosis, secretion, and membrane fluidity in macrophages, probably through calcium and PKC-dependent rearrangement of the actin cytoskeleton (Cohn, 1988). The synthesis of MARCKS and MacMARCKS proteins is stimulated by LPS (Aderem et al., 1986), and these two proteins integrate calcium and PKC signal transduction pathways in the reorganization of the macrophage cytoskeleton after LPS stimulation (Aderem, 1992).

LPS stimulates expression of the MacMARCKS gene within minutes in macrophages. Nuclear run-on experiments demonstrate that this mRNA induction is not due to an increase in the rate of transcription. MacMARCKS transcripts are also rapidly induced by cycloheximide, and cycloheximide and LPS together superinduce MacMARCKS mRNA levels. These results indicate that LPS induction of MacMARCKS expression occurs at the post-transcriptional level, and suggest several plausible mechanisms for the molecular basis of LPS priming in macrophages. The RNA polymerase II system may be subjected to regulation by selective block of elongation at specific sites within the gene. Control over the elongation step of transcription is well documented for a number of genes, including c-fos (Fort et al., 1987) and TNF-α (Biragyn and Nedospasov, 1995). In this model, a transcriptional pause site at the 5' end of the MacMARCKS gene would cause most of the RNA polymerase II complexes to terminate prematurely. These prematurely terminated transcripts are unstable and are rapidly degraded. As a result, there is a high flux of nascent transcripts through the 5' region of the MacMARCKS gene but only a few transcription complexes proceed through to the 3' end of the gene. Addition of LPS abolishes the pause site, so that the pol II complexes proceed through to the 3' end, resulting in increased levels of full-length transcripts. Cycloheximide functions like LPS to eliminate the pause, and the two together have an additive effect. The
observation that cycloheximide increases MacMARCKS mRNA so rapidly suggests that a labile protein might occupy the pause site, and that protein synthesis is required to maintain this factor. Since only the full-length MacMARCKS probe was used in the nuclear-run on analysis, it was not possible to confirm this mechanism of regulation. 5' and 3' MacMARCKS probes are required to determine whether elongation termination is the post-transcriptional mechanism regulating MacMARCKS gene expression. Alternatively, the MacMARCKS message could be very unstable and most transcripts generated are rapidly degraded by a labile factor. Both LPS and cycloheximide repress this factor, allowing accumulation of MacMARCKS mRNA. Many immediate early genes contain conserved AU-rich motifs in the 3' region of their mRNA which confer mRNA instability (Shaw and Kamen, 1986). MacMARCKS, however, does not have these sequences in its 3' untranslated region (data not shown). The MacMARCKS message is also stable up to 6 hrs, indicating that unlike most immediate-early gene transcripts, the MacMARCKS message is probably not regulated by degradation.

Functional analysis of 5' deletion clones indicate that even the shortest promoter fragment tested, 7'-10 luc, possesses full LPS responsive activity. This construct contains a sequence only 113 bp upstream of the transcriptional start site which possess 3 potential SP-1 binding sites. This observation is somewhat surprising, since most LPS response elements (LREs) previously described do not contain SP-1 sites. The LREs of a large number of genes induced by LPS contain NF-κB and AP-1 binding sites (Shakov et al., 1990). While the MacMARCKS promoter contains two AP-1 sites and an imperfect NF-κB site, deleting all three elements did not abolish the LPS responsiveness of either p6 luc or 7'-10 luc. Recently, Hume et al have reported that SP-1 response elements can function as LREs, and the induction of MacMARCKS mRNA by LPS could be mediated through its multiple SP-1 sites.
MacMARCKS is expressed in microglia in both embryonic and the adult CNS (see Chapter 6), and the MacMARCKS protein is induced in purified microglia stimulated with LPS. A 4 kb MacMARCKS 5' flanking sequence is sufficient to direct lacZ expression in microglia but not in peritoneal macrophages, suggesting that the MacMARCKS gene is differentially regulated in these cell types. Microglial cells are resident macrophages of specialized function derived from hematogenous monocytes which invade the developing CNS during the late embryonic and early prenatal period (Perry and Gordon, 1991). These ameboid microglial cells differentiate into ramified microglia which are widely distributed throughout the white and grey matter of the adult CNS. These cells remain quiescent until they are confronted with pathologically induced changes in the CNS. Under these circumstances microglial cells are activated, change their cellular distribution and immunophenotype, and begin phagocytosing dying cells (Perry and Gordon, 1991; Miguel et al., 1995). Activated microglia also undergo morphological alterations that are driven by a complex series of coordinated changes in the underlying cytoskeleton. It is tempting to speculate that MacMARCKS, an actin-associated cytoskeletal protein, is required for the morphological changes associated with microglial activation by pathological insults.
CHAPTER SIX

EXPRESSION OF THE MacMARCKS GENE DURING MOUSE DEVELOPMENT

INTRODUCTION

This chapter describes the characterization of MacMARCKS expression in the mouse in various embryonic and adult tissues. Temporal and spatial patterns of MacMARCKS expression during embryonic development were determined by RNase protection, in situ hybridization and immunohistochemistry. To define cis-regulatory elements in the MacMARCKS promoter that specify MacMARCKS expression patterns during central nervous system (CNS) development, transgenic mice carrying MacMARCKS promoter-lacZ constructs were generated (see Chapter 5, Fig. 5-5). The results of these studies indicate that MacMARCKS is highly expressed in the developing CNS, consistent with it playing a role during neural development.

Neurulation, the formation of the neural tube, begins in the E7.5 mouse embryo with transformation of the midline epiblast (a totipotent stem cell population) into a pseudostratified columnar epithelium at the ventral midline which forms the neural plate (reviewed in Copp et al., 1990). This transformation is mediated by the underlying notochord and paraxial mesoderm. Formation of the neural plate leads to elevation of its edges above the midline to convert the plate into a groove. Apposition of the edges results in neural fold fusion along the dorsal midline to create the roof plate at approximately E9. The ventral midline of the neural tube lying directly over the notochord is termed the floor plate. This structure has ventral patterning activity within the neural tube and is important for axonal guidance (Yamada et al., 1991; Hatta, 1992). Concomitant with neural tube formation, neural crest cells originate from the cells of the neural folds and then emigrate to
give rise to skeletal structures of the head and the peripheral nervous system. By E9.5, the primitive brain can be demarcated into five regions. The forebrain is composed of the rapidly expanding telencephalon and the diencephalon. The diencephalon is connected to the midbrain, or mesencephalon, which in turn connects to the hindbrain. The hindbrain is divided into the rostral metencephalon and the caudal myelencephalon. Finally, the myelencephalon connects with the spinal cord.

To understand the molecular mechanisms underlying neurulation and neural patterning in the mouse, it is necessary to identify genes that are expressed during this stage of development. Many genes involved in developmental regulation and pattern formation in *Drosophila* have been functionally conserved in the mouse, and several are expressed during neurulation (for review see Beddington and Smith, 1993). This suggests that the mechanism underlying pattern formation may itself be evolutionarily conserved. Some of these genes include *HNF-3β*, which encodes a fork head domain DNA binding motif (Sasaki and Hogan, 1993), the *Engrailed (En)* family of homeobox containing genes (Davis *et al.*, 1988), and the *Wnt* gene family which encode secreted cell signaling glycoproteins (for review see McMahon, 1992).

*HNF-3β* is expressed during gastrulation in the node and notochord, and during neurulation in the notochord, floorplate and gut (Sasaki and Hogan, 1993). The node is crucial in the organization and patterning the midline axis of the embryo (Ang and Rossant, 1994), and cells from the node gives rise to the notochord. Mice homozygous for the *HNF-3β* mutation do not develop a node or notochord and die during embryogenesis. However an abnormal neural tube without floor plate cells does form, indicating that *HNF-3β* is required for the formation of the node and notochord, but is not absolutely required for neural tube induction (Weinstein *et al.*, 1994; Ang and Rossant, 1994).

The *En* genes are expressed in a highly specific pattern in all vertebrate embryos examined (Joyner *et al.*, 1991). During and after neural tube closure, *En-1* and *En-2* are
expressed in the midbrain-hindbrain border as a specific stripe of En-positive cells (Davis et al., 1988). In the adult, En-2, but not En-1, is expressed in granular cells of the cerebellum. Targeted deletion of En-1 results in a deletion of most of the cerebellum, colliculi, and pons (Wurst et al., 1994). Deletion of En-2 generated normal embryos, while adults showed only subtle cerebellar patterning defects (Joyner et al., 1991). These results suggest that En-1 can substitute for En-2 function during embryonic development, but not vice versa.

The Wnt-1 gene is expressed rostrally throughout the neural folds of E9 embryos, while its caudal expression is restricted to the lateral lips of the neural plate (Wilkinson et al., 1987). By E9.5, expression is seen in the dorsal midline of the midbrain, hindbrain, and along the length of the neural tube into the spinal cord. This expression pattern remains unchanged late into development. Targeted deletion of Wnt-1 results in deletion of the midbrain and part of the metencephalon, while the rest of the neural tube and all other tissues are normal (McMahon and Bradley, 1990). This result suggests that Wnt-1 may be a determinant required for specifying the midbrain during development, and functional redundancy with the Wnt-3a gene may explain why the hindbrain and spinal cord are normal in Wnt-1 mutants (McMahon et al., 1992). Interestingly, the expression patterns of En-1 and Wnt-1 overlap during development, and in Wnt-1 null embryos the first identifiable phenotype appears to be a loss of anterior En expression in the midbrain at E8.5. By E9.5, En expression is completely abolished, and this loss coincides with a deletion of neural tissue (McMahon et al., 1992). This implies that Wnt-1 activity is required to maintain both En expression and the generation or maintenance of midbrain structures. A similar role has been proposed for the Drosophila ortholog wingless (wg) during segmentation -- secreted wg gene product is required to regulate the expression of En in neighboring cells (DiNardo et al., 1988).
Neurulation is driven by a complex series of developmentally coordinated changes in cell shape, and these changes in cell morphology are in turn dependent upon changes in the underlying cytoskeleton. Transcription factors such as HNF-3β and En must therefore activate genes encoding factors which organize the cytoarchitecture of differentiating cells. In Drosophila, wg organizes pattern formation by activating armadillo, which encodes a homologue of β-catenin, a cadherin-associated protein (Peifer and Wieschaus, 1990). Cadherins are a molecular superfamily of cell adhesion proteins that associate with the catenins. This association is required for normal cadherin function. Both cadherins and catenins localize to the cell-cell adherens junctions and anchor the actin cytoskeleton. Disruption of armadillo function in Drosophila germ cells leads to a collapse of the cortical actin cytoskeleton and distortion of cell shape, consistent with the role for armadillo and the adherens junction in anchoring the actin cytoskeleton (Peifer, 1995). In the mouse, E-cadherin is expressed at E9 in the diencephalon and mesencephalon, in regions that parallel the expression of Wnt-1. αN-catenin is expressed at the roof plate of the diencephalon and mesencephalon, coinciding with Wnt-1 expression (Shimamura et al., 1994). In Wnt-1 null mice, E-cadherin expression is upregulated, while αN-catenin expression is suppressed (Shimamura et al., 1994). These results suggest that Wnt-1 is involved in the regulation of E-cadherin and αN-catenin expression in specific domains of the embryonic brain.

MARCKS and MacMARCKS are actin-associated cytoskeleton proteins (Hartwig et al., 1992; Aderem, unpublished data). MARCKS is highly expressed in the brain and spinal cord of fetal and adult animals (Albert et al., 1986; Patel and Kligman, 1987; Lobach et al., 1993), and functional deletion of the MARCKS gene is lethal and results in a number of neuronal defects that affect cortical patterning (Stumpo et al., 1995). This is perhaps not surprising considering that MARCKS is a prominent PKC substrate; most members of the PKC family are expressed in brain and are implicated in neural development (Nishizuka, 1990).
By Northern analysis, MacMARCKS is highly expressed in the adult brain, as well as in the testis, uterus and spleen (Lobach et al., 1993).

