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Membrane localization of Myristoylated Alanine-Rich C Kinase Substrate in C6 glioma and CHO-K1 cells

by

Donna Nabeha Douglas

B.Sc. (Hons.) Carleton University, 1991
M.Sc. (Chem.) Carleton University, 1993

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Dalhousie University

Department of Biochemistry
Dalhousie University
Halifax, NS

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The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "Membrane localization of Myristoylated Alanine-Rich C Kinase Substrae in C6 glioma and CHO-K1 cells"

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Signature of Author

Dedicated to my parents

Errol and Alia

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Abstract

MARCKS (Myristoylated Alanine-Rich C Kinase Substrate) is known to interact with calmodulin (CaM), actin filaments, and anionic phospholipids at a basic effector domain and phosphorylation within this domain by PKC has been shown to negatively influence all these interactions. In the present study, cytochalasin D (CD) and CaM antagonists were used to examine the influence of F-actin and CaM on membrane interaction of endogenous MARCKS in C6 glioma cells. Both CD and CaM antagonists potentiated PKC-induced translocation of MARCKS without affecting further the level of MARCKS phosphorylation. CD and the PKC inhibitor staurosporine increased membrane association of MARCKS in a PKC-independent manner since both these compounds did not affect phorbol ester-induced PKC translocation or MARCKS phosphorylation. On the other hand, CaM antagonists had little effect on the cellular distribution or phosphorylation of MARCKS. Thus F-actin and CaM might serve to independently modulate PKC-regulated localization of MARCKS at cellular membranes. Addition of [γ^{32}P]ATP to the membrane fraction of digitonin-permeabilized, cytosol depleted C6 cells resulted in phosphorylation and solubilization of MARCKS which was unaffected by EGTA and downregulation of cPKCs and nPKCs (by >90%). However, inhibition by PKC-specific inhibitors and a MARCKS peptide containing PKC phosphorylation sites implicate a PKC-like kinase. Furthermore, phosphoamino acid analysis and limited proteolytic digestion followed by peptide mapping indicated phosphorylation sites similar to those phosphorylated by activated PKC *in vivo*. These findings support an alternate mechanism by which MARCKS may be phosphorylated and translocated by an atypical PKC isoform *in vivo*. Finally, site-directed mutagenesis and expression of MARCKS in Chinese hamster ovary cells has revealed that the serines known to be phosphorylated by PKC *in vivo* may have distinct roles in targeting MARCKS to specific cellular locations. Taken together, these data show that factors other than phosphorylation may influence the association of MARCKS with membranes.

Abbreviations

AA	arachidonic acid
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
βTPA	4β isomer of 12-*O*-tetradecanoylphorbol 13-acetate
CaM	calmodulin
CB	cytochalasin B
CD	cytochalasin D
CHO	Chinese hamster ovary
CMZ	calmidazolium
DAG	diacylglycerol
dATP	dideoxyadenosine triphosphate
DMEM	Dulbecco's modified essential medium
DMSO	dimethylsulfoxide (GF 109203X)
EGF	epidermal growth factor
EGTA	ethylene glycol-(*bis*)(β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid
F-actin	filamentous actin
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GAP	growth-cone associated protein
HBS	Hepes-buffered saline

Hepes	N-[2-hydroxyethyl]peperazine-N-[2-ethanesulfonic acid]
IC_{50}	inhibition concentration (causing 50% inhibition)
Ins (1,4,5)P_3	inositol (1,4,5) triphosphate
LysoPC	lysophosphatidylcholine
MARCKS	Myristoylated Alanine-Rich C Kinase Substrate
MH2	MARCKS homology 2
PA	phosphatidic acid
PBS	phosphate-buffered saline
PG	phosphatidylglycerol
PH	pleckstrin homology
P*i*	inorganic phosphate
PIP_2	phosphatidylinositol-4,5-*bis*phosphate
PKA	protein kinase A
c, n, a PKC	conventional, novel, atypical protein kinase C
PKM	protein kinase M
PL	phospholipid
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PP*i*	inorganic diphosphate
PS	phosphatidylserine
S152A	serine 152 to alanine replacement

S156A	serine 156 to alanine replacement
SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel electrophoresis
STS	staurosporine
TBS	Tris-buffered saline
TFP	trifluoperazine
TM	transmembrane
TPCK	L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone-treated trypsin
Wt	wild type

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I. General Introduction

A. Overview: Protein Kinase C-Mediated Signal Transduction and MARCKS

Overall, a cell must have complex control to enable it to mediate the events that begin with the recognition of an extracellular signal and end with the selective activation of genes. While there are many such 'signal transduction' pathways, complexity emerges from different combinations of a limited set of pathways that are evolutionarily conserved and ubiquitously distributed amongst living organisms. A schematic overview of a generic signal transduction pathway is shown in *Figure 1*. It is imperative that the components of signal transduction have exquisite feedback and crosstalk mechanisms which if left unchecked, could otherwise lead to uncontrolled cell signaling and proliferation or even cell death. That most, if not all, known proto-oncogenes have turned out to represent proteins involved in signal transduction pathways at all levels (i.e. ligands, receptors, transducers and effectors) demonstrates the importance of team work amongst the information relay components of a signal transduction cascade (for review see Hunter, 1991). At the protein level, strategies to secure regulation include covalent modifications and subsequent changes in conformation or subunit number. Protein phosphorylation, catalyzed by a multitude of both tyrosine- and serine/threonine-specific protein kinases, is a fundamental process for the regulation of cell growth and other diverse cellular processes due to its rapid, economical and reversible nature (Edelman *et al.*, 1987; Yarden and Ullrich, 1988 ; Hanks *et al.*, 1988 ; Ben-David *et al.*, 1991). It is not surprising then

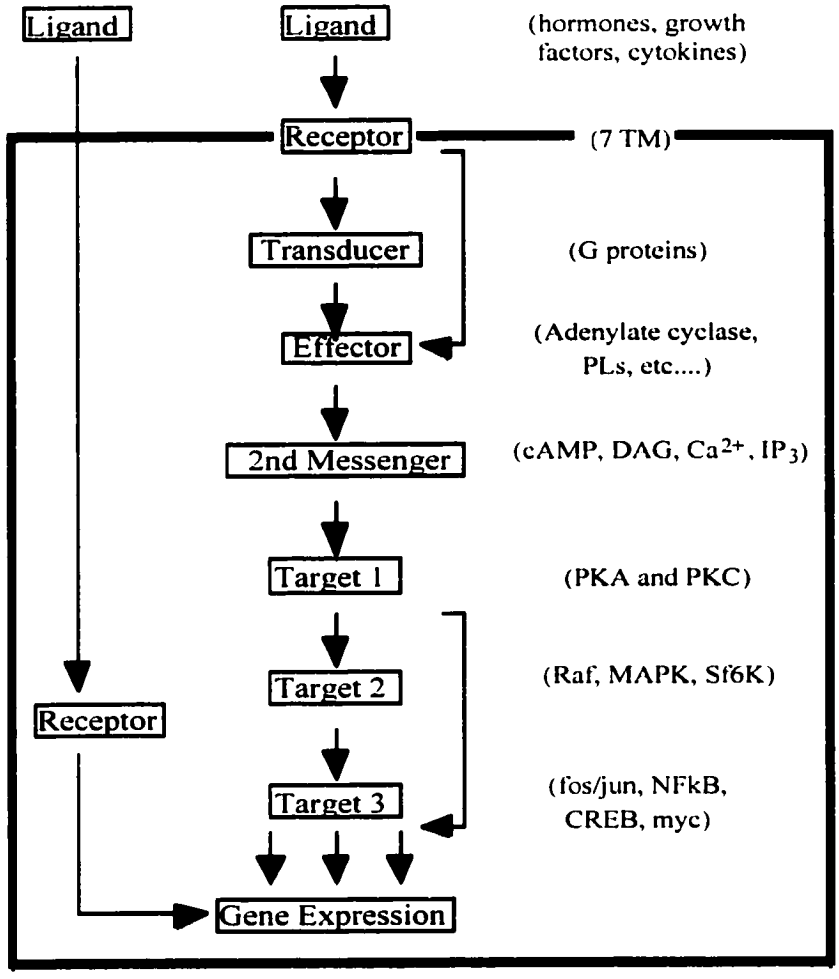


Figure 1. **Generic signal transduction pathways.** Extracellular signals (ligands) either bind to the extracellular domain of cell-surface receptors or can penetrate cellular membranes to interact with intracellular receptors. These ligand 'activated' receptors can directly, or through their association with 'transducers', activate effectors. Effectors then modulate the distribution of 'second messengers', which in turn activate target proteins. Target proteins, as such or by acting on further 'downstream' targets, finally modulate gene expression at both transcriptional and translational levels.

that phosphorylation represents the majority of covalent modifications observed within receptor-mediated signal transduction. Phosphorylation and dephosphorylation events mediate the amplification and propagation cascade of extracellular signals that ultimately control the transcriptional activity of genes (Hunter and Karin, 1992).

Two prominent serine/threonine-specific kinases, both activated by second messengers, play a central role in signal transduction: the cyclic AMP-dependent protein kinase A (PKA) (for review see Taylor *et al.*, 1990) and the Ca^{2+}/phospholipid-activated protein kinase C (PKC). The idea that the latter is a key component in signal transduction has emerged from the observation that diverse signalling pathways result in the generation of diacylglycerol (DAG), a major *in vivo* activator of PKC (see below). PKC has been implicated in such diverse cellular responses as differentiation, cell motility, secretion, membrane trafficking and mitogenesis (Nishizuka, 1984, 1986, 1992; Goodnight *et al.*, 1994). However, the mechanisms by which PKC controls these processes, or the precise roles of many phosphorylated substrates of PKC, is still not understood. *In vitro* studies by [^{32}P]orthophosphate labelling of cells treated with phorbol ester or other stimuli of PKC-mediated signalling pathways have revealed a large number of putative physiological substrates (for a review see Kikkawa and Nishizuka, 1986) and it is rather difficult to assess their significance in PKC-mediated cellular events. The widely expressed MARCKS protein, the primary focus of this thesis, is one of the most prominent of these cellular PKC substrates and is phosphorylated by PKC under several conditions such as macrophage activation or growth factor-dependent mitogenesis. However, despite its nearly ubiquitous distribution, a definitive physiological role for MARCKS is not yet

known.