RESULTS

RNase Protection Analysis of MacMARCKS Expression In Tissues:

To examine expression profiles of the MacMARCKS gene, total RNA from various organs from both E16.5 embryonic and adult mouse tissues was used in the RNase protection assay (RPA). This analysis utilized an antisense MacMARCKS probe which specifically protected a 254 bp transcript. An antisense actin probe was used as an internal loading control to normalize expression levels. In the E16.5 embryo, MacMARCKS expression was highest in the CNS, particularly in the forebrain and midbrain (Fig. 6-1A). In the adult, MacMARCKS expression was highest in the testis, uterus and spleen, with lower levels of expression in the CNS (Fig. 6-1B). To determine whether the down-regulation of MacMARCKS mRNA in the CNS took place pre- or postnatally, RPA was performed on total RNAs from embryonic, P0 and adult brains. Down-regulation of MacMARCKS mRNA in the CNS occurred primarily postnatally, with high levels of MacMARCKS expression seen in the embryonic and newborn (P0) CNS; expression in the adult CNS was approximately 10-fold less than the embryonic levels (Fig. 6-1C). The pattern of MacMARCKS expression suggests that it may be involved in embryonic CNS development.

MacMARCKS Expression Patterns During Mouse Development:

To investigate the temporal and spatial pattern of MacMARCKS expression, whole mount in situ hybridization was performed with a MacMARCKS riboprobe. MacMARCKS expression was seen throughout the embryonic portion of the egg cylinder
Figure 6-1. RNase protection analysis of MacMARCKS expression in tissues.

a. MacMARCKS expression in E16.5 embryonic tissues. Total RNA was isolated from various tissues and analysed by RNase protection. 5 μg of total RNA was hybridized to a 315 bp run-off transcript complementary to the MacMARCKS coding sequence. A 250 bp β-actin antisense RNA probe was included as an internal control. Protected fragments were resolved in a 6% sequencing gel and detected by autoradiography. Lanes: 1, total brain; 2, forebrain; 3, hindbrain; 4, midbrain; 5, eye; 6, heart; 7, intestine; 8, limb; 9, liver; 10, lung; 11, skull; 12, spine.

b. MacMARCKS expression in adult tissues. Total RNA was isolated from various organs and processed as described in (a). Protected fragments were resolved in a 6% sequencing gel and detected by autoradiography. Lanes: 1, total brain; 2, eye; 3, kidney; 4, liver; 5, lung; 6, muscle; 7, spine; 8, spleen; 9, sm. intestine; 10, testis; 11, uterus.

c. MacMARCKS expression in brain is developmentally regulated. Total RNA isolated from developmentally staged brains were analysed by RNase protection as described in (a). Protected fragments were resolved in a 6% sequencing gel and detected by autoradiography. Lanes: 1, E12.5; 2, E14.5; 3, E16.5; 4, E18.5; 5, P0; 6, adult.
as early as the mid-gastrulation (E7.5) stage (Fig. 6-2a). By E8.5, MacMARCKS transcripts were detected throughout the head folds (Fig. 6-2b). Anterior MacMARCKS expression was confined to the cranial neural plate, a layer of thickened neural epithelium that proliferates to eventually close the neural tube. MacMARCKS expression was also detected along the entire length of the neural tube (Fig. 6-2b). Specific enrichment of MacMARCKS transcripts was seen in the lateral edges of the neural plate (Fig. 6-2b) and at the floor plate, the ventral portion of the neural tube (Fig. 6-2c). In situ hybridization of cross sections through an E8.5 embryo indicated that while MacMARCKS transcripts were detected in the surrounding cerebral mesenchyme, they were more abundant in the lateral folds of the neural epithelium (Fig. 6-2d). Hybridization signals were also detected in the notochord (data not shown).

In situ hybridization of sagittal embryo sections from E12.5 to P0 indicated that strong MacMARCKS expression was detected throughout the brain and spinal cord beginning at E12.5 (Fig. 6-2e). Expression was seen in the dorsal root ganglia and other neurons of peripheral nervous system beginning at E14.5 (Fig. 6-2f). At E16.5, MacMARCKS expression was also detected in the nuclear layer of the developing retina (Fig. 6-2g). Examination of MacMARCKS expression at P0 indicated that elevated levels were restricted to the cerebral neocortex and olfactory bulb, with lower levels detected in the cerebellum and dorsal spinal cord (Fig. 6-2h). At this stage, MacMARCKS expression in other organs such as the kidney and spleen was also detected (data not shown). In all cases, hybridization with a sense control probe detected no signals above background (data not shown). In the adult mouse brain, high levels of MacMARCKS transcripts were detected in specific areas, including the cerebral cortex, hippocampus, dentate gyrus and to a lesser extent, the primary olfactory cortex and cerebellum (Fig. 6-2I and data not shown). These results suggest that the down-regulation of MacMARCKS expression in the adult brain results from restricting MacMARCKS expression to specific regions of the brain.
Figure 6-2. MacMARCKS expression patterns during mouse development.

a. Whole mount in situ hybridization of E7.5 embryo using MacMARCKS antisense riboprobes labeled with digoxygenin-UTP. MacMARCKS transcripts were detected throughout the embryonic (e) portion of the egg cylinder, while no staining was seen in the extraembryonic (ee) portion. ps, primitive streak.

b. Side view of whole mount in situ hybridization of an E8.5 embryo processed as described in (a). MacMARCKS was detected at the lateral edges of the head fold (hf) and in the neural tube (nt).

c. Frontal view of the embryo shown in (b). MacMARCKS transcripts were enriched throughout the head folds (arrowheads) and in the floor plate (arrow).

d. Transverse section through the prospective forebrain (left) and the prospective hindbrain (right) of E8.5 mouse embryo was hybridized with an antisense $^{35}$S-UTP labeled MacMARCKS RNA probe. MacMARCKS transcripts were detected throughout the head folds (hf) and in the mesenchymal cells (m). ng, neural groove.

e. Sagittal section of an E12.5 embryo hybridized with an antisense $^{35}$S-UTP-labeled MacMARCKS riboprobe. MacMARCKS transcripts were detected throughout the CNS, including the telencephalon (t), cerebellum (cb) and spinal cord (sc). A sense probe showed no signal above background (not shown).

f. Sagittal section of an E14.5 embryo processed as in (e). MacMARCKS transcripts were detected throughout the CNS and PNS, including the dorsal root ganglia (drg) and glossopharyngeal ganglion (IX).

g. Sagittal section of an E16.5 embryo processed as in (e). MacMARCKS transcripts were concentrated throughout the CNS and the nuclear layer of the retina (nl). Abbreviations are as in (e).

h. Sagittal section of a P0 brain, processed as in (e). MacMARCKS transcripts were localized preferentially to the olfactory bulb (of) and cortex (ctx), with some signals in the cerebellum (cb) and dorsal spinal cord (sc).

i. Sagittal section of an adult brain, processed as in (e). MacMARCKS transcripts are concentrated in the cortex (ctx), hippocampus (h) and dentate gyrus (dg). MacMARCKS signal was also detected in the cerebellum and olfactory bulb (not shown).
k. Dorsal view of an E9.5 embryo processed as in (j). MacMARCKS protein was present along the entire neural tube (arrows) and spinal cord (sc).

l. Side view of an E10.5 embryo stained with an anti-MacMARCKS antibody. MacMARCKS immunoreactivity was present in the telencephalon (t), mesencephalon (m), neural tube (nt) and notochord (nc).
Immunohistochemical analysis of E8.5 embryos using an anti-MacMARCKS antibody indicated that MacMARCKS protein was enriched in the lateral edges of the neural folds, consistent with its mRNA distribution pattern (Fig 6-2j). At E9.5, MacMARCKS protein was detected throughout the spinal cord (Fig. 6-2k). In the E10.5 embryo, MacMARCKS protein was detected in the telencephalon, the ventral mesencephalon, the neural tube and the notocord (Fig. 6-2l). These data indicate that initiation of MacMARCKS expression in the nervous system occurs very early during embryogenesis, and suggest a role for MacMARCKS in neural development.

β-galactosidase Expression Patterns in Embryos:

To define cis-regulatory elements which determine MacMARCKS expression during CNS development, transgenic mice carrying the 4 kb MacMARCKS promoter-lacZ constructs (lines 4 kb-7 and 4 kb-13; Fig. 5-6A) were examined for β-galactosidase activity beginning at E7.5. Expression was first detected in specific cells surrounding the periphery of the morphologically distinct node found at the very tip of the primitive streak (Fig. 6-3a). This pattern of β-galactosidase activity was more restricted than the distribution of the endogenous MacMARCKS transcript in E7.5 embryos (Fig. 6-2a) and strongly resembles the expression pattern of nodal, a node-specific marker (Zhou et al., 1993). At E8.5, intense β-galactosidase activity was detected in the CNS at the ventral midline of the midbrain and along the presumptive spinal cord extending into the tail (Fig. 6-3b). Frontal views revealed that lacZ expression was at the ventral portion of the closing cranial neural folds at the forebrain-midbrain junction. This region corresponds to de novo closure point two (Fig. 6-3c; see below). Compared with the distribution of the endogenous MacMARCKS transcript at this stage of development, the pattern of β-galactosidase activity in transgenic E8.5 embryos appeared to be ventrally restricted. LacZ expression in transgenic embryos correlated with endogenous MacMARCKS expression at the floor plate.
**Figure 6-3.** Expression of β-galactosidase in 4 kb-lacZ transgenic mice during development.

**a.** Lateral view of an E7.5 embryo showing specific lacZ-expressing cells around the node (n).

**b.** Lateral view of an E8.5 embryo. β-galactosidase activity was detected in the mesencephalon (m), rhombencephalon (rh) and notochord-floorplate (nc-fp). Notochord-floor plate expression extended almost the entire body length. p, prosencephalon.

**c.** Frontal view of E8.5 embryo, showing lacZ expression at the floorplate (arrow) of the closing neural tube. The site of lacZ expression coincided with closure point two.

**d.** Coronal section through the rhombencephalon of the E8.5 embryo shown in (b) indicated that strong β-galactosidase activity was observed in the floor plate (fp) and notochord (nc). nf, neural fold; fg, foregut.

**d’.** Coronal section through the tail of the E8.5 embryo shown in (b). LacZ expression was specific to the notochord. Abbreviations: fp, floor plate; nt, neural tube; s, somites.

**e.** Lateral view of an E9.5 embryo. LacZ expression was concentrated in the ventral mesencephalon (m), rhombencephalon (rh), floor plate-notochord (fp-nc), and within the otic vesicle (o). t, telencephalon; di, diencephalon.

**f.** Dorsal view of embryo shown in (e).

**g.** Lateral view of an E10.5 embryo. β-galactosidase activity was detected throughout the CNS and neural tube (nt). Abbreviations are as in (e).

**h.** Frontal view of embryo shown in (g). Intense lacZ expression was detected in the spinal cord (sc) and somites (arrowhead).

**i.** Dorsal view of same embryo as in (g). Expression is most intense in the spinal cord (sc).

**j.** Lateral view of an E12.5 embryo. LacZ activity was diffuse throughout the CNS and neural tube (nt).

**k.** High magnification view of embryo in (j) indicated that β-galactosidase activity was present in the dorsal root ganglia (drg) and spinal neurons (s).
of the closing neural tube (compare Fig. 6-2c with Fig. 6-3c). Cross sections through the presumptive midbrain of the embryo confirmed that the rostral limit of β-galactosidase activity was localized to the ventral forebrain-midbrain junction (data not shown). A more caudal section through the hindbrain region revealed specific β-galactosidase expression in the notochord and in the ventral midline cells of the floor plate (Fig. 6-3d; d'). The floor plate is involved in regulating dorsoventral patterning of the neural tube (Hatta et al., 1991). In contrast, lacZ expression was not detected in the lateral edges of the neural plate, a region of high endogenous MacMARCKS concentration (Fig. 6-2c). β-galactosidase activity was not detected in any other region of the embryo nor in nontransgenic littermates (data not shown).

In E9.5 embryos, intense lacZ expression was restricted to the mesencephalon, extending from the diencephalon-mesencephalon boundary to the mesencephalon-rhombencephalon border (Fig. 6-3e). This area, termed the germinal zone, is where cells divide to generate either post-mitotic neurons or glioblasts which migrate out of the germinal zone to populate cell-sparse regions around the outer surface of the neural tube (Davis et al., 1988). β-galactosidase activity in the spinal cord was continuous with activity in the midbrain and was restricted to the floor plate and the notochord (Fig. 6-3e; f). In addition, LacZ expression was also detected in the ventral portion of the otic placode (Fig. 6-3e; f). LacZ expression at E9.5 resembled the distribution pattern of the MacMARCKS protein, except that β-galactosidase activity was not detected in the telencephalon (compare Fig. 6-3g with Fig. 6-2l).

At E10.5, lacZ expression at the diencephalon-mesencephalon boundary became weaker. Instead, β-galactosidase expression extended throughout the developing CNS, with intense staining seen in the telencephalon, diencephalon and throughout the entire spinal cord (Fig. 6-3g; h; i). β-galactosidase activity was also detected in the otic and optic vesicles, the presomitic mesoderm, and in the somites (Fig. 6-3g; h; i). Expression was not
detected in the caudal somites, probably reflecting different stages of somite maturation (Fig. 6-3h). In E12.5 embryos, β-galactosidase activity was detected in the CNS, the entire rostral-caudal extent of the neural tube (Fig. 6-3j), the dorsal root ganglia, and the sclerotome (Fig. 6-3k). From E9.5 onward, the pattern of lacZ expression correlates well with the distribution of endogenous MacMARCKS transcripts and protein (Fig. 6-2e; I). This was in contrast with the earlier stages of development, where β-galactosidase activity was neural specific but defined more restricted patterns of expression when compared to whole mount *in situ* expression patterns (compare Fig. 6-2a; b; c to Fig. 6-3a; b; c). These results demonstrate that sequences which are sufficient for spatial regulation of the MacMARCKS gene from E7.5 to E12.5 are located within the 4 kb MacMARCKS 5' flanking region.

LacZ expression was examined in sagittal sections of E16.5 transgenic embryos with a monoclonal antibody specific for β-galactosidase. LacZ expressing cells were detected throughout the brain and spinal cord, consistent with the endogenous distribution of MacMARCKS transcripts (data not shown). β-galactosidase positive cells were also detected in the liver and intestines, probably reflecting expression of the transgene in resident macrophage populations (data not shown). In the developing eyes, MacMARCKS is specifically expressed in both the developing outer and inner nuclear layers of the retina (Fig. 6-4a; 6-2g). A similar lacZ expression pattern was also observed in the eyes of E16.5 transgenic embryos (Fig. 6-4b).

**LacZ Expression in P₀ and Adult Brain:**

LacZ expression was first detected at P₀ as weak β-galactosidase staining of the cerebellum (Fig. 6-4c). β-galactosidase activity was also detected in the ventral midline of the hindbrain as a single stripe (data not shown). Sagittal section through this structure revealed intense β-galactosidase activity localizing to axon tracts throughout the spinal
Figure 6-4. LacZ expression patterns in the embryonic retina and the brains of P0 and adult transgenic mice.

MacMARCKS and β-galactosidase immunoreactivity colocalized to the retina of E16.5 4 kb-lacZ transgenic embryos. (a) An affinity-purified anti-MacMARCKS antibody and a TR-anti-rabbit secondary antibody were used to detect MacMARCKS in the inner nuclear layer (inl) and outer nuclear layer (onl) of the developing retina. (b) LacZ expression in the retina was detected with an anti-β-galactosidase mAb. Embryos were sectioned to 20μ.

c. Dorsal view of the P0 brain of a 4 kb-lacZ transgenic mouse, showing β-galactosidase activity in the cerebellum (cer).

d. Sagittal 3 mm section of brain in (c), revealing lacZ expression in the cerebellum and fiber tracts (ft) in the spinal cord. Abbreviations for (c) and (d): ctx, cortex; m, mesencephalon; my, mylencephalon.

e. Sagittal 5 mm section of an adult brain of a 4 kb-lacZ transgenic mouse. β-galactosidase activity was most intense in the cortex (ctx) and hippocampus (h). Staining was also observed in the white matter tracts of the mylencephalon (my).

f. Horizontal 20 μ section through the cerebellum of an adult 4 kb-lac Z transgenic mouse, showing intense lacZ expression in cells localized exclusively to the white matter (wm).

g, h. Bright-field (g) and dark-field (h) views of a 20 μ horizontal section of the brain of an adult 4 kb-lacZ transgenic mouse. β-galactosidase activity was detected throughout the ca1 and ca2 regions of the hippocampus (h), the entorhinal cortex (ec), and the cerebral cortex (cc).
cord, brainstem, and pons (Fig. 6-4d). The existence of lacZ expression in fiber tracts suggests that MacMARCKS might be expressed in glial cells.

In the adult brain, β-galactosidase activity was detected throughout the cerebral cortex in well-defined layers, and in the olfactory cortex and hippocampus (Fig. 6-4e and data not shown). This pattern of lacZ expression showed strong concordance with the distribution of endogenous MacMARCKS transcripts as detected by in situ hybridization (compare Fig. 6-4e with 6-2i). This result indicates that the 4 kb 5' sequence was sufficient to confer the complex temporal and spatial patterns of expression of endogenous MacMARCKS in the adult brain. The lacZ expression pattern in these regions reflected the distributions of neurons, as prominent white matter tracts such as the corpus callosum were not stained (Fig 6-4e). Higher magnifications revealed that the transgene was specifically expressed in CA1 and CA2 pyramidal cells of the hippocampus but not the dentate gyrus (Fig. 6-4g; h). Expression was also detected in the neighboring entorhinal cortex (Fig. 6-4g; h). LacZ expressing cells were also found in the brain stem and spinal cord (data not shown).

Thin sections of the cerebellum revealed that lacZ expression specifically localized to a population of large, intensely staining cells confined to the white matter (Fig. 6-4f). Regions of the cerebellar cortex that contained high concentrations of neuronal perikarya were completely devoid of lacZ-positive cells (data not shown). This pattern of lacZ expression suggests that MacMARCKS might be expressed in glial cells in the cerebellum.

**Identification of β-Galactosidase Expressing Cells In The CNS:**

Examination of lacZ-positive cells in the cerebral cortex indicated that β-galactosidase activity was detected throughout the cortical layers and was restricted to the cell bodies of neurons (Fig. 6-5a). To determine the identity of the lacZ-expressing cells in
**Figure 6-5.** Identification of β-galactosidase-expressing cells in the brains of 4 kb-LacZ transgenic mice.

**a, b.** β-galactosidase reactivity was detected in neurons. Horizontal 20μ sections through the cortex were first stained with X-gal to detect lacZ activity (a), and then stained with the neural specific NOVA antibody (b). β-galactosidase activity was detected primarily in cells that also stained with NOVA.

**c, d.** 20 μ sections through the cerebellum were double-labeled with a monoclonal antibody to β-galactosidase (c) and a polyclonal antibody against GFAP (d). The radial processes stained by GFAP do not colocalize with lacZ-positive cells.

**e, f.** 20 μ sections through the cerebellum were double-labeled with a anti-β-galactosidase antibody (e) and the macrophage/microglia F4-80 antibody (f). Both antibodies stained cells with morphologies resembling microglia.

**g, h.** 30 μ sections through the rat cerebellum were double-labeled with the neuronal-specific anti-synaptophysin antibody (g) and an affinity-purified anti-MacMARCKS antibody (h). The anti-MacMARCKS antibody detected large, ramified cells resembling those observed in (e) and (f). These cells did not stain with the anti-synaptophysin antibody (g).
transgenic lines, thin sections from several brain regions were probed with an antibody against NOVA, a neuronal-specific antigen (Bucanovitch et al., 1995). β-galactosidase activity was detected histochemically in these NOVA-labeled sections. β-galactosidase activity reaction products appeared as large dots which colocalized with NOVA-positive cells (Fig. 6-5a; b). While not all NOVA-positive cells were positive for β-galactosidase activity, all lacZ expressing cells were labeled with the anti-NOVA antibody. This result indicates that the MacMARCKS-lacZ transgene is expressed in cortical neurons.

Populations of cells that were lacZ positive but NOVA-negative were scattered throughout the brain. Brain sections were double-labeled with antibodies specific for β-galactosidase, and for one of two different markers: glial fibrillary acidic protein (GFAP) for astrocytes, and F4-80 for microglia. In sections that were labeled with GFAP, radial processes labeled intensely, but of 238 β-galactosidase-positive cells counted, none were positive for the astrocyte marker (Fig. 6-5c; d). In contrast, in sections that were labeled with the microglial marker F4-80, 135 out of 166 β-galactosidase positive cells also labeled with the F4-80 antibody. These large cells displayed characteristic microglial aborizations and were also detected in E16.5 brains (Fig. 6-5e; f and data not shown). Moreover, while an affinity-purified anti-MacMARCKS antibody labeled neurons, it also strongly labeled cells that displayed morphology similar to microglia (Fig. 6-5h; Miguel et al., 1995). These cells ans cell processes do not label with an antibody against synaptophysin, a neuron-specific marker (Fig. 6-5g). These results indicated that in addition to neurons, both endogenous MacMARCKS and the 4 kb-lacZ transgene were expressed in a subpopulation of microglial cells (see also Chapter 5). The identity of cells that were lacZ positive but did not label with any of the markers, including the population of cells in the cerebellar white matter, remains unknown.
Expression of B-galactosidase in 1.8 kb-lacZ Transgenic Mice:

Deletion analysis was carried out to further define the location of cis-regulatory elements within the 5' flanking region. Digestion of the 4 kb-TM construct with PstI generated the 1.8 kb-TM construct (Fig. 5-6), which was used to generate three founder lines that expressed the transgene. Since all three lines gave the same phenotype, only results for line 1.8 kb-6 are presented. Examination of E8.5 transgenic embryos indicated that patchy β-galactosidase activity localized specifically to the lateral edges of the cranial neural folds, with no activity detected in the floor plate and notochord (Fig. 6-6a; b). This pattern of β-galactosidase distribution partially correlated with the endogenous MacMARCKS expression at E8.5 (Fig. 6-2b; c), with the exception that ventral expression was not detected in the 1.8 kb transgenic embryos. Thus at E8.5 the β-galactosidase expression pattern in 1.8 kb transgenic lines was opposite to the pattern seen for embryos carrying the 4 kb-lacZ transgene. β-galactosidase activity was not detected in E9.5 embryos (data not shown). However, at E10.5 and E11.5, β-galactosidase activity was seen in the hindbrain, spinal cord and somites. A stripe of lacZ expressing cells was also detected in the diencephalon-mesencephalon boundary (Fig. 6-6c; d). This pattern of β-galactosidase activity was similar to the expression pattern observed in the 4kb-lacZ transgenic lines at E12.5, with the exception that β-galactosidase activity was seen throughout the brain in the 4kb-lacZ lines (compare Fig. 6-6c; d with Fig. 6-3f).

DISCUSSION

The cellular and molecular bases of neural tube formation are poorly understood, but it is well documented that the actin cytoskeleton is involved in this process (reviewed by Copp et al., 1990; Schoenwolf and Smith, 1990). Circumferential microfilament bands are present in the apices of neuroepithelial cells, and they appear increasingly dense during bending of the neural plate (Morriss-Kay et al., 1994). It is proposed that the
**Figure 6-6.** Expression of the 1.7 kb-lacZ transgene during mouse development.

- **a.** Lateral view of an E8.5 transgenic embryo. β-galactosidase activity localized exclusively to the lateral edges of the head folds (arrow).
- **b.** Dorsal view of the same embryo in (a). Arrow indicates LacZ expression in cranial neural folds.
- **c.** Lateral view of an E10.5 embryo. LacZ expression was detected in the diencephalon/mesencephalon border (arrow), rhombencephalon, and dorsal root ganglion (drg).
- **d.** Lateral view of an E11.5 embryo. β-galactosidase activity was detected throughout the neural tube, including the drg, and was prominent in the diencephalon/mesencephalon border (arrow) and rhombencephalon.
microfilaments contract gradually in a purse-string like fashion, constricting the apices of neuroepithelial cells, thereby transforming them from columns into wedges that aid in the elevation of the neural folds (Schoenwolf and Smith, 1990). As a regulator of the dynamic actin cytoskeleton, it is tempting to speculate that MacMARCKS may play a role in the regional organization of the neural plate and subsequent neural patterning of the CNS. The temporal and spatial pattern of MacMARCKS expression during embryonic development is consistent with this hypothesis.