B. Protein Kinase C

i. Structure

PKC was first discovered by Nishizuka and coworkers in 1977 as a histone protein kinase from rat brain that could be activated by limited proteolysis (Inoue *et al.*, 1977), Ca^{2+} and phospholipids (Takai *et al.*, 1979) or by phorbol esters and phospholipids (Castagna *et al.*, 1982). While early biochemical studies and preparations revealed at least three isoforms (α, β, γ) (Huang *et al.*, 1986a), the discovery that PKC represents a large multi-gene family of isoforms with notable differences in their structures and tissue distribution emerged with the advent of molecular cloning (Knopf *et al.*, 1986).

To date, 11 members of the protein kinase C family have been identified; these can be grouped in two major classes according to their dependence on Ca^{2+} ion. The first group, the conventional PKCs (cPKCα, βI, βII, and γ), require phosphatidylserine (PS) and DAG (or phorbol ester) in the presence of Ca^{2+} for their activation (Nishizuka, 1992), whereas the second group, the novel PKCs (nPKCδ, ε, η(L) and θ), require phospholipid and DAG (or phorbol ester) but are Ca^{2+}-independent (Ono *et al.*, 1988; Osada *et al.*, 1990; Osada *et al.*, 1992). More recently, however, two novel members of the PKC family have been identified that do not fit into the above classification and might represent members of an independent subgroup. They have been classified as atypical

PKCs (aPKCζ, λ and τ) because while they are dependent on PS, they are not affected by DAG, phorbol ester, or Ca^{2+} (Nishizuka, 1992).

All PKCs share an N-terminal regulatory region (approximately 20-40 kDa) and a highly conserved catalytic kinase domain in the C-terminal region (approximately 45 kDa) within a single polypeptide (Nishizuka, 1992; Hug and Sarre, 1993). Cloning of the first isoforms in the mid-1980s revealed four conserved domains (C1-C4) (Coussens *et al.*, 1986), each representing a functional module which is shared by many other unrelated proteins (Newton, 1995), that are separated by variable regions (V1-V5) (Coussens *et al.*, 1986) (*Figure 2*). The regulatory (V1-C2) and catalytic (C3-V5) domains are separated by a hinge region (V3) that becomes proteolytically labile when the enzyme is membrane-bound.

While relatively little is known about the roles of the variable regions, extensive biochemical and mutational analysis of the conserved domains has provided insight into their functions. The C1 domain is common to all PKC isoforms and contains two zinc finger motifs (except aPKCs which contain only one); each with a cluster of six cysteine residues, a common DNA-binding motif found in transcription factors such as GAL4 (Pan and Coleman, 1990). Like GAL4, each of the two cysteine-rich clusters coordinates two Zn^{2+} ions (Quest *et al.*, 1992). Although DNA-binding activity has not been detected for PKC isoforms, the regulatory subunit alone (generated by proteolytic cleavage in the V3 hinge region) has the capacity to bind DNA (James and Olson, 1992). While extensive deletion mutant analysis revealed a functional role for the C1 region in binding DAG and

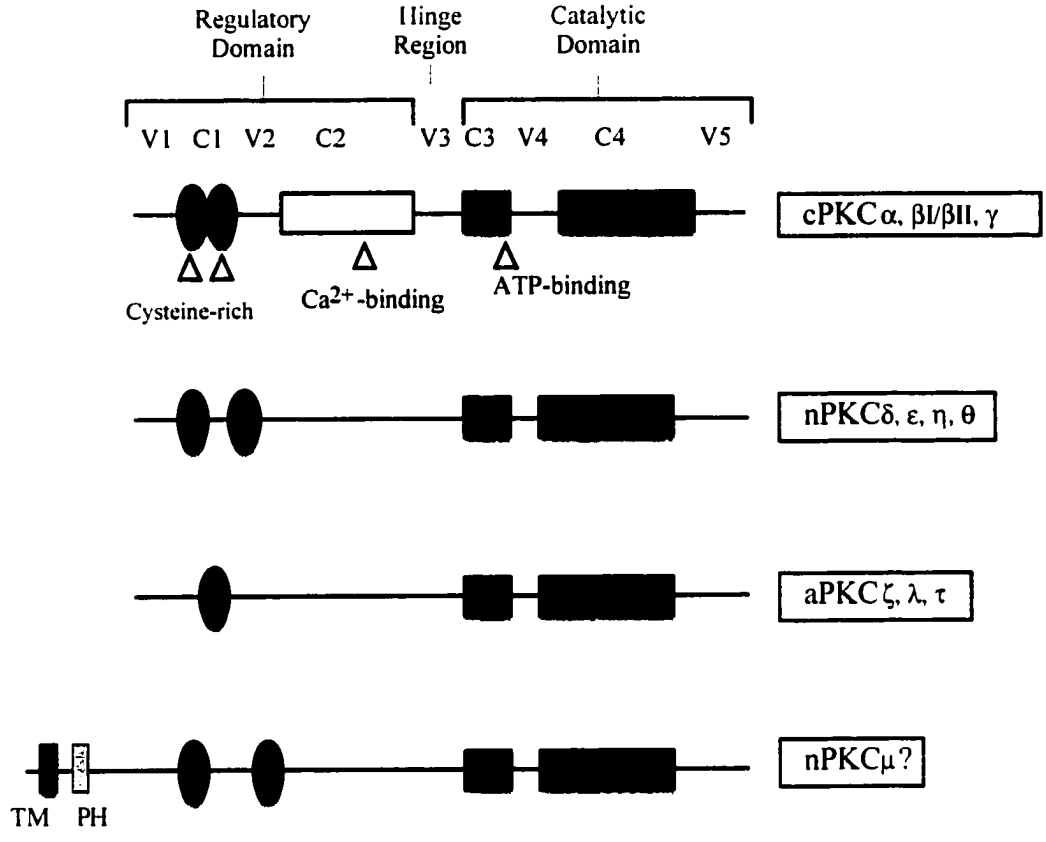


Figure 2. **PKC isoform domain structure.** All PKCs have an N-terminal regulatory region and a highly conserved C-terminal catalytic kinase domain. There are four conserved domains (C1-C4) separated by variable regions (V1-V5). The presence of a transmembrane (TM), domain signal peptide and a pleckstrin homology (PH) domain within the regulatory domain of nPKCμ represent unique structural motifs.

phorbol esters (Muramatsu *et al.*, 1989; Kaibuchi *et al.*, 1989; Burns and Bell, 1991), subsequent structural (Zhang *et al.*, 1995) and biophysical studies (Mosior and Epand, 1993; Mosior and Newton, 1995; Newton and Keranen, 1994) have provided more insight into the mechanism of action of C1 ligands on PKC function. Thus, DAG and phorbol esters behave as hydrophobic anchors, recruiting PKC to the membrane by increasing the enzyme's membrane affinity (Mosior and Newton, 1995). Structural analysis revealed that the C1 region of PKC contains a hydrophilic groove and phorbol ester, once bound to the C1 region, fills in this groove. The consequence of ligand binding in this case would, from a topological point of view, increase the surface hydrophobicity of PKC, and hence its membrane affinity, without actually inducing a conformational change in the enzyme (Zhang *et al.*, 1995).

In addition to increasing PKC's membrane affinity, C1 ligands might also contribute to stabilization of the active conformation of the enzyme since DAG doubles the catalytic efficiency of PKCs bound to PS (Mosior and Epand, 1993; Newton and Keranen, 1994; Hannun *et al.*, 1986; Newton and Koshland, 1989). Finally, since Ca^{2+} is also implicated in increasing membrane affinity of cPKCs by a separate mechanism (see below), C1 ligands also reduce the Ca^{2+} requirement for PS-dependent activation of PKC (Nishizuka, 1984). On the other hand, aPKCζ contains only one zinc finger and does not bind DAG or phorbol ester (Ono *et al.*, 1989a; McGlynn *et al.*, 1992). In fact, aPKCζ, at least *in vitro*, exhibits constitutive protein kinase activity (Liyanage *et al.*, 1992; McGlynn *et al.*, 1992; Nakanishi and Exton, 1992).

The cysteine-rich cluster(s) of all PKC isoforms is immediately preceded (by a distance of 15 amino acids) by an autoinhibitory pseudosubstrate motif. This motif is similar to the consensus sequence xRxxS/TxRx found in the phosphorylation sites of prominent PKC substrates (House and Kemp, 1987; Graff *et al.*, 1989a; Kemp and Pearson, 1990; House and Kemp, 1990) with the exception that alanines replace serine or threonine residues in the pseudosubstrate motif of all PKC isozymes. Several studies have established that this motif exerts its autoinhibitory features by sequestering the catalytic site from substrate. Indeed, pseudosubstrate peptides are rather efficient inhibitors of PKC both in cell culture and *in vitro* (House and Kemp, 1987; Eicholtz *et al.*, 1990; Shen and Buck, 1990) and proteolytically generated kinase domain (PKM), extricated from the hindrance imposed by the pseudosubstrate, is constitutively active (Inoue *et al.*, 1977). More recent studies demonstrate that activation is accompanied by removal of the pseudosubstrate domain from the catalytic core. Specifically, the pseudosubstrate is protected from proteolysis when the enzyme is not catalytically active but becomes highly sensitive to proteolytic digestion upon activation (Orr *et al.*, 1992a). Furthermore, incubation of PKC with an antibody directed against the pseudosubstrate was shown to activate the enzyme, presumably by removing the pseudosubstrate from the catalytic core (Makowske and Rosen, 1989).

The molecular basis for the functional heterogeneity between Ca^{2+}-dependent and Ca^{2+}-independent PKCs is provided by the conserved C2 region, which was originally thought to be absent from the nPKC subfamily (Ono *et al.*, 1988; Osada *et al.*, 1990; Osada *et al.*, 1992). While the C2 region does not appear to contain a sequence motif that

resembles a known Ca^{2+}-binding site such as the classical E-F hand binding motif, it does contain many acidic amino acids which are thought to participate in Ca^{2+} binding (Ohno *et al.*, 1987). Regardless, other Ca^{2+}-binding proteins have been shown to have homology with the C2 domain of the cPKCs. Some of these include phospholipase A2 (Clark *et al.*, 1991), phospholipase Cγ (Stahl *et al.*, 1988) and two synaptic vesicle Ca^{2+}-dependent proteins (Perin *et al.*, 1990; Geppert *et al.*, 1991). The crystal structure of the C2 domain of synaptotagmin (Sutton *et al.*, 1995) revealed that 5 aspartate residues within the core of this domain form the Ca^{2+}-binding site. Binding of Ca^{2+} to this domain increases the affinity of cPKCs for negatively charged lipids (Bazzi and Nelsesteun, 1987), with no apparent selectivity for headgroup other than the requirement for negative charge (Newton and Keranen, 1994). Sossin and Schwartz (1993) first noted that nPKCs indeed contain a C2 domain but the coordinating oxygens in the Ca^{2+}-binding site are essentially absent (Newton, 1995).