**MacMARCKS expression during embryonic development:**

Several lines of evidence indicate that MacMARCKS activity may be important during neural development. Whole mount *in situ* hybridization indicates that MacMARCKS is expressed as early as E7.5. By E8.5, expression is seen throughout the head folds and the presumptive spinal cord. Specific enrichments are seen in the lateral lips of the elevating neuroepithelium, and in the floor plate of the neural tube. Unfortunately, analysis of late- stage embryos by this technique is complicated by the high background staining seen in the epithelium of the embryo, which obscures internal structural details. Immunohistochemical analysis of E8.5 embryos indicates that MacMARCKS distribution correlates with its mRNA expression pattern, and in E9.5 embryos, MacMARCKS protein is detected in the telencephalon, the ventral diencephalon, the neural tube and the notochord.

By E12.5, MacMARCKS is highly expressed throughout the CNS, and analysis of later embryonic stages by both *in situ* hybridization and by RPA indicates that MacMARCKS expression in the CNS continues to be high until birth. In the P0 brain, high levels of MacMARCKS expression are restricted to the forebrain. MacMARCKS expression decreases to low levels throughout the adult brain, with high levels of expression seen only in specific regions, including the hippocampus, olfactory bulb, and
the cortex. This pattern of adult MacMARCKS expression is in general agreement with previous results (Kato, 1990; Umekage and Kato, 1991), with the exception that MacMARCKS was detected in the dentate gyrus of the hippocampus, but not in CA1 or CA2 regions, in the earlier studies. This may be due to the greater specificity of the RNA probes and higher sensitivity of the detection method employed in the present study (Duncan et al., 1994). Umekage and Kato used single stranded DNA probes and autoradiography onto X-ray film, methods that have been shown to be less sensitive (Hogan et al., 1994).

Expression of the MacMARCKS-LacZ transgene during neural development:

A lacZ reporter construct containing 4 kb of 5' flanking sequence reproduced the neural-specific MacMARCKS expression pattern during embryonic development in two independent transgenic mouse lines; however, its expression pattern appears to be ventrally restricted. At E7.5, endogenous MacMARCKS transcripts are detected throughout the embryonic egg cylinder with no specific sites of concentration. In contrast, the transgene is specifically expressed around the node. The node is a bilaminar structure with both dorsal and ventral cell layers, and cells in the ventral layer give rise to the notochord (Hogan et al., 1994). The 4 kb-LacZ expression in the node is consistent with its later expression in the notochord, suggesting that β-galactosidase activity in the node is restricted to the ventral cells. The lacZ expression pattern is reminiscent of nodal expression (Shawlot and Behringer, 1995). Nodal is a member of the TGF-β family and is expressed at the node during gastrulation. Ectopic expression of nodal induces a secondary axis in zebrafish embryos, suggesting that it has a role in embryonic pattern formation (Toyama et al., 1995).

In E8.5 transgenic embryos, β-galactosidase activity is restricted to the ventral midline of the midbrain, the floor plate, and notochord. A high level of expression is seen
at the ventral region of the neural tube between the forebrain and midbrain, coinciding with closure point two. During mouse neural development, four sequential points of de novo neural tube closure in the developing brain have been described (McDonald et al., 1989; Sakai, 1989; Golden and Chernoff, 1993). The neural tube first fuses at the hindbrain-spinal cord boundary at E8.0. Closure of the neural tube then spreads in both caudal and cranial directions in a zipper-like fashion. The second site of neurulation occurs at the midbrain-forebrain boundary at E8.5-9.0. During this developmental stage, the neural folds of the midbrain and hindbrain converge at the midline to form the closed neural tube. Closure point three occurs at the extreme rostral end of the forebrain, and closure point four begins at the caudal end of the hindbrain (see Chapter 7 for more details). It is interesting that both endogenous MacMARCKS and the transgene product are localized to this region of the neural tube, suggesting that MacMARCKS may be involved in neural tube closure. The fact that the 4 kb-lacZ transgene does not express in the lateral lips of the neural fold implies either that an element exists within the 4 kb flanking sequence that restricts the expression of the transgene to ventral regions, or that additional genomic sequences from the intron or the 3' flanking region are required for appropriate transgene expression in the lateral folds. Support for the former hypothesis comes after examining the expression patterns of transgenic embryos carrying the 1.8 kb-lacZ construct. At E8.5, embryos from lines expressing this 5' deletion construct express lacZ at the lateral neural folds in a pattern indistinguishable from the endogenous MacMARCKS transcripts. However, expressions in the ventral lips, floor plate and notochord were not detected in these embryos. These results imply that the 1.8 kb region alone is sufficient to confer expression of the reporter construct to the lateral lips of the folding neural plate, and that the upstream 2.3 kb sequence inhibits this activity. This region probably also contains elements that direct the expression of the transgene to ventral structures and confers expression in E9.5 embryos,
since the 1.8 kb-lacZ construct is not expressed at E9.5. The 4 kb-lacZ construct is expressed at E9.5, but its expression is restricted to the ventral midbrain, with no staining in the telencephalon as seen by immunohistochemistry. By E10.5-12.5, the 4 kb-lacZ transgene expression pattern strongly resembles the endogenous MacMARCKS expression pattern. The 1.8 kb-lacZ transgene shows a similar caudal expression pattern in the hindbrain, spinal cord and dorsal root ganglia, but its rostral expression is restricted to the diencephalon-mesencephalon border, with no expression in the telencephalon.

In adult brain, both in situ hybridization and RPA indicate that down regulation of the MacMARCKS gene has occurred. Analysis of the 4 kb-lacZ expression pattern revealed that, like the endogenous gene, the transgene is only expressed in specific regions of the brain. Although there is a strong correlation between the distribution of MacMARCKS and the observed expression of β-galactosidase in the transgenic lines, some differences are observed. It is interesting that β-galactosidase activity is seen only in CA1 and CA2 pyramidal cells of the adult hippocampus, while in situ hybridization reveals that the MacMARCKS transcript is detected throughout the hippocampus. β-galactosidase activity was also not detected in the dentate gyrus, another site where endogenous MacMARCKS transcripts are found. These results suggest that while the 4 kb upstream sequence can confer the correct temporal and spatial regulation to most regions of the adult brain, it is not enough to recapitulate the complete phenotype.

Regulatory elements that drive neuron-specific expression in transgenic animals have been described for several genes, including those for human amyloid precursor protein (Wirak et al., 1991), Purkinje cell protein-2 (Vandaele et al., 1991) and α-tubulin (Gloster et al., 1994). No specific sequences have been identified in these genes that direct neuron-specific expression, although all the required elements are located in the 5' flanking region and share some sequence similarities (Vandaele et al., 1991). A role for cis-acting elements in the introns and 3' flanking regions is described for the GAP-43 and the Thy-1
genes. Inclusion of a stretch of the first intron results in neural-specific expression of GAP-43-lacZ transgenic constructs by suppressing ectopic transgene expression in non-neural tissues, indicating that some of the neural specificity of GAP-43 expression is due to repression of inappropriate expression in other tissues (Vanselow et al., 1994). The Thy-1 gene contains elements responsible for brain, thymus, kidney and spleen expression all located downstream of the transcription-initiation site, with the neural-specific element residing within the first intron (Vidal et al., 1990). Endogenous MacMARCKS mRNA is detected in non-neural tissues such as the testis and spleen, but MacMARCKS-lacZ transgenic mice do not express β-galactosidase in these tissues. This result suggests that elements located outside the 4 kb 5’ flanking region, either further upstream, within the intron, or at the 3’ flanking region are needed for proper expression outside the CNS.

**MacMARCKS regulation during embryonic development:**

At present, there are few clues as to the nature of cis-regulatory factors that govern MacMARCKS expression. Two copies of retinoic acid response element (RARE) half-sites (Schule et al., 1990) are found within the 1.8 kb MacMARCKS 5’ flanking region at positions -1646 and -186 (Fig. 5-1a), suggesting that MacMARCKS transcription is regulated by retinoic acid (RA) during development. The vitamin A derivative retinoic acid is involved in vertebrate anterior-posterior (AP) patterning along the body axis and has been shown to induce the expression of a large number of genes implicated in early embryonic development, including members of the Wnt (Schuuring et al., 1989), and Hox (LaRosa and Gudas, 1988) families. RA induces P19 embryonic carcinoma cells to differentiate into neurons, and stimulates the expression of a number of genes involved in neural development, including MacMARCKS (Jonk et al., 1994). This result suggests that MacMARCKS expression is at least partially controlled by RA-dependent mechanisms. RA is a morphogen and exogenous application induces AP transformation of the developing
CNS (Durston et al., 1989), and localized application of RA into the chick limb bud causes duplication of the normal AP pattern (Thaller and Eichele, 1987). RA exerts its effects by binding to a family of nuclear RA receptors (RARs), ligand inducible transcription factors which bind to RAREs in the promoter regions of target genes (Rossant et al., 1991). RARs are expressed throughout the nervous system in the developing embryo, and the pattern of RAR-α strongly resembles MacMARCKS embryonic expression (Ruberte et al., 1990; 1991). These results suggest that MacMARCKS gene regulation by RA during embryonic development could be mediated through interactions with RAR-α.

Since most neural patterning genes examined to date are transcription factors, it may be informative to correlate MacMARCKS expression patterns during embryonic development with those of known regulatory genes. At E8.5, the MacMARCKS expression pattern resembles that of Wnt-1 and En-1 (Wilkinson et al., 1987; McMahon et al., 1992). Wnt-1 and En-1 transcripts are first detected at E8, at the anterior head fold of the neural plate of the presumptive midbrain. More caudal sections show a restriction of expression to the lateral lips. At E9.5, the MacMARCKS expression pattern begins to resemble the expression pattern of HNF-3β. At this stage of development, HNF-3β is expressed along the ventral midline in the floor plate and notochord. Like MacMARCKS, HNF-3β expression begins in the anterior and then progresses caudally (Sasaki and Hogan, 1993). While no evidence exists that the En-1, Wnt-1 or HNF-3β gene products are activating MacMARCKS transcription in vivo, it is tempting to speculate that the pattern of MacMARCKS expression in the embryo reflects the expression pattern of these regulatory genes, since transcription factors such as HNF-3β and En must act upstream of the proteins that organize the cytoarchitecture of differentiating cells during development.

In later periods, MacMARCKS expression patterns strongly resemble those of GAP-43. GAP-43 is a neural specific, palmitoylated PKC substrate that binds calmodulin in the absence of Ca²⁺ and bears strong functional similarities to MARCKS and
MacMARCKS (reviewed by Aderem, 1992). GAP-43 is highly expressed during neural development and axonal regeneration, suggesting a role in axonal growth (Skene and Willard, 1981). GAP-43-lacZ transgene constructs are expressed throughout the brain and spinal cord of transgenic mice beginning at E12.5, in a pattern that mimics the pattern of expression of the 4 kb MacMARCKS-lacZ transgenic lines (Vanselow et al., 1994 and this study). GAP-43-lacZ expression peaks perinatally and is then down regulated in the adult brain, with high levels of expression in the entorhinal cortex, the hippocampus, and olfactory bulb. This pattern of expression is similar to that of MacMARCKS, and analysis of GAP 43-lacZ lines indicate that inclusion of the first intron is necessary to prevent ectopic expression of β-galactosidase in non-neural tissues (Vanselow et al., 1994). This is in contrast to the MacMARCKS promoter-lacZ lines, in which even the 1.8 kb 5' flanking element is sufficient to direct neural-specific expression in tissues.
INTRODUCTION

Embryonic development of the brain and spinal cord, a process called neurulation, results in the formation of the neural tube. This structure is the precursor of the central nervous system, and failure of neurulation results in neural tube defects (NTDs) which are among the most common congenital malformations (reviewed in Copp et al., 1990; Golden and Chernoff, 1995). In chordates, neurulation occurs in two distinct phases. The first phase, primary neurulation, can be subdivided into several stages. First, the flat neural ectoderm is induced to thicken by interactions with the underlying mesoderm, resulting in the formation of the neural plate (reviewed by Ruiz I Altaba and Jessell, 1993). The edges of the induced neural plate then elevate and bend inward, fusing at the dorsal midline to convert the neural plate into the neural tube (Schoenwolf et al., 1988; Copp et al., 1990). After closure of the caudal neuropore, secondary neurulation takes place in the caudal spinal cord. This occurs by a totally different mechanism without prior formation of the neural folds (Schoenwolf, 1984).

During normal mouse development, four sequential points of de novo neural tube closure in the developing brain have been described (McDonald et al., 1989; Sakai, 1989; Golden and Chernoff, 1993). The neural tube first fuses at the hindbrain-spinal cord boundary during the seven somite stage (E8.5). Closure of the neural tube then spreads in both caudal and cranial directions in a zipper-like fashion. Caudal closure forms the thoracic neural tube (the spine) and the posterior neuropore. Rostral closure proceeds to the level of the otic vesicles (caudal hindbrain). The second site of neurulation occurs at the
midbrain-forebrain boundary in the 11 somite stage (E9.0). During this developmental stage, the neural folds of the midbrain and hindbrain converge at the midline to form the closed neural tube. Caudally, closure point two progresses over the midbrain and terminates at the rostral hindbrain. Rostrally, closure two proceeds over the forebrain to meet closure three. Closure point three occurs at the extreme rostral end of the forebrain, and fusion proceeds caudally to meet closure two, closing the anterior neuropore in the process. Finally, closure point four begins at the caudal end of the hindbrain and proceeds unidirectionally in a rostral direction to meet the caudal aspect of closure point two (see Fig. 7-4e; Golden and Chernoff, 1993).

The cellular and molecular bases of neurulation are poorly understood, and there is evidence that different mechanisms of neurulation exist at different levels of the body axis (Copp et al., 1990; Schoenwolf and Smith, 1990). During neural fold elevation, the shape of the neuroepithelial cells in the floor plate change from columnar to pyrimidal -- a process known as cell wedging. Localized cell wedging drives neural plate furrowing at specific sites, eventually bending the lateral edges of the neural plate toward the midline to close the neural tube (Schoenwolf and Smith, 1990).

It is well documented that the actin cytoskeleton is involved in cell wedging (for review see Copp et al., 1990; Schoenwolf and Smith, 1990). Transmission electron microscopy demonstrates that circumferential microfilament bands are present in the apices of neuroepithelial cells of all species examined, and they appear increasingly dense during bending of the neural plate (Morriss-Kay et al., 1994). The distribution of actin in the neuroepithelium is consistent with its role during neurulation. Prior to neural fold elevation, no actin is observed in the primitive streak stage of the embryo. At the two somite stage (E8), when the neural folds are beginning to elevate, actin is localized along the basal aspects of the neuroepithelial cells. At the five somite stage (E8.5), when neuroepithelial cells are undergoing active cell wedging, actin redistributes from the basal to the apical
region of the cells (Sadler et al., 1981). Actin binding contractile proteins such as myosin and fodrin are also present in the apices of neuroepithelial cells (Sadler et al., 1986). Finally, Ca\(^{2+}\), a regulator of microfilament contractility, is localized to coated vesicles in the apices of neuroepithelial cells (Nagele and Lee, 1980) and is released during neurulation (Moran, 1976). These results strongly implicate actin as the driving force for apical constriction of neuroepithelial cells. It is proposed that the microfilaments contract gradually in a purse-string like fashion, constricting the apices of these cells and thereby transforming them from columns to wedges that aid in the elevation of the neural folds (Schoenwolf and Smith, 1990).

Results of experiments with the drug cytochalasin D (CD, which depolymerizes actin microfilaments) underscore the importance of the actin cytoskeleton during neurulation. Disassembly of microfilaments by CD causes the collapse of the cranial neural folds (Morriss-Kay and Tuckett, 1985). Elevation of the neural folds in the chick embryo is retarded after CD treatment, and neural fold fusion across the midline is always abolished (Schoenwolf et al., 1988). However, it is unclear whether contraction of the microfilament bundles causes, or stabilizes the wedging of the neuroepithelium during neural fold elevation (Copp et al., 1990).

Several lines of evidence indicate that MacMARCKS might be involved in neural development. Analysis of MacMARCKS promoter-lacZ transgenic mouse lines as well as the endogenous murine MacMARCKS expression pattern indicates that MacMARCKS is highly expressed in the developing central nervous system, consistent with it playing a role during neural development (Chapter 6 of this thesis). MacMARCKS is expressed as early as E7.5, and by E8.5 specific expression is seen in the lateral aspects of the elevating neuroepithelium, the floor plate of the neural tube, and the notochord. Peak MacMARCKS expression corresponds to closure point two of the developing cranial neural tube. By E9.5, MacMARCKS expression becomes restricted to the midbrain-hindbrain boundary,
and by E10.5 intense expression is seen throughout the brain and spinal cord. Furthermore, MacMARCKS protein is also found to be enriched at the edges of the neural plate. MacMARCKS expression persists in the adult brain, with high levels of expression in the cortex, hippocampus, olfactory bulb and cerebellum.

The most straightforward way to address the role of a single gene during mouse development is to generate targeted mutation of the gene in the mouse germline by homologous recombination, and to examine homozygous null animals for defects. This chapter describes the targeted deletion of the MacMARCKS gene in the mouse. Disruption of the MacMARCKS gene prevents cranial neural tube closure, resulting in exencephaly. This result suggests a role for MacMARCKS and the PKC signal transduction pathway during neural development, and supports the hypothesis that actin-based motility directs cranial neural tube closure.

RESULTS

Targeted Disruption of MacMARCKS and Germ Line Transmission:

To functionally delete the MacMARCKS gene, a positive-negative selection strategy was used (Mansour et al., 1988; Chen et al., 1996). A MacMARCKS clone isolated from a 129/sv mouse genomic DNA library was used to construct the targeting vector, which was generated in the pPNT vector containing the neomycin (neo) and herpes simplex virus thymidine kinase (tk) cassettes for positive and negative selection, respectively (Tybulewicz et al., 1991). Homologous recombination of this construct with the endogenous genomic DNA would result in the replacement of most of the MacMARCKS exon 1 and the entire exon 2 with the neo gene (Fig. 7-1a). A HindIII site was introduced just 5' of the neo gene to aid in the identification of the mutant allele.
Figure 7-1. Targeted disruption of MacMARCKS and germline transmission.

a. Structure of the MacMARCKS gene and the gene targeting construct. Exon 1, exon 2 and the intron of the MacMARCKS gene were replaced with a pgk-neo cassette and flanked by 7.5 kb of 5' and 1.3 kb of 3' MacMARCKS genomic sequence (thick black lines). After homologous recombination in ES cells, the mutant allele was detected by a HindIII (H) site which was engineered into the neo<sup>f</sup> cassette. The expected sizes of the HindIII restriction fragments for both the wild type (7.8 kb) and the mutant allele (4.2 kb for probe A and 3.6 Kb for probe B) are indicated.

b. Germline transmission of the MacMARCKS null allele. Genomic DNAs from tails of F2 siblings were digested with HindIII and analyzed by Southern blot using probe A. The presence of a single 4.2 kb fragment indicates a homozygous mutant (−/−). A DNA sample from a C57/B16 (B6) mouse was included as a control for the HindIII fragment polymorphism between C57/B16 (6.5 kb) and 129SV (7.8 kb) mice.

c. Absence of MacMARCKS mRNA in MacMARCKS null mice. RNase protection analysis (RPA) was performed on RNA samples isolated from the brains of wild type (+/+), heterozygous (+/−), and homozygous (−/−) embryos. MacMARCKS transcripts were undetectable in (−/−) embryos, and reduced by half in the heterozygotes.

d. Absence of MacMARCKS protein in MacMARCKS null mice. 50 μg of heat-treated E16.5 embryonic brain lysate from wild type (+/+), heterozygous (+/−), and homozygous (−/−) embryos were resolved by 10% SDS-PAGE. Immunoblots were probed with anti-MacMARCKS and MARCKS antiserum. MacMARCKS protein was not detected in the null embryos and was reduced by 50% in the heterozygotes. The level of the MARCKS protein was unaffected by the MacMARCKS deletion.
a. Exon1 Exon2

Genomic MacMARCKS

Targeting Vector

Mutant Allele

probes B Neo A 1 Kb

Wt

Mutant

b. B6 +/- +/- -/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/- +/-vero

Homologous Recombination

b. c. d.
The targeting vector was introduced into J1 (Li et al., 1992) mouse embryonic stem (ES) cells. To screen for homologous recombinants, ES cell DNA was digested with HindIII and probed with sequences located 5' (probe B) and 3' (probe A) of the sites of recombination. The wild-type locus showed a 7.7 kb fragment when analyzed with both probes, while the disrupted locus revealed a 3.6 kb band when probed with probe B, and a 4.2 kb band when probed with probe A (data not shown). To demonstrate single-site integration, a probe derived from the neo gene was used (data not shown). Five out of 108 G418 and FIAU resistant colonies screened by both Southern blot (using probes A, B and Neo) and PCR analyses (using oligonucleotides P1 and P2) contained the mutant allele and had a single homologous integration at the MacMARCKS locus (a homologous recombination frequency of 4.6%). The five positive J1 clones were injected into C57/B16 blastocysts (Bradley, 1987) by the Rockefeller University transgenic facility and germline transmission of the disrupted allele was obtained for two male founders. Heterozygous siblings were intercrossed to generate homozygous MacMARCKS null mice. Genomic DNAs from tails of F2 siblings were digested with HindIII and analyzed by Southern blot using probe A (Fig. 7-1b). The presence of a 4.2 kb fragment (and not the 6.5 kb fragment) indicated a MacMARCKS<sup>–/–</sup> homozygous mutant. A DNA sample from a C57/B16 (B6) mouse was included as a control for the HindIII fragment polymorphism between C57/B16 (6.5 kb) and 129SV (7.8 kb) mice.

RNase protection analysis of total RNA from the brains of wild-type, heterozygous, and homozygous E16.5 mice demonstrated that the MacMARCKS transcript was undetectable in the MacMARCKS<sup>–/–</sup> animals (Fig 7-1c). MacMARCKS was expressed at about half the normal level in the MacMARCKS<sup>+/–</sup> animals, as compared to the MacMARCKS<sup>+/+</sup> animals (Fig. 7-1c). Similarly, immunoblot analysis demonstrated the absence of the MacMARCKS protein in homozygous null animals, while MacMARCKS
levels in heterozygotes were approximately half that of their wild-type counterparts (Fig. 7-1d). All three genotypes expressed identical levels of the related protein, MARCKS.

**Cranial Neurulation Is Disrupted In MacMARCKS Null Embryos:**

MacMARCKS heterozygous mutant mice had no detectable abnormalities, and were intercrossed to generate offspring. Among 132 pups from these matings (hybrid and isogenic genetic backgrounds), 15 homozygous MacMARCKS ±/− mice were born, while 68 MacMARCKS +/+ and 49 MacMARCKS ±/+ offspring were obtained (Table. 7-1). This non-mendelian ratio suggested that a portion of the MacMARCKS ±/− embryos were dying in utero. To characterize the morphology of the mutants and to determine the time of embryonic lethality, embryos were dissected at various stages of development. The genotypes of all embryos were confirmed by PCR. When embryos were examined between 8.5 and 10.5 days of development (E8.5-E10.5), the genotype of the embryos segregated with the expected mendelian ratios (Table 7-1), indicating that some of the homozygous embryos die after E10.5 and were resorbed by the mother.