The primary structure of the catalytic core of cPKCs is approximately 40% identical to that of PKA (Knighton *et al.*, 1991). The C3 and C4 regions within the core of the catalytic domain contain the sites of interaction with ATP and substrate, respectively (Taylor and Radzio-Andzelm, 1994). The C3 region contains the ATP-binding motif $xGxGx_2Gx_{16}Kx$ (where x is any other amino acid) conserved in most protein kinases (Taylor *et al.*, 1990; Kemp and Pearson, 1990). Only aPKCζ differs slightly from the consensus ATP-binding motif in that it has an alanine in place of a glycine. Regardless, human aPKCζ purified from recombinant baculovirus-infected insect cells does show kinase activity (Liyanage *et al.*, 1992; McGlynn *et al.*, 1992; Kochs *et al.*, 1993a). The

C4 region contains the substrate binding site and the phosphate transfer region. The central element in the phosphate transfer region is the sequence DFG which is also highly conserved amongst protein kinases (Taylor *et al.*, 1990; Kemp and Pearson, 1990). The aspartate residue is thought to be responsible for the transfer of the phosphate group to substrates. Once again, only aPKCζ differs at the level of the phosphate transfer region; phenylalanine is substitued for a tyrosine within the consensus sequence DFG.

Most recently, the cloning and characterization of a novel PKC gene, designated PKCμ, revealed unique structural motifs relative to other PKC isozymes (Johannes *et al.*, 1994). Like nPKCs, PKCμ lacks the functional C2 domain observed in cPKCs. PKCμ also possesses two highly conserved zinc-finger-like domains, characteristic of both nPKC and cPKC subgroups. However, PKCμ has an atypically long spacing of 74 amino acids between the two zinc finger domains which has been associated with inefficient phorbol ester binding and apparently constitutive *in vitro* kinase activity. The divergence of PKCμ from other members of this kinase family becomes further evident by the unusually large size of the molecule, the presence of two unique hydrophobic amino-terminal domains comprising a putative signal peptide and transmembrane domain and an apparent lack of a typical pseudosubstrate site. Furthermore, the presence of a pleckstrin homology (PH) domain within the regulatory domain (Gibson *et al.*, 1994) is so far unique within the PKC family.

ii. Activation

With the discovery that PKC represents a (phospho)lipid-dependent protein

kinase, a model of activation (for at least cPKCs) involving the metabolic breakdown products of cellular glycerolipids soon emerged (Berridge, 1987; Berridge; 1989; Meldrum *et al.*, 1991). Extracellular signals that stimulate members of the large families of G protein-coupled receptors, tyrosine kinase receptors, or non-receptor tyrosine kinases can cause DAG production by activation of specific phospholipase Cs (PLC). In this model, receptor-mediated PLC activation leads to the generation of DAG and inositol-(1,4,5)-triphosphate (IP_3) from the hydrolysis of plasma membrane-associated phosphatidylinositol(4,5)bisphosphate (PIP_2). IP_3 stimulates the release of Ca^{2+} from intracellular storage sites, which in turn binds to the C2 region of PKC and causes the translocation of the enzyme to the plasma membrane. Here the enzyme becomes activated by DAG and PS, both of which are constitutively present in the membrane (see Bell and Burns, 1991, for review). In this model Ca^{2+}, phospholipid and DAG act in concert to activate cPKC. Alternatively, DAG production can occur more slowly by the action of phospholipase D (PLD) on membrane phospholipids (PL) to generate phosphatidic acid (PA) which can be converted to DAG (Asaoka *et al.*, 1992; Nishizuka, 1992; Nishizuka, 1995). In addition, fatty acid generation by phospholipase A2 activation modulates PKC activity (Nishizuka, 1995). Thus, multiple receptor pathways feeding into multiple phospholipid degradation pathways have the common end result of activating PKC by production of putative lipid second messengers (*Figure 3*).

In 1982, Nishizuka and coworkers reported that PKC in association with phospholipid could be activated by phorbol esters, pharmacological agents known to promote oncogenic transformation of cells both *in vivo* and in culture (Hecker, 1978), by

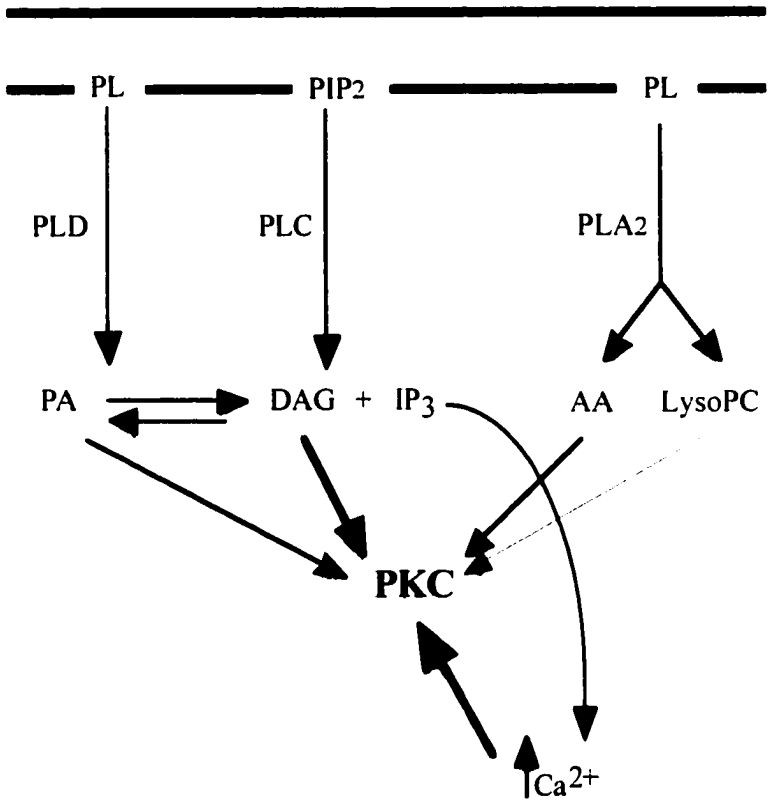


Figure 3. **Lipid networks in PKC regulation.** Signals that lead to the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) through the activation of phospholipase C (PLC) result in the generation of the second messenger DAG (an *in vivo* activator of PKC). Alternatively, DAG can be generated more slowly through phosphatidic acid (PA) which is produced by the hydrolysis of phospholipid (PL) by phospholipase D (PLD). In addition, the activity of PKC is also modulated by arachidonic acid (AA) and lysophosphatidylcholine (LysoPC) which are generated by the action of phospholipase A2 (PLA_2) (Hannun *et al.*, 1996).

the same mechanism as activation by DAG. Shortly thereafter, PKC was shown to be a direct 'receptor' protein for phorbol esters (Blumberg *et al.*, 1984). In fact, early research on PKC showed that phorbol esters could activate the enzyme with much higher potency than DAG (Castagna *et al.*, 1982) and also compete with the binding of DAG to the enzyme (Sharkey *et al.*, 1984). While phorbol esters are DAG analogues, they differ by virtue of their longer biological half-life. Thus, treatment of cells with these molecules results in prolonged activation of PKC. As a result, phorbol esters have proved invaluable in indentifying PKC-catalyzed phosphorylations *in vivo*.

iii. Autophosphorylation

All of the known PKCs undergo rather pronounced autophosphorylation which is often but not necessarily linked to a modulation of kinase activity (Pears *et al.*, 1992; Kochs *et al.*, 1993a,b,c; Flint *et al.*, 1990; Patel and Stabel, 1989; Fiebich *et al.*, 1990; Ogita *et al.*, 1992; Koide *et al.*, 1992; Saido *et al.*, 1992; Osada *et al.*, 1990; Dekker *et al.*, 1992; Osada *et al.*, 1992; Johannes *et al.*, 1995). Autophosphorylation is an intramolecular reaction at serine and threonine residues on both the regulatory and catalytic domains (Huang *et al.*, 1986b; Mochly-Rosen and Koshland, 1987; Newton and Koshland, 1987) and is strictly dependent on the presence of activators, having a Km value for ATP about 10-fold lower than that for substrate (Huang *et al.*, 1986b; Newton and Koshland, 1989). As such, autophosphorylation is not likely a prerequisite for PKC activity, but rather a concomitant event. Neverthless, as one of the immediate early events of PKC activity,

autophosphorylation may be used as a potential measure of activation (Mitchell *et al.*, 1989; Molina and Ashendel, 1991; Pfeffer *et al.*, 1991).

Mutational analysis has further expanded the role of phosphorylation and autophosphorylation in PKC activation (Pears *et al.*, 1992; Cazaubon *et al.*, 1994; Zhang *et al.*, 1994) and suggest that an early step of PKC activation requires PKC to be phosphorylated by a PKC kinase. At the molecular level, modelling has suggested that this phosphorylation occurs at a conserved "activation loop" (Orr and Newton, 1994). Thus a model has been proposed in which PKC is translated as an inactive kinase, modified by PKC kinase(s) to prime it for co-factor dependent activation and then autophosphorylated to produce the fully active kinase *in vivo* (Pears *et al.*, 1992). The PKC kinase has not been identified; however, PKC has been shown to be a substrate for casein kinase II (Tominaga *et al.*, 1991).

iv. Downregulation of PKC

The use of phorbol esters in a variety of contexts has been considerably effective in implicating PKC isotypes in regulating a myriad of biological processes. The ability of phorbol esters to induce prolonged membrane association and sustained activation of PKC isotypes at any or all locations in the cell has spatial and temporal consequences which surpass those of the more short-lived (locally produced) DAG second messenger. One consequence of this sustained activation is increased susceptibility to degradation, a phenomenon commonly referred to as downregulation. Indeed, PKC isotypes become

downregulated in an isotype-specific manner in those physiological settings where sustained DAG generation is maintained (Kiley *et al.*, 1991; Olivier and Parker, 1994).

Studies in intact cells have demonstrated that the dominant mechanism governing the loss of steady-state DAG-responsive PKC protein upon chronic exposure to phorbol ester is a net increase in proteolysis without a change in the rate of synthesis (Young *et al.*, 1987). There is at least one exception where a change in the rate of mRNA and protein synthesis occurs for PKCβ in T cells (Obeid *et al.*, 1992). The promoter of PKCβ is phorbol ester-responsive. Thus, chronic exposure of T cells to phorbol ester leads to a poorly downregulated PKCβ presumably due to the compensatory increase in the rate of mRNA and protein synthesis.

Exactly how sustained activation of certain PKCs results in their increased susceptibility to proteolysis *in vivo* is not clear. *In vitro* however, activation of particular PKC isotypes by phorbol esters/lipids induces a conformational change which exposes the hinge region between the regulatory and catalytic domains (V3 region) thus increasing their susceptibility to proteolysis (Kishimoto *et al.*, 1989). Perhaps a similar rationale could explain phorbol ester-induced downregulation *in vivo*. The observation that degradation appears to be a passive event independent of PKC isotype activity supports this hypothesis. Thus, kinase-defective forms of PKC can also be downregulated (Pears and Parker, 1991; Freisenwinkel *et al.*, 1991), at least on expression of these mutants in the context of other active PKC isotypes.

The PKC proteases *in vivo* are thought to be Ca^{2+}-activated neutral protease I and II (calpains) which are active in the micromolar and millimolar concentration range of

Ca^{2+}, respectively (Pontremoli *et al.*, 1988; Inoue *et al.*, 1977; Kishimoto *et al.*, 1983). Calpains cleave PKC in the V3 hinge region and thus produce two distinct protein fragments, the regulatory domain and the kinase domain (designated as PKM) which is catalytically active in the absence of any activators (Kishimoto *et al.*, 1989; Saido *et al.*, 1992). However, studies with linker-region mutants of PKCα displaying a complete spectrum of susceptibility to m-calpain demonstrated that a broader-based mechanism may exist since one mutant resistant to m-calpain was still susceptible to phorbol ester-induced downregulation in COS cells (Junco *et al.*, 1994). The linker swaps involved in these mutations were such that it is unlikely that any single protease with sequence specificity could be responsible for the downregulation of this PKC. Proteasome-mediated and lysosome-based degradation are obvious candidates for such a non-discriminating degradation.

On the other hand, studies of mammalian PKC expression in *Schizosaccharomyces pombe* suggested a general mechanism of downregulation. As in mammalian cells, PKCγ, δ and η downregulate on exposure to phorbol ester and as expected the atypical PKCζ does not (Goode *et al.*, 1994). By contrast, PKCε, which will downregulate in mammalian cells in response to both physiological and pharmacological agents (Olivier and Parker, 1992; Olivier and Parker, 1994), does not downregulate in *S. pombe* (Goode *et al.*, 1994). In addition, some, but not all of these PKC isotypes will induce an up-regulation of vesicle traffic in *S. pombe* and there seems to be a correlation between the ability of PKC isotypes to downregulate and their ability to induce vesicle traffic. These observations have led to the proposal that vesicle

trafficking may provide the mechanism of PKC downregulation. In this particular model, phorbol ester-sensitive PKCs which do not induce vesicle traffic would not downregulate and those that do would be carried by the locally induced traffic for sorting/degradation.

This is contradicted by observations in COS cells where kinase-deficient forms of both PKCα and PKCγ are downregulated (Pears and Parker, 1991; Freisewinkel *et al.*, 1991). However, COS cells express an endogenous PKCα (among other PKC isotypes) and it is possible that once membrane associated, a kinase deficient mutant might move with the up-regulated traffic induced by endogenous PKCs. This 'dominant' effect of one PKC over another has been tested in *S. pombe* and indeed shown to be the case (Goode *et al.*, 1995). Thus, expression of a kinase-defective form of PKCδ does not induce vesicle traffic in *S. pombe* and does not downregulate in response to phorbol ester. However, introduction of wild-type PKCδ into these cells expressing the kinase-defective mutant induces vesicle traffic and confers phorbol ester-induced downregulation on the inactive mutant. Thus it seems that PKC isotypes 'fastened' to membrane compartments by virtue of their stable interaction with phorbol ester are somehow sorted for degradation at a rate that increases as a function of increased vesicle traffic.

v. Tissue distribution and expression

Soon after the identification of the various PKC isoforms it became clear that there are tissue-specific patterns of expression. PKCα, βI/βII, δ, ε and ζ seem to be ubiquitously distributed, e.g. in brain, lung, spleen, thymus and skin (Nishizuka,1988; Wada *et al.*,

1989; Yoshida *et al.*, 1988; Schaap *et al.*, 1989; Ohno *et al.*, 1991; Wetsel *et al.*, 1992), whilst PKCγ is restricted to cells of the central nervous system (Nishizuka, 1988; Ohno *et al.*, 1991; Wetsel *et al.*, 1992). PKCη is strongly expressed in skin and lung and only slightly in brain and spleen (Osada *et al.*, 1990; Bacher *et al.*, 1991). PKCθ is predominantly expressed in skeletal muscle and to a lesser extent in brain and spleen (Osada *et al.*, 1990). It is not yet known why all PKC isoforms are expressed in brain. It is possible that the ubiquitously distributed isoforms may substitute for each other in different tissues with respect to identical or similar function. PKCα and PKCε seem to be absent in liver (Schaap *et al.*, 1989; Rogue *et al.*, 1990), where PKCβ is the major isoform (Rogue *et al.*, 1990). By contrast, no PKCβ could be detected in kidney, though PKCα, δ, ε and ζ are present (Huwiler *et al.*, 1992). No tissue has yet been found in which PKCα and PKCβ are both absent (Mischak *et al.*, 1991; Grabarek *et al.*, 1992; Duyster *et al.*, 1992).

Not only are there tissue-specific patterns of expression but there are also variations of the amount and number of PKC isoforms within a given tissue depending on its developmental stage (for review see Nishizuka, 1988). Thus, in order to guarantee the ordered sequence of proliferation and differentiation events which leads to and maintains the characteristics of a given tissue, there may be a distinction between isoforms for house-keeping functions and those for distinct roles in a differentiated, specialized cell.

vi. Subcellular localization and translocation

The treatment of cells of various origins with specific growth promoting agents (e.g. growth factors, hormones or cytokines) or unspecific agents (e.g. serum, phorbol esters) results in a redistribution of PKC from the cytosol to the membrane. The distribution, translocation and downregulation of PKC can be monitored by analysis of the so called particulate (i.e. membrane) and soluble (i.e. cytosolic) fractions of the cells, by means of PKC activity or immunoblotting (using isoform-specific antibodies).

Indeed, translocation of a particular PKC isoform to (the) cellular membrane(s) appears to be the hallmark of its activation. However, in various cell types, rather significant portions of certain PKC isoforms are constitutively present in the particulate fraction. It has been proposed that constitutive membrane localization indicates a permanent and persistent activation or activity (Bazzi and Nelsestuen, 1988; Burgoyne, 1989).

Immunofluorescent and cellular fractionation studies have shown that different PKC isoforms are localized to distinct compartments of the cell (Goodnight *et al.*, 1995). The most common localizing sites have been cytoskeletal elements and the nuclear fraction. However, it is important to note that the subcellular distribution of a particular PKC isoform appears to vary depending on cell type and conditions analyzed. PKCβII has been localized to to the nucleus and cytoskeleton using both immunological and biochemical detection methods (Goodnight *et al.*, 1995). PKCα appears to be associated with the cell periphery in rat pancreatic β cells, the nucleus in COS cells, and translocates to elements associated with the cytoskeleton in rat pancreatic β cells. In macrophages,

PKCα has been localized to phagosomes (Allen and Aderem, 1995).

C. Myristoylated Alanine-Rich C Kinase Substrate (MARCKS)

i. Discovery: An historical perspective

The first account of MARCKS (originally termed "80K") was in 1982 when Greengard and colleagues (Wu *et al.*, 1982) observed the presence of an acidic 87-kDa protein in rat brain synaptosomes. That study demonstrated the increased phosphorylation of this protein in response to depolarization-induced Ca^{2+}-influx after treatment of a cytosolic extract from rat brain with Ca^{2+} and phospholipid. Since this initial observation, numerous investigators have also observed the increased phosphorylation of an acidic 80-87-kDa protein by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in response to treatment of intact cells with a variety of growth-promoting agents that stimulate phosphatidylinositol (PI) turnover (Erusalimsky *et al.*, 1991).

The first real evidence that 80K/MARCKS was a substrate for PKC came one year after its initial discovery. In 1983, Rozengurt and colleagues demonstrated that downregulation of PKC in Swiss 3T3 fibroblasts led to a loss in the ability of phorbol esters to induce phosphorylation of 80K (Rozengurt *et al.*,1983). Subsequently, it was shown that the loss of phorbol ester-induced phosphorylation in PKC-depleted fibroblasts could be rescued by the addition of exogenous PKC to these cells (Rodriguez-Pena and Rozengut, 1986). Moreover, agents that are unable to stimulate PI turnover, e.g.,

insulin, were unable to induce phosphorylation of 80K/MARCKS. Similarly, cAMP-elevating agents (e.g., dibutyryl-cAMP, cholera toxin, cGMP or exogenous Ca^{2+}) were unable to alter the phosphorylation state of this protein (Rodriguez-Pena and Rozengurt, 1986; Erusalimsky *et al.*, 1988).

Since these initial studies, MARCKS has been shown to be one of only a few proteins identified to be a specific *in vitro* and *in vivo* substrate for PKC. As such, the phosphorylation of MARCKS has been exploited as a marker of PKC activation *in vivo*.

ii. Properties and structure of MARCKS

Other 60-90 kDa PKC substrates related to the original 80K protein found in Swiss 3T3 fibroblasts have been identified in a wide variety of cell and tissue types (Aderem, 1992a; Blackshear, 1993). There is 93% identity with the deduced amino acid sequence of the cDNA clone encoding rat brain 80K (Erusalimsky *et al.*, 1989) and the original Swiss 3T3 fibroblast 80K (Brooks *et al.*, 1991b). Furthermore, the deduced amino acid sequence of 80K was identical to a 68 kDa PKC substrate cloned from murine macrophages (Seykora *et al.*, 1991). However, comparison of rodent sequences with those deduced from the cDNA sequence of bovine and human 87kDa PKC substrates (originally termed "MARCKS"), showed only 66-74% amino acid identity (Brooks *et al.*, 1991b; Herget *et al.*, 1992). While this demonstrates a considerable degree of divergence between species, there are certain domains and features of the 80K/MARCKS protein that have been shown to be highly conserved among species. Thus, in addition to being

'alanine-rich', 80K/MARCKS is also particularly rich in glycine, proline, and glutamic acid residues. Consequently, 80K/MARCKS is an extremely acidic protein with an isoelectric point ranging from 3.5 to 4.4. It is also heat-stable and does not precipitate when heated, a feature which facilitates the isolation of MARCKS from other contaminating proteins. The structure of 80K/MARCKS is rod-shaped and the calculated Mr of 80K/MARCKS based on its primary structure is 28 to 32 kDa, depending on the species (Erusalimsky *et al.*, 1991; Li and Aderem, 1992a). However, 80K/MARCKS migrates anomalously on SDS-PAGE as a 65-87 kDa protein, depending on species and gel composition. An apparently high axial ratio together with its acidic nature, rod-like structure and extensive post-translational modification, e.g., myristoylation and glycosylation (Aderem, 1992a; Erusalimsky *et al.*, 1991) may be factors which contribute to this phenomenon.