Exencephaly is defined as a failure of neural tube closure followed by outward expansion of neural tissue by eversion of the neural plate (Sah et al., 1995). All MacMARCKS ±/− embryos examined (66/66; 100% penetrance) demonstrated a failure to close the cranial neural tube. Comparison of brain development in wild-type versus MacMARCKS mutant embryos illustrates the developmental differences which result in exencephaly in MacMARCKS ±/− embryos. Prior to E8.5 there was no discernible difference in neural plate shaping when comparing wild-type and mutant embryos: in both cases the neuroepithelium in the forebrain and midbrain proliferated and elevated around a median hinge point (MHP). By E8.5 in wild-type embryos, the neural plate began converging towards the midline around paired dorsolateral hinge points (DLHP), resulting in convergence and subsequent neural tube closure (Fig. 7-2a, right; Schoenwolf, 1991). In
Table 7-1

Genotypes of embryos from MacMARCKS heterozygote matings

<table>
<thead>
<tr>
<th>Age</th>
<th>Total Mice</th>
<th>-/-</th>
<th>+/-</th>
<th>+/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>E8.5-E10.5</td>
<td>236</td>
<td>58</td>
<td>120</td>
<td>58</td>
</tr>
<tr>
<td>E12.5-E16.5</td>
<td>43</td>
<td>8</td>
<td>29</td>
<td>6</td>
</tr>
<tr>
<td>Newborn</td>
<td>132</td>
<td>15</td>
<td>68</td>
<td>49</td>
</tr>
</tbody>
</table>
**Figure 7-2.** Disruption of MacMARCKS prevents cranial neural tube closure and results in exencephaly.

a. Dorsal view of E8.5 MacMARCKS null (left) and wild-type (right) embryos. The neural plates of the wild type embryo bend toward the midline thereby closing the cranial neural tube. The neural folds of the MacMARCKS null embryos splay abnormally outward from the midline (arrows).

b, c. Histological comparisons of horizontal sections of E8.5 MacMARCKS null (b) and wild-type (c) embryos. The headfold (HF) of the MacMARCKS null embryo failed to bend inward at the lateral hinge points (arrows), while the head folds of the wild-type embryo bent normally. An abnormal groove was also seen at the floor plate (arrowhead) of the null embryo (b). The cytoarchitecture of the neuroepithelium (ne) and the mesenchymal cells (ME) of the null embryo appeared normal. The notochord (N) of the wild-type embryo is indicated.

d. Side view of E9.5 MacMARCKS null (left) and wild-type (right) embryos. The MacMARCKS null embryo has an open anterior neural tube. The approximate closure points four (large arrow), two (arrowhead) and three (small arrow) are indicated. Abbreviations: P, prosencephalon; M, mesencephalon; Rh, rhombencephalon.

e. Transverse section of E9.5 MacMARCKS null embryo at the level of the otic vesicles. The neural folds of the prosencephalon (P) and rhombencephalon (Rh) are elevated but do not approach each other and fail to fuse. The spinal cord (SC) and the first (I) and second (II) branchial arches are indicated.

f. Side view of E10.5 MacMARCKS null (left) and wild-type (right) embryos. Arrows and arrowhead designations are as in (d). O, otic vesicle. Abbreviations: T, telencephalon; D, diencephalon; M, mesencephalon; Rh, rhombencephalon.

g. Horizontal sections at the level of the eyes (arrows) of the head of E10.5 MacMARCKS null (left) and wild-type (right) embryos. The rhombencephalon (Rh) of the null embryo was persistently open, and both the telencephalon and diencephalon adopted an abnormal cruciform structure. In contrast, the rhombencephalon (Rh) of the wild-type embryo closed normally.
h. Whole mount *in situ* hybridization of E10.5 embryos with the telencephalon marker *Emx-2*. Frontal view of MacMARCKS null (left) and wild-type (right) embryos. The arrow indicates Emx-2 expression at the lateral edges of the presumptive telencephalon.

i. Whole mount *in situ* hybridization of E10.5 embryos with the telencephalon marker *En-2*. Side views of MacMARCKS null (left) and wild-type (right) embryos. Arrows indicate *En-2* expression at the midbrain-hindbrain junction.
MacMARCKS null embryos, while the neuroepithelium appeared to thicken at the normal rate, the lateral edges of the neural plate splayed abnormally outward from the midline, and the neural folds appeared to be unable to approach each other to form the neural tube (Fig. 7-2a, left). Histological analysis of mutant embryos indicated that the neuroepithelium failed to bend toward each other at the DLHP, and an abnormal groove was seen at the MHP (Fig. 7-2b). In contrast, the neural folds in wild-type embryos underwent convergence at the DLHP, and the MHP appeared normal (Fig. 7-2c).

By E9.5 the primitive brain consists of five major regions. The prosencephalon (forebrain) is composed of the rapidly expanding telencephalon, which gives rise to the cerebral cortex and the basal ganglia, and the diencephalon, which later forms the thalamus. The diencephalon is connected to the mesencephalon (midbrain) which in turn connects to the rhombencephalon (hindbrain). The rhombencephalon is divided into the metencephalon and mylencephalon. The metencephalon will form the cerebellum dorsally and the pons ventrally, and the mylencephalon gives rise to the medulla oblongata. By E9.5, severe exencephaly which involved the prosencephalon, mesencephalon and rhombencephalon was apparent in MacMARCKS-/- embryos (Fig 7-2d, left). Neurulation at the diencephalon-mesencephalon boundary (closure point two), at the extreme anterior end of the neural plate (closure point three) and at the metencephalon (closure point four) all failed to occur, resulting in a persistently open cranial neural tube (Fig 7-2d). Histological analysis of E9.5 mutant embryos indicated that while the neuroepithelial layer appeared normal, fusion of the neural folds at the prosencephalon and rhombencephalon failed to occur (Fig. 7-2e). In contrast, wild-type littermate’s cephalic neural tube was completely closed at this stage of development (Fig 7-2d, right). By E10.5 the two telencephalic hemispheres (which are normally fused together) were completely separated in MacMARCKS null embryos but continue to grow (Fig. 7-2f; h, left). However, initiation of closure point one seemed to have taken place, because the caudal aspect of the hindbrain
up to the level of the otic vesicle appeared normal (Fig. 7-2f, left). Examination of histological coronal sections indicated that the rhombencephalon was completely open at E10.5, while the telencephalon and diencephalon had folded into an abnormal cruciform-like structure (Fig. 7-2g, left). By E11.5 the anterior neuropore closed, indicating that closure point three proceeded in a delayed manner to close the anterior forebrain (data not shown). Interestingly, posterior neuropore closure in the region of the spinal cord appeared normal in MacMARCKS null embryos (not shown), suggesting that cellular events required for posterior neural tube formation and subsequent establishment of the dorsal-ventral axis via inductive events were normal and did not require MacMARCKS function. No sex preference in exencephalic mice between E8.5 and newborn was detected by PCR using primers for the male specific sry gene (male 50.0%, female 49.9%; n=53).

Two homeobox (Hox) genes that normally show region-specific expression patterns along the anterior-posterior (AP) axis in the brain were used to examine their boundaries of expression in both MacMARCKS<sup>−/−</sup> and wild-type embryos. If normal neural patterning of the MacMARCKS<sup>−/−</sup> embryos was disrupted, one would expect that patterns of expression of the Hox genes would change. Emx-2, a marker of the developing telencephalon (Simeone et al., 1992), and En-2, a marker of the mesencephalon-metencephalon (Davis et al., 1988), were analysed in E10.5 mutant and wild-type embryos. In the wild-type embryos Emx-2 was highly expressed in the rostral telecephalon (Fig. 7-2h, right). In the MacMARCKS mutants, Emx-2 expression was localized to the lateral edges of the two unfused telencephalic hemispheres (Fig. 7-2h, left). En-2 expression in the wild-type embryo was in the caudal mesencephalon-rostral metencephalon boundary (Fig. 7-2i, right), while expression in MacMARCKS mutants was also highest in the same area (Fig. 7-2i, left). These results indicate that while normal brain development in MacMARCKS<sup>−/−</sup> embryos is disrupted, A-P patterning of the CNS is normal.
Brain Malformations In MacMARCKS Null Embryos:

To further characterize the mutant phenotype, E18.5 embryos were removed just prior to birth. By E18.5, normal CNS development is advanced, and several convenient morphological landmarks distinguish brain regions. These landmarks allow unambiguous identification of the affected areas, as well as the degree of normal development of the tissues. All mutant animals recovered were alive and responded to tactile stimuli, but displayed grossly abnormal brains that were completely exposed to amniotic fluid. The skull and the overlying skin were completely absent (Fig. 7-3a, left; b). Higher magnification indicated that the brains of the mutants appeared small and flattened, with extensive necrosis seen throughout (Fig. 7-3b). In the MacMARCKS mutants normal brain landmarks were not apparent. Sagittal sections through a wild-type fetus indicated a well developed cerebral hemisphere, diencephalon, and midbrain (Fig. 7-3d). Additionally, the cerebellum has formed from the metencephalon. Behind this structure, the posterior choroid plexus marks the metencephalic-mylecephalic boundary (Fig. 7-3d). In contrast, E18.5 mutant brains displayed such extensive perturbations of normal architecture that firm identifications of brain structures were very difficult. Sagittal sections of E18.5 mutant brains indicated that the cerebral hemispheres were replaced with cortical tissues that appeared to be folded inside-out and displaced rostrally, although laminations characteristic of the cortex were present (Fig. 7-3c; e). Coronal sections demonstrated that forebrain structures that were dorsal in the wild-type animal were lateral in the mutant (Fig. 7-3G, left). The midbrain was completely replaced with a fold of tissue that extended over the presumptive cortex (Fig. 7-3c; g), and this tissue did not make contact with the diencephalon. Extensive necrosis was seen as a pseudolaminar pattern throughout the ependymal and subependymal layers of this structure (data not shown). The necrotic zone ended in the mylencephalon. The cerebellum, which is derived from the caudal midbrain and rostral metencephalon, was absent, confirming that the disturbance extended into the
Figure 7-3. Brain malformations in MacMARCKS null embryos.

a. Side view of E18.5 MacMARCKS null (left) and wild-type (right) embryos. Arrowhead indicates the malformed brain. Scale bar: 0.8 cm.

b. Higher magnification of the head of the MacMARCKS null embryo depicted in (a). The skull and skin overlying the brain is missing, and severe necrosis is seen throughout the MacMARCKS null brain. Scale bar: 0.2 cm.

c, d. Histological comparisons of sagittal sections of E18.5 MacMARCKS null (c) and wild-type (d) embryos. In the wild-type embryo, the skull and normal brain architecture was evident, while the MacMARCKS null brain was completely abnormal. Arrow in (c) indicates the presumptive forebrain. Abbreviations: OB, olfactory bulb; FB, forebrain; MB, midbrain; Cer, cerebellum; CP, choroid plexus.

e, f. H/E staining of coronal sections through the level of the eyes of E18.5 MacMARCKS null (left) and wild-type (right) embryos. The arrow in (e) indicates the position of the laterally displaced cortical rudiments, seen at higher magnification in the inset. The arrowhead indicates the absence of the skull. The arrowhead in (f) indicates the skull of the wild-type embryo.

g, h. H/E staining of coronal sections through the level of the cerebellum of E18.5 MacMARCKS null (left) and wild-type (right) embryos. The arrowhead in (g) points to the presumptive midbrain. Abbreviations: MB, midbrain; Cer, cerebellum; CP, choroid plexus.
metencephalon (Fig. 7-3c; g). However, the pons (which like the cerebellum is a metencephalon derivative) was present, indicating that not all metencephalic tissue was missing (Fig. 7-3g; h). Moreover, the posterior choroid plexus was present, indicating that disturbances due to MacMARCKS deletion lie rostral to the metencephalic-mylencephalic boundary (Fig. 7-3c). No overt abnormalities were detected outside the CNS.

**Mice Lacking MacMARCKS Are Born Anencephalic and Die Soon After Birth:**

MacMARCKS\(^{-/-}\) mice were born anencephalic, and all died soon after birth. The empty cranial cavity showed no evidence of any brain structures, and the overlying skull and skin were absent (Fig. 7-4a; b). The eyes of the mutant animals seemed to be abnormally displaced toward the midline. Gross examinations of other internal organs (including heart, lung, liver and kidney) indicated that they were normal. Since the brains of MacMARCKS mutant embryos were present prior to birth, it appears that when the anencephaly observed at birth was due to a combination of *in utero* necrosis and birth-related trauma.