MARCKS possesses three distinct domains that appear to be conserved among a variety of species *(Figure 4)*. These are the N-terminus, which contains the consensus sequence for myristoylation and contributes to the association of MARCKS with the plasma membrane (George and Blackshear, 1992); a MARCKS homology (MH2) domain containing a consensus sequence for alternate splicing (Blackshear, 1993); and an internal effector domain consisting of 25 amino acids (13 of which are very basic and none of which are acidic), representing a very basic motif within a highly acidic molecule. The effector domain contains the only putative PKC phosphorylation sites (Heemskerk *et al.*,

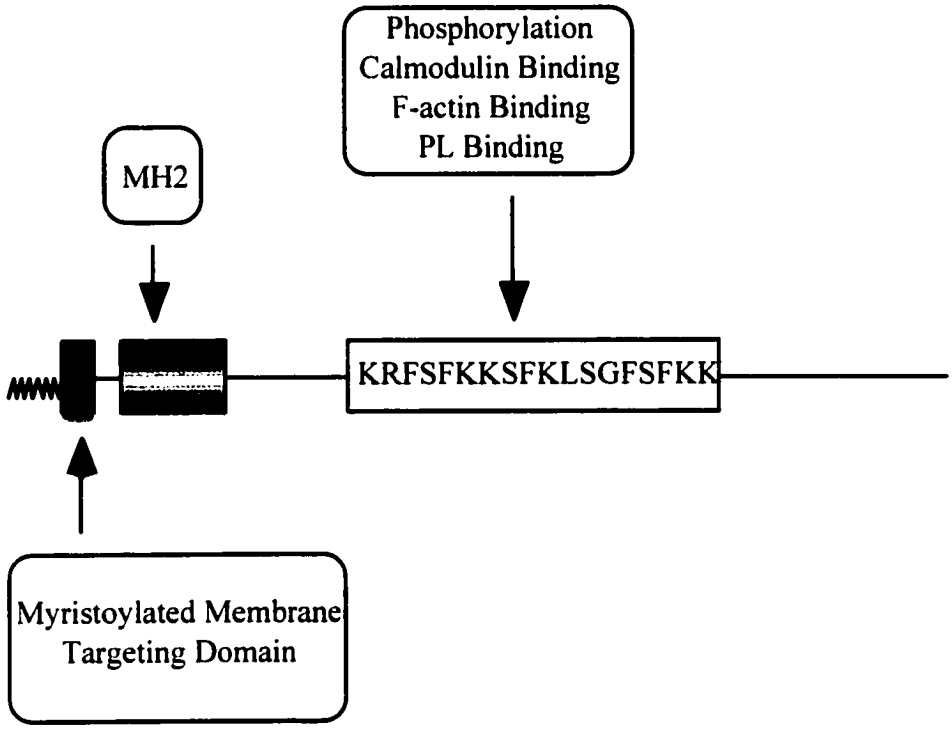


Figure 4. **Domain structure of MARCKS.** MARCKS is a rod-shaped molecule containing at least three domains: an N-terminal myristoylated domain, which participates in membrane binding, a highly conserved MH2 domain of unknown function, and a basic effector domain, which contains the PKC phosphorylation sites as well as calmodulin- and actin-binding sites (Aderem, 1995).

1993) and is also a site of interaction with both calmodulin, filamentous actin and phospholipids (see below).

Southern blot and PCR analyses have demonstrated that only 80K is present in the rodent genome and that only MARCKS is present in the bovine and human genomes (Herget *et al.*, 1992). Interestingly, however, the tissue distribution of 80K in the rodent was identical to that of MARCKS in bovine or human tissue. These observations taken together would indicate that rodent 80K and bovine and human MARCKS represent equivalent substrates in different species rather than distinct members of a gene family (Herget *et al.*, 1992). This conclusion was confirmed by chromosomal mapping studies (Blackshear *et al.*, 1992; Sakai *et al.*, 1992) of the mouse (*Macs*) and human (*MACS*) 80K/MARCKS genes. In these studies, the mouse gene was shown to localize to chromosome 6p. Further analysis using cDNA from a patient with del(6)(q21) demonstrated that the human gene localizes to 6q21 in the human genome (Blackshear *et al.*, 1992). The anacronym MARCKS has become the paradigm for this PKC substrate in different species.

MARCKS Related Protein is a relative of MARCKS cloned by screening a mouse cerebellar library for transcripts preferentially expressed in the cerebellum (Umekage and Kato, 1991; Mangels and Gnegy, 1990) and has an amino acid composition and domain structure similar to MARCKS (Blackshear *et al.*, 1992; Li and Aderem, 1992). It is considerably smaller (20 kDa vs. 28-32 kDa for MARCKS), migrates very anomalously on SDS-PAGE and is also heat stable, acidic and alanine-rich. In addition, it is myristoylated in intact cells and serves as a substrate for co-expressed N-

myristoyltransferase in *E. coli* (Blackshear *et al.*, 1992). It is also a substrate for a mixture of rat brain PKC isozymes and binds CaM in a similar manner as MARCKS (Blackshear *et al.*, 1992). Although the MRP gene (*Mrp*) and *Macs* are located on different chromosomes (Graff *et al.*, 1989), *Mrp* appears to be regulated in ways similar to *Macs*. For example, *Mrp* is also rapidly induced in macrophages following lipopolysaccharide stimulation (Li and Aderem, 1992). However, the two proteins are differentially expressed, suggesting that they have different functions. Unlike MARCKS which is widely expressed, MRP is restricted in expression, is highly enriched in macrophages, and appears to be much more highly expressed in reproductive tissues (Graff *et al.*, 1989). Nonetheless, MRP and MARCKS are members of a small gene family of heat-stable, acylated, alanine-rich, calmodulin-binding and probably actin-binding PKC substrates. Other members include GAP-43 (or neuromodulin) (Gordon-Weeks, 1989) and adducin, a PKC substrate that promotes the association of actin with spectrin in a CaM-regulated manner (Joshi *et al.*, 1991).

iii. Regulation of MARCKS expression

Two mRNA species for MARCKS can be detected by Northern blot analysis in Swiss 3T3 fibroblasts – a major transcript of 2.25 kb and a minor one of 3.9 kb (Brooks *et al.*, 1991b). Since only a single gene product is produced, the larger message has been proposed to be an unprocessed form of the 2.25 kb transcript (Stumpo *et al.*, 1989; Herget *et al.*, 1992). Interestingly, stimulation of PKC in fibroblasts by biologically active

phorbol esters reduced the steady state levels of both transcripts of MARCKS to 8.8% of control values within 5 to 7 h of exposure (Brooks *et al.*, 1992). This effect was dose-dependent and was abolished by prior depletion of PKC by chronic treatment of cells with phorbol ester for 48 h. The phorbol ester-induced downregulation of MARCKS mRNA levels was transient and recovery was shown to coincide with loss of PKC activity. The effects of phorbol ester on MARCKS mRNA levels were also observed at the protein level. Thus, phorbol ester treatment of Swiss 3T3 fibroblasts induced a transient reduction in the levels of MARCKS protein within 14 to 18 h of first exposure (Brooks *et al.*, 1991a). In a more recent study, these same investigators demonstrated that expression of both MARCKS mRNA and protein could be down regulated via both PKC-dependent and -independent pathways (Brooks *et al.*, 1992). However, the time courses and duration of MARCKS downregulation was shown to vary depending upon the pathway involved. Thus, bombesin (PKC-independent) induced a dramatic and sustained downregulation of MARCKS mRNA and protein levels to a minimum of 5% of control values within 8 and 48 h of exposure. In contrast to treatment of cells with phorbol ester, bombesin did not lead to depletion of PKC activity. Cyclic-AMP elevating agents, e.g., prostaglandin E1 and forskolin, induced a response similar to bombesin on MARCKS levels. Some agonists, e.g., platelet-derived growth factor, were shown to induce downregulation of MARCKS expression both by PKC-dependent and -independent pathways, depending upon the dose administered. Other agents such as insulin and Ca^{2+} ionophore failed to affect MARCKS expression, suggesting that the response is specific for certain agonists/signaling pathways. The effects of all agents were shown to be

unrelated to the rate of transcription of the MARCKS gene, implying that downregulation of MARCKS occurs via a post-transcriptional mechanism (Brooks *et al.*, 1992).

A similar conclusion was reported by Lindner *et al.* (1992) who showed, using a polyclonal antiserum against MARCKS, that phorbol ester induced a rapid downregulation of MARCKS protein in Swiss 3T3 fibroblasts. Since this response could be inhibited by the kinase inhibitor staurosporine, the authors proposed that this downregulation was phosphorylation-dependent. However, this hypothesis has been shown to be incorrect since downregulation of MARCKS could also be induced by agents which do not stimulate the phosphorylation of MARCKS (e.g., cAMP elevating agents) (Brooks *et al.*, 1992).

iv. Phosphorylation

Activation of PKC leads to rapid (within seconds) phosphorylation of MARCKS, and this response has been used by numerous investigators as a marker of PKC activation *in vivo* (e.g., Rodriguez-Pena and Rozengurt, 1986; Brooks *et al.*, 1991a). Although PKC exists as a family of isoforms that differ from each other in terms of their tissue distribution and co-factor requirements for activation, it remains unclear whether specific PKC isoforms can phosphorylate MARCKS preferentially. The fact that MARCKS is expressed ubiquitously in almost all tissues and cell types, whereas some PKC isoforms are distributed more specifically, would suggest that this protein can be used as a substrate by most, if not all, PKC isozymes.

MARCKS has four potential PKC phosphorylation sites situated at S152, S156, S160, and S163 (numbering according to the rat sequence). However, phosphorylation has been shown only to occur on three of these residues, S152, S156, and S163 (Heemskerk *et al.*, 1993). Since phosphorylation by PKC is optimal when the target serine residue has basic residues on either side of it, it is possible that S152, S156, and S163 are initially in a more favorable conformation for phosphorylation and, once phosphorylated, they become more negatively charged and reduce the availability of S160 for phosphorylation. Blackshear and colleagues (Graff *et al.*, 1989b) showed that both myristoylated and non-myristoylated forms of MARCKS are phosphorylated on the same serine residues by PKC.

v. Membrane association

MARCKS is associated with membranes to a significant extent in all cells examined thus far. For example, MARCKS co-localizes in adhesion plaques with vinculin and talin (Rosen *et al.*, 1990) and is localized with phagosomes in macrophages (Allen and Aderem, 1995). Although the physiological function of MARCKS is not yet known, the association of MARCKS with the plasma membrane or cytoskeleton (see below) has been proposed to be essential for its function. A striking feature of MARCKS is its stimulation-dependent translocation from the membrane (Rosen *et al.*, 1990; Thelen *et al.*, 1991; Wang *et al.*, 1989). It has been suggested that MARCKS is capable of binding to phospholipids without interaction with membrane proteins (Taniguchi and Manenti,

1993; Kim *et al.*, 1994; Newton and Koshland, 1989). The myristic acid moiety at the N-terminus is a good candidate for contributing to the binding of MARCKS to membranes by hydrophobic interactions. While myristoylation has been shown to influence protein conformation (Nadler *et al.*, 1993) as well as protein-protein interactions (Linder *et al.*, 1991) and protein-ligand interactions (Franco *et al.*, 1995), the most obvious function of the myristoyl moiety is to mediate membrane binding. Evidence for the latter function has been presented for several proteins through the analysis of the subcellular localization of myristoylation deficient mutants (Busconi and Michel, 1994; Yu and Felsted, 1992; David-Pfeuty *et al.*, 1993; Hallak *et al.*, 1994). When the N-terminal glycine of MARCKS is substituted by an alanine, the result is a mutant which cannot be acylated cotranslationally by N-myristoyltransferase and is consequently localized to the cytosol when expressed in COS cells (Graff *et al.*, 1989b). In addition, nonmyristoylated MARCKS binds to fibroblast membranes in a cell free system with much less affinity than the myristoylated wild-type counterpart (George and Blackshear, 1992). At least one study has demonstrated a pool of nonmyristoylated (or demyristoylated) MARCKS *in vivo* which was exclusively localized to the cytosolic compartment of rat brain synaptosomes, thus further supporting a role for the myristic acid moiety in mediating protein-membrane interactions (McIlhinney and McGlone, 1990; Manenti *et al.*, 1993).

The dominant mechanism by which myristic acid is thought to mediate protein interaction with membranes is via insertion of the hydrophobic carbon chain into the lipid bilayer. However, the Gibbs free binding energy of myristoylated glycine to membranes (approximately 8 kcal/mol) is barely enough to attach proteins (Peitzsch and McLaughlin,

1993). The presence of a pool of myristoylated MARCKS in the cytosol of cells (James and Olson, 1989; Manenti *et al.*, 1992) and the ability of nonmyristoylated MARCKS to bind membranes (George and Blackshear, 1992), albeit with less affinity, suggests that other factors can influence the membrane affinity of MARCKS. *In vitro* studies with purified MARCKS have demonstrated that the protein has a greater affinity for acidic rather than neutral lipid vesicles (Nakaoda *et al.*, 1995; Kim *et al.*, 1994a). As MARCKS is a very acidic protein with only a single stretch of basic residues comprising the central effector domain, it is likely that the effector domain participates in the preferential association of MARCKS with acidic lipids. This hypothesis was confirmed in a study which showed that binding of a peptide, derived from the effector domain, to phospholipid vesicles increased sigmoidally with increased content of acidic phospholipids (Kim *et al.*, 1994b).

It seems then that the capacity of MARCKS to cycle between the membrane and the cytosol is a result of the cooperative binding energies contributed by both the insertion of the myristic acid moiety into the lipid bilayer, and by the electrostatic interaction of the basic effector domain with acidic phospholipids (Kim *et al.*, 1994b; Taniguchi and Manenti, 1993a). The mechanism by which MARCKS partitions between membrane and cytosol has been termed the myristoyl-electrostatic switch (McLaughlin and Aderem, 1995) and the paradigm relies on several considerations. First, myristic acid is necessary but not sufficient for membrane binding since mutation of the N-terminal glycine to alanine prevents myristoylation and abrogates membrane binding (Graff *et al.*,1989b; George and Blackshear, 1992; Swierczynski and Blackshear, 1995). Second, the

introduction of phosphate groups into the effector domain partially neutralizes the electrostatic interaction of MARCKS with the membrane resulting in decreased affinity of MARCKS for the membrane. When all the serine residues of MARCKS which are known to be phosphorylated by PKC both *in vivo* and *in vitro* are substituted for alanines, MARCKS can neither be phosphorylated nor released from the membrane in phorbol ester-stimulated cells (Allen and Aderem, 1995a). Third, decreasing the distance between the myristic acid moiety and the basic effector domain would increase the membrane binding affinity of MARCKS (Kim *et al.*, 1994b; McLaughlin and Aderem, 1995). By the insertion of myristate into the bilayer, the effector domain becomes confined in the vicinity of the membrane, increasing the probability that the effector domain will associate electrostatically with acidic lipids (McLaughlin and Aderem, 1995). This prediction was supported by the observation that MARCKS was not displaced from the membrane by PKC-dependent phosphorylation when the intervening sequence between the myristoyl moiety and the effector domain was deleted (Seykora *et al.*,1996). Finally, the model considers that the reversible nature of membrane binding may be a consequence of the fact that myristic acid provides barely enough energy to anchor the protein to the bilayer (Kim *et al.*, 1994b; Pietzsch and McLaughlin, 1993; Silvius and l'Heureux, 1994. A MARCKS chimera in which the myristoylation domain of MARCKS was replaced by the palmitoylation sequence of GAP43 (a related PKC substrate) was not released from the membrane upon phosphorylation (Seykora *et al.*, 1996). This indicates that the membrane binding energy contributed by two palmitic acid moieties is sufficient to anchor the molecule to the membrane without the participation of the basic effector domain.

Indeed, spontaneous desorption of doubly acylated peptides from artificial membranes is much slower than their singly acylated counterparts (Shahnian and Silvius, 1995).

vi. Calmodulin binding

The first evidence that MARCKS can bind calmodulin (CaM) came from studies with a peptide corresponding to the central effector domain of MARCKS (Graff *et al.*, 1989a). Subsequently, CaM binding to intact purified MARCKS was demonstrated (Verghese *et al.*, 1994) and formation of the CaM-MARCKS complex was shown to require Ca^{2+}, the hallmark of classical CaM ligands. While there is still no direct evidence for an interaction between CaM and MARCKS *in vivo*, at least two separate studies have provided compelling evidence that such a complex indeed exists in the cell. When the freshwater protozoan *Paramecium* undergoes an action potential, membrane depolarization occurs and a period of backward swimming ensues. The organism will return to forward swimming as a consequence of membrane repolarization which in turn requires the presence of CaM. Thus, microinjection of a peptide spanning the phosphorylation and CaM-binding domain of MARCKS demonstrated that only those peptides that are able to complex with endogenous CaM could affect the biological response (observed as an increase in the duration of backward swimming) (Hinrichsen and Blackshear, 1993). Furthermore, the overexpression of MARCKS in Rat 1 cells resulted in a significant decrease in the concentration of CaM antagonists required to inhibit DNA synthesis, the initiation and progression of which requires CaM (Herget *et al.*,1994a).

Unphosphorylated MARCKS is able to bind to CaM following a rise in intracellular calcium concentrations (reviewed in Aderem, 1992a; Nairn and Aderem, 1992). Conversely, a decrease in intracellular calcium concentrations leads to loss of CaM binding. The sites of phosphorylation by PKC and CaM binding are located within the effector domain and phosphorylation within the effector domain prevents binding of CaM (Aderem, 1992a; Nairn and Aderem, 1992). The reciprocal relationship between CaM and phosphorylation of MARCKS has led to the suggestion that MARCKS functions to sequester CaM that could be released upon activation of PKC. The first indication that MARCKS might mediate cross-talk between CaM and PKC came from the observation that CaM inhibited the Ca^{2+}/phospholipid-dependent phosphorylation of MARCKS in crude extracts of rat brain synaptosomal cytosol (Wu *et al.*, 1982). More recently, Chakravarthy *et al.* (1995b) have demonstrated that MARCKS might mediate a CaM-dependent regulation of the PKC signal transduction pathway *in vivo.* In this case, treatment of intact C6 rat glioma cells with ionomycin, a Ca^{2+} ionophore, activated PKC without stimulating MARCKS phosphorylation. Pretreatment with calmidazolium, a CaM anatagonist, prevented ionomycin from blocking MARCKS phosphorylation by activated PKC.

Evidence that the MARCKS-CaM complex actively participates in signal transduction was provided in an interesting report by Sawai *et al.* (1993). In cultured mast cells, phorbol esters stimulate MARCKS phosphorylation and potentiate carbacyclin-induced cAMP formation. A role for CaM in adenylate cyclase activation and hence cAMP formation was confirmed by the observation that W-7, a CaM antagonist,

suppressed the phorbol ester enhancing activity. Interestingly, MARCKS inhibited the CaM-induced activation of adenylate cyclase in cells permeabilized with saponin and this inhibition could not be overcome by the addition of excess CaM. The implication was that sequestration of CaM by MARCKS was not responsible for the inhibitory effect of MARCKS but rather the CaM/MARCKS complex might actively regulate the cAMP signal transduction pathway in a PKC-dependent manner.

vii. Filamentous actin binding

Non-phosphorylated MARCKS can bind to and crosslink actin filaments (F-actin), suggesting a role for this protein in cytoskeletal organization (Hartwig *et al.*, 1992). This crosslinking effect is lost when MARCKS is phosphorylated, although it does not result in loss of F-actin binding (Hartwig *et al.*, 1992). The actin filaments are however less rigid (Aderem, 1992b), perhaps an important requirement for cell motility.

viii. Potential physiological roles

MARCKS has been observed in almost all tissues and cell types investigated so far with the highest expression levels found in brain. In macrophages, MARCKS has a punctate distribution and many cellular structures associated with MARCKS are found at the substrate-adherent surfaces of pseudopodia/filopodia suggesting a role in cell motility. Furthermore, many of these cellular structures also contain talin and vinculin, two components of focal contacts, suggesting that MARCKS may be involved in the initial,

transient adhesion complex formed at this site in locomoting macrophages and other motile cells (Aderem, 1992b). Further evidence for a role in motility comes from the observation that chemotactic agents (e.g., f-Met-Leu-Phe or interleukin 8) cause MARCKS to shuttle between membrane and cytosol in neutrophils (Thelen *et al.*, 1991).