By E16.5 the developing retina can be divided into two layers, the inner and outer nuclear layers. MacMARCKS is expressed in both layers at high levels (Chapter 6, Fig. 6-4a). Compared to wild-type embryos, both retinal layers of the MacMARCKS null embryos were markedly thinner (Fig. 7-4c). This was especially evident in the inner nuclear layer, where marked hypocellularity was observed.

**DISCUSSION**

Our initial studies examining MacMARCKS expression indicates that this gene is highly expressed in the developing neural tube from the head fold stage (E.7.5) to the late gestational stages (see Chapter 6), with expression continuing in the adult in specific
Figure 7-4. Disruption of the MacMARCKS gene results in anencephaly and retinal defects.

a. Dorsal view of newborn MacMARCKS null (left) and wild type (right) pups. MacMARCKS null mice are born anencephalic.
b. Side view of newborn MacMARCKS null (left) and wild type (right) pups, showing absence of brain, skull and overlying skin.
c. Cross-section of wild-type retinal layers. The retinal pigment epithelium (rpe) is detached due to a fixation artifact.
d. Cross-section of retinal layers of MacMARCKS null mice. Both the inner and outer nuclear layers are markedly thinner than the wild-type retina, and the inl contained many picnotic nuclei (pn). Abbreviations: inl, inner nuclear layer; onl, outer nuclear layer; s, segments; rpe, retinal pigment layer.
e. Diagrammatic representation of the normal cranial neural tube closure points seen in the wild-type embryo at E8.5 to E9.5.
f. Diagrammatic representation of closure points seen in the MacMARCKS null embryos. Closure points two and four do not initiate in these embryos, resulting in a persistently open cranial neural tube. Closure point three showed delayed initiation. Abbreviations: P, procencephalon; M, mesencephalon; R, rhombencephalon.
Normal Closure  MacMARCKS -/-
regions of the CNS. These data strongly implicate a role for MacMARCKS in the morphogenesis of the neural tube in the developing mouse embryo. To directly address this possibility, the MacMARCKS gene was disrupted by homologous recombination in ES cells. Cells carrying the integrated targeting vector were injected into blastocysts to produce chimeric mice. These mice were then crossed to generate homozygous mice lacking the MacMARCKS gene.

Examinations of MacMARCKS<sup>-/-</sup> embryos indicate that neural tube defects were apparent as early as E8.5. In these embryos the cranial neural folds were unable to approach each other, thereby causing a defect in neural tube closure. By E9.5, malformations in the brain of MacMARCKS mutant embryos were manifested as an exencephaly extending from the forebrain to the hindbrain regions. It is apparent in MacMARCKS<sup>-/-</sup> embryos that <i>de novo</i> closure points two, three and four (Copp <i>et al.</i>, 1990; McDonald <i>et al.</i>, 1989; Golden and Chernoff, 1993) were affected, leading to exencephaly extending from the very rostral point of the anterior forebrain to the rostral hindbrain. In addition, the rostral movement of neural tube fusion continuing from closure point one in MacMARCKS mutant embryos was severely affected, leading to an open hindbrain extending to the level of the otic vesicles. However, by E11.5 the anterior neuropore closed, indicating that closure point three was delayed but not abolished in MacMARCKS null mice. Despite these serious defects, the A-P patterning of the anterior head structure appeared normal, as determined by the correct expression patterns of anterior markers such as <i>Emx-2</i> and <i>En-2</i>. Interestingly, lumbrosacral spina bifida was never observed in the mutant embryos, even though MacMARCKS is highly expressed in the spinal cord beginning at E10.5 (Chapter 6, Fig. 6-2e). This indicates that normal spinal neural tube closure does not require MacMARCKS function to proceed.

At E18.5, MacMARCKS mutant embryos showed severely disrupted brain architecture. A forebrain structure, resembling the cortex from its lamination pattern, was
visible in coronal sections, but it was displaced laterally and appeared to be folded inside-out. Midbrain structures were absent and appeared to be replaced with a flap of necrotic tissue extending from the mylencephalon over the presumptive forebrain. The cerebellum also completely failed to develop, although structures caudal to it, like the choroid plexus, developed normally. Thus, the E18.5 neural phenotype was in complete concordance with the anatomical domains affected in the E9.5 mutant embryos which defined the A-P limits of MacMARCKS requirement from the rostral forebrain to the metencephalic-mylencephalic boundary. The regions of MacMARCKS requirement also appeared to be dorsoventrally restricted, since dorsally derived tissues such as the cerebellum were undetectable, whereas the ventrally derived pons was present and appeared normal. Finally, MacMARCKS null mutants were born anencephalic, but the trunk and tail regions developed normally.

Several other mouse mutants with neural tube defects (NTD) that lead to exencephaly have been described (reviewed in Copp et al., 1990). In mice with trisomies 12 and 14, midbrain and hindbrain exencephaly results from the failure of the edges of the neural folds to turn medially and fuse at the midline (Putz and Morriss-Kay, 1981). In the SELH/Bc mouse strain, initiation of neural tube closure at closure point two is disrupted, but in most embryos de novo closure at closure point three occurs and proceeds caudally through the entire midbrain region to generate a completely normal neural tube (McDonald et al., 1989). A delayed closure point three is similarly observed in MacMARCKS null embryos, but unlike in the case of the SELH/Bc mouse strain, closure point three cannot compensate for disruptions of closure points two and four. In the open brain (opb) mutation, closure point two is disrupted, although closure points one and three appear normal (Gunther et al., 1994). In these mutants, exencephaly extends from the caudal forebrain to the hindbrain regions. In addition, widespread defects in the development of the spinal cord, dorsal root ganglia, eye and the axial skeleton are observed. The genetic
bases for trisomy 12, 14, SELH/Bc and opb have not been identified. In addition, disruption of several genes with known functions also results in exencephaly. Targeted deletions of two helix-loop-helix transcription factors, twist and mammalian hairy and Enhancer of split homolog-1 (HES-1) results in exencephaly beginning at E8.5 (Chen and Behringer, 1995; Ishibashi et al., 1995). In twist null embryos closure points two and four are disrupted, and the embryos die by E11.5 (Chen and Behringer, 1995). Twist is normally expressed in the head mesenchyme and not in the neural tube, and twist null embryos also exhibit defects in head mesenchyme formation. Chimeric analysis demonstrate that the head mesenchyme is required for neural tube closure (Chen and Behringer, 1995). While the exact mechanism is unknown, the head mesenchyme could support neural tube morphogenesis by maintaining the shape of the neural folds, perhaps coordinating the bending and apposition processes. In HES-1 mutants, neurogenesis is accelerated in the telencephalon, indicating that the normal role of HES-1 is to negatively regulate neural development. Accelerated neurogenesis could lead to neural tube closure defects by depleting the population of dividing neuronal progenitors (Ishibashi et al., 1995). A new mouse mutation, jumonji (jmj), was recently generated by gene trapping (Takeuchi et al., 1995). The jmj mutation is an embryonic lethal, and 46% of the homozygous embryos develop NTDs at E10.5. Mutant jmj embryos showed a failure of neurulation at closure point two, although closure points one and three appeared normal. Jmj encodes a protein with partial homology to the retinoblastoma binding protein RBP-2, although the function of this protein during CNS development is unknown.

Finally, functional deletion of the MARCKS gene results in a number of developmental defects in the mouse CNS, including exencephaly (Stumpo et al., 1995). However, only 25% of the MARCKS<sup>−/−</sup> animals exhibited exencephaly, compared to 100% of the MacMARCKS mutants. The onset of exencephaly of MARCKS mutant embryos during neural development was not characterized, and the pups were born with malformed
brains. Moreover, neither skull or skin covered the brain. This is in contrast with the MacMARCKS mutant, in which the pups were born without a brain. In addition, subtle CNS defects, including decreased brain size, disrupted commissures, abnormal separation of the cerebral hemispheres and poor cortical laminations were observed in MARCKS null brains (Stumpo et al., 1995). Thus the MacMARCKS mutation generated CNS defects that were far more disruptive than the MARCKS mutation, since normal brain landmarks were not observed in MacMARCKS null brains. It would be interesting to see the neural defects of mouse embryos in which both the MARCKS and MacMARCKS genes are deleted, and preliminary results indicate that embryos heterozygous for both genes die in utero and are exencephalic (Chen et al., unpublished results).

NTDs in humans are second only to congenital heart defects as a cause of perinatal mortality due to birth defect and affect approximately 1 per 715 conceptions in the U.S. (Golden and Chernoff, 1995). Little is known about the etiology of NTDs in humans, although complex multifactorial genetic and environmental components are involved (Lemire, 1988). NTDs most commonly manifest as spina bifida and anencephaly; the incidence of anencephalic births in the U.S. is about 0.3 per 1000 live births. Anencephaly stems from a failure of anterior neural tube closure, resulting in an open brain that is degraded by exposure to amniotic fluid. The fetus is born without a brain, however, the spinal cord of the fetus remains intact. Recent studies have demonstrated that anterior neural tube closure during human neural development also occur at the same four fusion sites as described for the mouse, and that anencephaly results from a failure of closure points two and four to initiate fusion (Van Allen et al., 1993; Golden and Chernoff, 1995). This result suggests that mouse models for NTDs such as the MacMARCKS null mouse will be helpful in investigating the pathogenesis of NTDs in humans. The homologies between the mouse and human genome permit the identification of human genes that when mutated cause NTDs, leading to DNA probes for linkage studies in families with recurrent
NTDs and possible prenatal screenings for NTDs. MacMARCKS maps to mouse chromosome 4 which is within a linkage group of genes that map to human chromosome 1p32-p35 (Lobach et al., 1993). The few patients with a deletion in this region of chromosome 1 exhibit several common clinical features, including facial dysmorphism, mental retardation, high arched palate, clonodactyly of the fifth finger, and heart defects (Howard and Porteus, 1990). It would be interesting to examine human anencephalic fetuses for the absence of the MacMARCKS gene by PCR; correlation of the anencephalic phenotype with a deletion of the MacMARCKS gene would represent the first step toward a genetic screen for anencephaly.

Numerous studies in rodents and humans have demonstrated that various teratogens can cause NTDs (reviewed in Copp et al., 1990). Retinoic acid (RA) induces neuroepithelial abnormalities during mouse development, leading to a wide variety of craniofacial defects. RA interferes with neural fold elevation by disrupting the actin-cytoskeleton of the neuroepithelium (Yasuda et al., 1987). The MacMARCKS gene contains retinoic acid receptor elements (RAREs; see Chapters 5 and 6) at the 5' flanking sequence, and MacMARCKS mRNA expression is highly induced by RA (Jonk et al., 1994). It is likely that RA functions as a teratogen by altering, either regionally or globally, the expression of genes important in neural patterning such as MacMARCKS. Thus, MacMARCKS may be a genetic link between teratogens and a variety of neural tube defects. The identification of MacMARCKS as a genetic factor involved in the development of RA-induced NTDs is extremely important, because the interactions between teratogens and the genetic background of the embryo is at present unknown.

Little is known about the signal transduction pathways involved in neural tube closure, but many experiments implicate a role for the actin cytoskeleton. Results presented here are consistent with these observations, since the disruption of an actin regulator prevents cranial neural tube closure. The specificity of this event is also of
interest; the fact that only anterior neural tube closure is abrogated emphasizes the unique role of this protein. Most of the mouse mutations that result in exencephaly as yet cannot be tied to any known biochemical or molecular mechanisms. In contrast, the MacMARCKS null mutation clearly links a very specific neurulation defect to an actin binding protein in a PKC-dependent signaling pathway, and implicates the actin cytoskeleton in this event. The MacMARCKS knockout mouse should therefore provide a very useful mouse model for investigating the etiology of anencephaly in humans.
EXTENDED DISCUSSION

To understand the intracellular signal transduction pathways that control cytoskeletal-mediated changes in cell physiology, it is necessary to understand the proteins regulating these events. Elucidating the roles of these proteins requires three complementary approaches. First, the cDNAs encoding these factors must be identified and cloned. Second, these proteins must be individually characterized as to their structure, chemical properties, and function. Finally, factors which are significant in vitro must be analyzed in the cell and ultimately in the organism.