The potential involvement of MARCKS in secretion and membrane recycling has been demonstrated. MARCKS is phosphorylated when rat brain synaptosomes are depolarized (Wu *et al.*, 1982; Wang *et al.*, 1989) and tumor necrosis factor and bacterial lipopolysaccharide induce MARCKS synthesis and simultaneously prime macrophages and neutrophils for enhanced secretion of inflammatory mediators and cytokines (Thelen *et al.*, 1990; Aderem, 1992a). Furthermore, McLaughlin and co-workers have shown by fluorescence digital imaging microscopy that MARCKS inhibits PLC-catalyzed hydrolysis of PIP_2 phospholipid vesicles by sequestering PIP_2 into lateral domains (Glaser *et al.*, 1996). Phosphorylation of MARCKS by PKC released the inhibition of PLC by MARCKS, producing a burst of DAG and IP_3.

Several observations would indicate that MARCKS is involved in mitogenesis and cellular transformation. Growth factors induce phosphorylation of MARCKS and transition of cells from G_0 to G_1 phase of the cell cycle (Rozengurt *et al.*, 1983). The downregulation of MARCKS protein expression occurs in NIH3T3 fibroblasts stably transfected with a variety of oncogenes, e.g., v-Ha-ras, v-fms, v-src and spontaneously transformed murine melanoma cells also have downregulated levels of MARCKS compared with normal melanocytes (Wolfman *et al.*, 1987; Oh-uchida *et al.*, 1990; Reed *et al.*, 1991; Joseph *et al.*, 1992; Goss and Brooks, 1993).

Finally, there is evidence that MARCKS may in fact be essential for normal development, particularly of the central nervous system. First, MARCKS is highly expressed in the brain and spinal cord of adult animals (Blackshear *et al.*, 1986; Albert *et al.*, 1986; Lobach *et al.*, 1993). In the bovine brain, its concentration has been estimated to be about 12 μM (Albert *et al.*, 1986). Second, its expression is much higher in fetal than in adult brain of rats (Patel and Kligman, 1987). Finally, most members of the PKC family are also expressed in brain (Nishizuka, 1988; Wetsel *et al.*, 1992) and have been suggested to play important roles in brain development. Indeed, targeted disruption of the gene encoding MARCKS in mice (*Macs*) indicates that expression of MARCKS during embryonic and fetal life in the mouse is necessary for normal development of the central nervous system as well as for postnatal survival (Stumpo *et al.*, 1994). While mice heterozygous for the disrupted gene (*Macs* +/-) appeared normal in all respects, homozygosity (*Macs* -/-) of the disrupted gene was associated with universal prenatal or perinatal death (i.e. within 4 hours of birth). These MARCKS deficient mice also exhibited high frequencies of: **(i)** exencephaly (25%) (otherwise termed "anencephaly" in humans), a severe birth defect in which the brain is malformed and in which the normal skin and skull covering the brain are absent; **(ii)** omphalocele (19%), a large herniation at the umbilicus, in which portions of the liver and other abdominal contents protrude through the abdomen; and **(iii)** runting (29%), defined as crown-rump length of less than about two-thirds that for most other pups in the litter. Other gross abnormalities associated with the MARCKS deficient mice included failure of the cerebral hemispheres to fuse, agenesis of the corpus callosum (and other forebrain commissures), and defective

retinal and cortical lamination.

D. Objective of Thesis

It is likely that the membrane association of MARCKS is essential to its proposed roles in PKC-mediated membrane-cytoskeletal alterations that accompany cell migration and vesicle trafficking. The objective of the work presented in this thesis was to further explore the nature of the interactions of MARCKS binding to membranes *in vivo* and *in vitro*. To do this, the focus was primarily on the effector domain of MARCKS as this domain appears to be essential for the regulation of membrane association at multiple levels. Since the effector domain interacts competitively with phospholipid, CaM and F-actin, and phosphorylation within this domain by PKC can reverse these interactions, competition for this domain may be key in the regulation of the association of MARCKS with membranes *in vivo*.

In one study, the effect of CaM and F-actin on membrane localization of MARCKS was examined (indirectly through the use of CaM antagonists and cytoskeletal disrupting agents) using a permeabilized cell system which allowed for the measurement of membrane affinity under a variety of conditions. This permeabilized cell system provided evidence for an alternate mode of regulation of MARCKS phosphorylation and translocation, namely by a phorbol ester- and Ca^{2+}-independent membrane-bound kinase. The second study presented in this thesis attempts to identify and characterize this kinase. Finally, the possibility that individual serine residues within the effector domain might differentially target MARCKS to various cellular locations as a result of

phosphorylation by PKC was examined by site directed mutagenesis of rat MARCKS cDNA and immunolocalization confocal microscopy of CHO-K1 cells stably expressing MARCKS.

II. Materials and Methods

A. Materials

[9,10-^{3}H]Myristic acid (16 Ci/mmol), carrier-free [^{32}P]orthophosphoric acid (5 mCi/ml), [γ-^{32}P]ATP (10 mCi/ml), [α-^{32}P]dATP (3000 Ci/mmol) and En^{3}Hance were purchased from Du Pont Canada Inc. (NEN Products, Lachine, Quebec). Triton X-100 (Surfact-Amps) was from Pierce (Chromatographic Specialties Inc., Brockville, Ontario). The 4β isomer of 12-*O*-tetradecanoylphorbol 13-acetate (βTPA), staurosporine (STS), dimethylsulfoxide (DMSO), trifluoperazine (TFP), calmidazolium (CMZ), cytochalasin D (CD), cytochalasin B (CB), *Staphylococcus aureus*V8 protease, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone-treated trypsin (TPCK-trypsin), phosphoamino acid standards and digitonin were from Sigma Chemical Co. (St. Louis, MO). *Bis*-indolylmaleimide I (bIM, Gö6850 or GF 109203X) was from Calbiochem-Novabiochem Intl. (La Jolla, CA, U.S.A). Go6976 was from BIOMOL Research Laboroatories, Inc. (Plymouth Meeting, PA). MARCKS peptides were synthesized by Dr. Sook Shin, Dept. of Biochemistry, Queen's University, Kingston, ON. Fluorescein phalloidin was obtained from Molecular Probes (Eugene, OR). G418 (Geneticin) and affinity purified rabbit polyclonal antibodies against peptide sequences unique to PKCα, δ, β and ε were purchased from Gibco BRL (Life Technologies Inc., Burlington, ON). Affinity purified rabbit polyclonal antibody raised against peptide sequences unique to PKCζ and PKCμ

were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Equipment and reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis were from BioRad Canada Ltd. (Mississauga, Ontario). Qiagen plasmid purification kit was purchased from Qiagen Inc. (Chatsworth, CA). Custom oligonucleotides were purchased from Cortec (DNA Service Laboratories, Inc., Queen's University, Kingston, ON). ^{T7}Sequencing kit was from Pharmacia Biotech Inc. (Baie d'Urfe, PQ). Altered Sites II *in vitro* Mutagenesis Systems was purchased from Promega Corporation (Madison, WI). Fluorescein-conjugated goat anti-rabbit IgG fraction (FITC-goat anti-rabbit) was purchased from Cappel (Organon Teknika Corp. West Chester, PA).

B. Methods

i. Cell culture

Stock cultures of C6 glioma cells (CCL-107) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere containing 95% air and 5% CO_2 at 37°C. For experiments, cells were seeded in 35-mm plastic petri dishes at 700 000 cells per dish in DMEM containing 10% FBS and used at 5 days when the cells were confluent.

Stock cultures of CHO-K1 (ATCC CCL61) cells were maintained in CHO-K1 medium (DMEM supplemented with 5% FBS and 34 μg ml^{-1} proline) in a humidified atmosphere containing 95% air and 5% CO_2 at 37°C. For transfections, cells were seeded

in 100-mm plastic petri dishes at 50 000 cells per dish in CHO-K1 medium for 24 h.

Stock cultures of MARCKS-expressing and mock transfected CHO-K1 cells were maintained in CHO-K1 medium containing 350 μg G418 ml^{-1}, but were subcultured in CHO-K1 medium without G418. MARCKS expression was routinely monitored by immunoblotting and immunofluorescence to ensure that at least 90% of the cells were stably overexpressing. For experiments, 2 x 10^5 cells were seeded into 35-mm dishes and were grown 3 days prior to labelling and treatments.

ii. Protein labelling

In some experiments, myristoylated proteins were prelabelled by incubating cells in 35-mm dishes with [^{3}H]myristic acid (50 μCi/ml) in 1 ml of fresh DMEM containing 10% FBS for 16 h. Protein phosphorylation experiments were performed by incubating cells with 20 μCi of [^{32}P]P_i in 1 ml of phosphate-free medium supplemented with 10% dialyzed FBS for 3h.

iii. Cell treatments

For cytoskeletal disruption, C6 cells were exposed to 10 μM CD for one hour by the addition of a 20 mM stock solution of CD (in DMSO) directly to the medium. Prior to permeabilization cells were treated as indicated in specific experiments with either βTPA, STS or both βTPA and STS simultaneously for 15 min. When desired, TFP or CMZ was also included with the above treatments. The final concentration of DMSO

never exceeded 0.3% (v/v). In some experiments, conventional and novel PKC isoforms were downregulated by addition of βTPA (2 μM final) for 18 h prior to permeabilization.

iv. Permeabilization and subcellular fractionation

To study the interaction of MARCKS with the membrane in a permeabilized cell system, conditions required to selectively extract cytosolic and membrane-bound proteins were optimized in C6 glioma cells (Douglas *et al.*, 1997) and CHO-K1 cells. Digitonin-induced permeabilization was assessed by lactate dehydrogenase (EC 1.1.1.27) as a cytosolic marker enzyme (Cook *et al.*, 1988). Incubation with digitonin resulted in time- and concentration-dependent release of lactate dehydrogenase into the medium. Incubation of C6 cells for 10 min with 120 μg digitonin ml^{-1} and CHO-K1 cells for 10 min with 10 μg digitonin ml^{-1} released greater than 80% of total lactate dehydrogenase activity and were thus the concentrations chosen for further studies.