This thesis has examined the biology of MacMARCKS, a PKC substrate which integrates signal transduction events with changes to the actin cytoskeleton. MacMARCKS is found in the neuroendocrine-derived PC12 cell line, and in rat brain synaptosomes, where it associates with a purified synaptic vesicle fraction. A phosphorylation-defective MacMARCKS mutant blocks depolarization-evoked exocytosis in PC12 cells. MacMARCKS is highly expressed in the CNS during mouse embryonic development. The 5' flanking sequences of MacMARCKS direct neural-specific expression of a lacZ reporter gene in transgenic mice during embryonic development. In vitro characterization of the MacMARCKS 5' flanking sequences localized LPS-response elements to a region only 113 bp upstream of the transcriptional start site. Finally, functional deletion of the MacMARCKS gene in mice results in cranial neural tube closure defects and exencephaly. The results of this work suggest some conclusions about MacMARCKS function during neural secretion and development.

The MacMARCKS effector domain interacts with several factors (Aderem, 1992). This region consists of only 31 amino acids, yet it is a substrate for PKC phosphorylation, it binds calcium-calmodulin in a calcium-dependent manner, and it interacts with actin. A theoretical consideration of how MacMARCKS might integrate
signal transduction events with the regulation actin-membrane interactions is discussed below. This model postulates that MacMARCKS associates with the plasma membrane through both its effector domain and myristic acid moiety. In its non-phosphorylated form, MacMARCKS cross-links actin into a rigid meshwork at the plasma membrane. Activation of the cell opens calcium channels, leading to an influx of calcium into the cell. This increase in local calcium concentration activates both calcium-calmodulin and PKC. Binding of calcium-calmodulin to the MacMARCKS effector domain prevents PKC phosphorylation, while PKC phosphorylation prevents MacMARCKS' interaction with calcium-calmodulin. However, both events disrupt MacMARCKS binding to actin. The dominant state depends on the equilibrium constants of the individual reactions, as diagrammed below:

\[
\text{MacMARCKS-actin} \\
\quad k_5 + k_6 \\
\quad \text{actin} + \quad \quad k_1 \\
\quad \text{MacMARCKS} + \text{Ca}^{2+}\text{-calmodulin} \quad \quad \text{MacMARCKS-calmodulin} \\
\quad k_2 \\
\text{PKC} \quad k_3 \quad \text{Phosphatases} \\
\text{MacMARCKS-Pi}
\]

In the presence of intracellular calcium, the equilibrium constants \( k_1 \) and \( k_3 \) will dominate, resulting in the formation of both a MacMARCKS-calmodulin complex and MacMARCKS-Pi. Because \( k_4 \) is very slow, MacMARCKS-Pi is essentially a terminal product. In PC12 cells, a portion of MacMARCKS-Pi dissociates from the plasma membrane.
membrane and translocates into the cytosol (Chang et al., 1996), removing it from sites of active interaction.

Since calcium levels in a cell are known to oscillate, the equilibrium constants $k_1$ and $k_2$ will shift with respect to the intracellular calcium concentrations. During phases of low intracellular calcium, the equilibrium constant $k_2$ dominates over $k_1$, resulting in the formation of free MacMARCKS, which can either interact with actin or become phosphorylated by PKC. Since the equilibrium constants $k_3$ and $k_6$ are postulated to be equal, eventually MacMARCKS-Pi product predominates. Over time, all MacMARCKS within a cell will be phosphorylated. Phosphatases such as phosphatase 1 and 2A eventually dephosphorylate MacMARCKS, allowing it to interact with calcium-calmodulin or actin. Within a cell, the actual rate constants are influenced by other considerations, including local concentrations of reactants and products and the diffusion rate constants of reactants and products, all of which will affect the ability of MacMARCKS to interact with other molecules.

PC12 cells contain both synaptic vesicle like microvesicles (SLMVs) and large dense-core vesicles (LDCVs; Thomas-Reetz and De Camilli, 1994). LDCVs store catecholamines and neural peptides whereas SLMVs store classical neurotransmitters such as acetylcholine and glycine (Thomas-Reetz and De Camilli, 1994). Both vesicle types contain synaptotagmin. In PC12 cells, SLMVs localize primarily to the cell body, and to a lesser extent are detected in the ends of processes, whereas dense-core granules are found predominantly in the tips of processes (Hudson et al., 1993; Liu et al., 1994). In PC12 cells, MacMARCKS colocalizes with the SLMV marker synaptophysin in the cell body and the tips of processes (Chang et al., 1996). Morphologic examination of PC12 cells by light microscopy suggests that MacMARCKS may be found on SLMVs, although its presence on LDCVs cannot be excluded at this level of resolution. The observation that MacMARCKS associates with a purified synaptic vesicle fraction in rat brain
synaptosomes further strengthens the argument that MacMARCKS is a component of SLMVs (Chang et al., 1996). Preliminary evidence indicates that MacMARCKS localizes to synaptic vesicles, as determined by electron microscopic observations of cryosections of rat brain synaptosomes.

Two functional assays indicate that MacMARCKS has a role in neural secretion of PC12 cells. In both the parental and wt-HA transfected PC12 cells, depolarization-induced exocytosis, as visualized by the anti-synaptotagmin\textsubscript{lum} antibody uptake (Matteoli et al., 1992), occurs prominently throughout neurite processes and around the periphery of the cell body. This observation is consistent with the previous finding that the anti-synaptotagmin\textsubscript{lum} antibody (syt\textsubscript{lum} Ab) uptake assay detects active exocytosis in dendritic processes and around the cell body of hippocampal neurons (Matteoli et al., 1992; Mundigl et al., 1995). The “hot spots” of exocytosis seen in these cells precisely coincide with the synaptic vesicle marker SV2 (Matteoli et al., 1992). These data imply that the syt\textsubscript{lum} Ab assay detects exocytosis from SLMVs in PC12 cells. PC12 cells expressing a phosphorylation-defective MacMARCKS mutant exhibit a profound inhibition of depolarization-dependent exocytosis. Little or no syt\textsubscript{lum} Ab uptake is observed in the processes or around the cell body. The PC12 cells expressing mutant MacMARCKS protein show profound alterations in their membrane cytoskeleton, suggesting that MacMARCKS has a role in regulating the actin cytoskeleton. The mutant protein colocalizes with actin to the periphery of the cells. Remodeling of the actin-cytoskeleton is necessary for exocytosis (Vitale et al., 1988), and it is tempting to speculate that in PC12 cells expressing mutant MacMARCKS, cortical actin cannot be efficiently remodeled because the mutant protein continues to cross-link actin in a phosphorylation-independent manner. This creates a physical barrier preventing synaptic vesicle fusion with the plasma membrane.
A second functional assay of exocytosis from PC12 cells involves $^3$H-NE prelabeling of LDCVs. PC12 cells are then stimulated with elevated potassium to induce $^3$H-NE release. Both wt transfected and parental cells rapidly secreted $^3$H-NE from LDCVs after potassium stimulation. Quantitation of $^3$H-NE released from the S-A transfected cells revealed that neurosecretion is greatly impaired in these cells. The rate of $^3$H-NE secretion was markedly delayed, with a lag time of approximately 6-7 min. Immunogold labeling and electron microscopy of the mutant MacMARCKS protein using an anti-HA antibody localizes the protein to LDCVs, and the cytosolic face of the plasma membrane. These results suggest that in PC12 cells expressing the phosphorylation-defective MacMARCKS mutant, exocytosis from LDCVs is also inhibited. One possible explanation would be that LDCVs are tethered to actin by mutant MacMARCKS. Depolarization-induced activation of PKC would not result in the phosphorylation of mutant protein, delaying exocytosis in these cells.

Analysis of the MacMARCKS promoter in RAW 264.7 cells localized a putative LPS response element to a region 113 bp upstream of the transcriptional start site. This promoter fragment is sufficient to confer full LPS inducibility to a luciferase reporter construct when transiently transfected into RAW264.7 cells. However, nuclear-run on analysis of RAW264.7 cells indicates that the basal rate of MacMARCKS transcription is already very high, and LPS treatment does not appreciably increase this rate. This apparent discrepancy between the two analyses can be resolved if a mechanism of transcriptional termination is evoked. This postulates that there is a pause site within exon one of the MacMARCKS gene and that the density of the RNA polymerase III at the 5' end of the MacMARCKS gene is very high. LPS induction of MacMARCKS may involve only a small proportion of the polymerase getting through the block, so when a full length MacMARCKS cDNA probe was used in the nuclear run-on assay (as was done in Chapter
5), little or no induction of transcription by LPS was detected. If this hypothesis is correct, the use of a 3’ specific cDNA probe will show an increase in MacMARCKS transcription after LPS stimulation. In the case of the MacMARCKS promoter-reporter constructs, the luciferase gene does not have a transcriptional termination site, so LPS induces LPS-responsive transcriptional factors to either increase the rate of formation of RNA polIII complexes, or the rate at which they move along the gene. The 113 bp MacMARCKS promoter fragment contains multiple SP-1 binding sites, and SP-1 response elements have been shown to confer LPS inducibility to heterologous genes (Hume et al., personal communication).

MacMARCKS is highly expressed throughout the central nervous system during embryonic development, suggesting that it has a role in neural development. To investigate this possibility, MacMARCKS was disrupted by homologous recombination in ES cells. Homozygous mutant mice lacking MacMARCKS have neural tube closure defects and exencephaly. The defects appear to involve cells in the dorsolateral hinge point (DLHP) of the developing neural tube. Cells within this region are known to undergo actin-dependent cell-shape changes in a process called cell wedging. Treatment of neurulating embryos with cytochalasin D disrupts cell wedging at the DLHP, abolishing neural tube closure (Schoenwolf and Smith, 1990). In the MacMARCKS null embryos, DLHPs do not form at neural tube closure points two and four, resulting in a persistently open brain. However, the median hinge point (MHP) forms normally in these embryos. Cell wedging at the MHP is required to bend the neural plate, but unlike cells in the DLHPs, cell wedging at the MHP is not an actin-based process (Schoenwolf et al., 1988). Analysis of MacMARCKS-lacZ transgenic mice indicates that the 4 kb MacMARCKS 5’ flanking sequence is expressed specifically in the MHP. The 4 kb sequence could be used to drive the expression of a diphtheria toxin-A gene in transgenic mice to specifically ablate cells within the MHP. (This approach has been used to ablate specific cell types in
the eye; Raymond and Jackson, 1995). Characterization of the predicted neural tube defects of transgenic mice lacking a functional MHP would complement analysis of the phenotype seen in the MacMARCKS knockout mice.

The work presented in this thesis further implicates MacMARCKS as an essential modulator of signal transduction-based changes to the actin-cytoskeleton. As discussed in the Introduction, modification of the actin-cytoskeleton is an essential aspect to exocytosis and development. Results presented in Chapters 3 and 4 demonstrate a role for MacMARCKS in neural secretion, an exocytotic event. Furthermore, as described in Chapters 5 and 6, analysis of the MacMARCKS promoter and endogenous expression patterns of both mRNA and protein indicate that it is developmentally regulated, consistent with a role during neural development. Finally, chapter 7 demonstrates that MacMARCKS has a crucial role during neurulation, as MacMARCKS null mice are born anencephalic. These data, therefore, strongly suggests that MacMARCKS is a pivotal integrator of signal transduction events and modulation of the actin-cytoskeleton.
BIBIOGRAPHY:


S. L. Ang and J. Rossant (1994) HNF-4β is essential for node and notochord formation in mouse development, *Cell* 78:


J. Li and A. Aderem (1992) MacMARCKS, a Novel member of the MARCKS family of protein kinase C substrates, Cell 70:791-801.


K. Meiri and P. Gordon-Weeks (1990) GAP-43 in growth cones is associated with areas of membrane that are tightly bound to substrate and is a component of a membrane skeleton subcellular fraction, *J. Neurosci.* 10:256-266.


End