After washing twice with 2 ml Na-Hepes buffer (15 mM Hepes, 140 mM NaCl, 1.2 mM KH_2PO_4, 4.7 mM KCl, 1 mM $MgCl_2$, 5.5 mM glucose, pH 7.4), cells were permeabilized with digitonin (120 or 10 μg ml^{-1}, as appropriate) in K-Hepes buffer (15 mM Hepes, 115 mM KCl, 1.2 mM KH_2PO_4, 1 mM $MgCl_2$, 10 mM KF, 4 mM Na-PP*i*, 5.5 mM glucose, 1 μM leupeptin, 200 μM phenylmethylsulfonyl fluoride, pH 7.0) by incubation on a rotary shaker (70 rpm) for 10 min at 25°C. In some cases, the permeabilization buffer also contained the PKC inhibitors STS (200 nM), bIM (0.001 μM - 10 μM as indicated), or Gö6976 (10 nM or 100 nM). The supernatant was removed

and the remaining particulate fraction was solubilized in K-Hepes buffer (1 ml) containing 1% (v/v) Triton X-100. In some cases, the Triton X-100 insoluble material (fraction s) was solubilized in RIPA buffer (50 mM Tris-Cl, pH 7.4 containing 1% v/v NP40, 0.25% v/v sodium deoxycholate, 0.1 % w/v sodium dodecylsulphate, 150 mM NaCl, 1mM EGTA, 200 μM phenylmethylsulfonyl fluoride and 1 μM leupeptin).

In some experiments (i.e Section IV), cytosol-depleted digitonin-permeabilized cells were incubated for an additional 15 min at 25°C in 1 ml fresh K-Hepes buffer (without digitonin) alone or containing [γ-^{32}P]ATP (40 μCi, 600 μM), EGTA (1 mM) and/or $CaCl_2$ (1 μM) and in some cases peptides corresponding to various regions of MARCKS.

v. SDS-PAGE and Western blot analysis

Radiolabelled MARCKS was enriched in cellular fractions by heat treatment (80°C for 10 min) and soluble proteins were precipitated with 14% (w/v) trichloroacetic acid (for ^{32}P-labelled MARCKS) or 5 volumes of cold acetone (for [^{3}H]myristate-labelled MARCKS). Equivalent cellular portions (by volume) of each fraction were separated by SDS-PAGE and stained with Coomassie Blue prior to fluorography or autoradiography using preflashed Amersham Hyperfilm MP. For immunoblot analysis, proteins from each fraction were acetone precipitated without prior heat treatment, separated by SDS-PAGE and transferred to nitrocellulose membranes (0.45 μm). Membranes were blocked with 5% skim milk powder in TBS-Tween buffer (25 mM Tris-HCl, pH 7.5, 125 mM NaCl, 0.1%

Tween 20) prior to incubation (1h, 25°C) with anti-rat MARCKS C-terminal peptide antisera (Rose *et al.*, 1994) at 1:3000 dilution or anti-PKCα, β, δ, ε, μ and ζ antibodies diluted to 2 μg/ml in TBS-Tween buffer. Nitrocellulose membranes were then washed four times with TBS-Tween buffer, incubated for 1 h in a 1:10,000 dilution of the appropriate secondary anti-IgG conjugated to horse radish peroxidase, and immunoreactive proteins were detected using the enhanced chemiluminescent (ECL) Western blotting kit.

vi. Densitometric analysis

Bands corresponding to immunoreactive or radiolabelled proteins were scanned using an Apple OneScanner with Ofoto software (Light Source, Inc., Greenbrae, CA, U.S.A.) and analyzed using NIH Image version 1.49 software (Research Services Branch, NIH, Bethesda, MD, U.S.A.). Data are presented as mean ± S.E.M. of at least three independent experiments, unless indicated otherwise. Student's t-test was used to analyze the results and statistical significance was set at $P<0.05$.

vii. Fluorescent staining of F-actin and fluorescence microscopy

C6 cells grown on glass coverslips, pretreated with or without CD as described above, were washed with PBS (140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH 7.4) then fixed in 3.7% formaldehyde in PBS for 10 min. The cells were washed twice more with PBS, then extracted with a solution of acetone at -20°C for 3 to 5

minutes and air dried. F-actin was labelled by 25 min incubation with 1 unit of fluorescein-phalloidin diluted in 200 µl PBS. Labelled cells were washed twice rapidly with excess PBS and mounted in a 1:1 solution of PBS and glycerol. Samples were observed and photographed with an Olympus fluorescence microscope with 100x magnification and oil immersion.

viii. Phosphoamino acid analysis

Phosphorylation reactions were performed as described above and the heat-stable ^{32}P-labelled proteins were separated by SDS-PAGE. Bands corresponding to ^{32}P-labelled MARCKS were excised and recovered from dried gels according to the method of Beeman and Hunter (1978). Briefly, the backing paper was scraped from the dried gel bands and the dried gel bands were cut up into small pieces and allowed to swell in a small volume of 0.05 M NH_4HCO_3 containing 0.1% SDS. The swollen pieces were homogenized with a glass dounce homogenizer, with more buffer being added when necessary. The homogenate was made 5% in 2-mercaptoethanol, boiled for 5 min, and then shaken overnight at 37°C in a rotary shaking water bath to elute the labelled proteins. The gel peices were removed by centrifugation. A 75 µg amount of bovine gamma globulin was added as carrier to the supernatant and the protein was then precipitated by making the solution 20% in trichloroacetic acid and leaving at 0°C for a minimum of 4 h. The precipitated protein was recovered by centrifugation and the pellet was washed successively with ethanol at -10°C and ethanol:ether (1:1) at -10°C. The dried pellet was

solubilized with 6 M HCl and the proteins were hydrolyzed by incubation at 110°C for 2 h. Following hydrolysis, HCl was removed under a stream of nitrogen gas and the residues were dissolved in 10 µl electrode buffer (acetic acid/88% formic acid/water, 78:25:897, v/v), mixed with authentic phosphoserine, phosphothreonine and phosphotyrosine standards (16 µg of each) and electrophoresed on Whatman cellulose thin-layer plates at 1000 V for 2 h. Standards were detected by ninhydrin staining and radiolabelled amino acids were detected by autoradiography.

viv. Peptide mapping by limited proteolysis

Phosphopeptide mapping was carried out using a previously described method (Dunkley *et al.*, 1986). Briefly, ^{32}P-labelled MARCKS obtained either by incubation of digitonin permeabilized cells with [^{32}P]ATP or by βTPA treatment of cells pre-labelled with [^{32}P]P_i was located on dried gels by autoradiography, excised and rehydrated. The gel pieces were then mounted onto a 15% acrylamide gel in the presence of *Staphylococcus aureus* V8 protease in sample loading buffer and electrophoresis was carried out at 100 V. When the tracking dye had reached the stacking gel/resolving gel interface, the power was discontinued for 20 min to permit incubation of the V8 protease with the ^{32}P-labelled MARCKS. After the incubation period electrophoresis was continued at 200 V until completion and the gel was then dried and subjected to autoradiography.

x. Two-dimensional tryptic peptide mapping

For tryptic peptide analysis, ^{32}P-labelled proteins prepared as above were separated by SDS-PAGE, transferred to a 0.45 μM nitrocellulose membrane (Herget *et al.*, 1993), and bands containing MARCKS were located by autoradiography and excised. The pieces of membrane were blocked with 0.5% polyvinylpyrrolidine-40 in 100 mM acetic acid at 30°C for 30 min, washed seven times with deionized water, and incubated for 18 h with 2 μg TPCK-trypsin in 20 mM NH_4HCO_3, pH 7.8 at 37°C as described (Boyle *et al.*, 1991). More than 80% of the radioactivity incorporated into MARCKS was released from the tryptic digest. The resulting phosphopeptides in the supernatant were repeatedly lyophilyzed and analyzed on Whatman cellulose thin layer chromatogram plates by two-dimensional peptide mapping. Electrophoresis in the first dimension was at 1000 V for 30 min in pyridine/acetic acid/water at pH 3.5 (1:10:189, v/v), and ascending chromatography in the second dimension was in n-butanol/pyridine/acetic acid/water (75:50:15:60, v/v) at room temperature. The plates were dried and phosphorylated peptides detected by autoradiography.

xi. Site-directed mutagenesis

The 1010 bp *KpnI-BamHI* fragment of rat MARCKS cDNA (Rose *et al.*, 1996) was subcloned into pAlter-1 and mutagenized according to the manufacturer's intstructions (Altered Sites II system; Promega) with the appropriate mutagenic

oligonucleotides (AAGCGCTTTGCCTTCAAGAAGT, corresponding to bases 445-466 and CTTCAAGAAGGCCTTCAAGCTGAGC corresponding to bases 456-480 of the coding strand (bases different than wild type are underlined). Two point-mutation mutants of MARCKS were generated; serine 152 was replaced with alanine (S152A) and serine 156 was replaced with alanine (S156A). Both mutants and wild-type (Wt) MARCKS were confirmed by dideoxy sequencing performed using the ^{T7}sequencing kit (Pharmacia) with a primer (AGTTCTTCTCGTTCTTCCTCCGG) derived from the non-coding 3' to 5' strand of the rat MARCKS cDNA and complementary to bases 491-513 of the coding strand. For transfection experiments (see below), mutated and wild-type *KpnI-BamHI* fragments were subcloned into pcDNA3.

xii. Transfection

Cells were seeded in 100-mm plastic petri dishes at 50 000 cells per dish in CHO-K1 medium 24 h prior to transfection by the calcium phosphate precipitation method. Briefly, 500 µl $CaCl_2$ (250 mM) containing 10 µg wild-type or mutant MARCKS cDNA in pcDNA3 purified with the Qiagen plasmid purification kit (according to the manufacturer's instructions) was added dropwise to 500 µl 2x HBS (50 mM Hepes (pH 7.1) containing 0.28 M NaCl and 1.5 mM Na_2HPO_4) while gently bubbling the 2x HBS solubtion with an automatic pipettor. The final 1 ml solution was left undisturbed at 25°C for 45 min to allow the DNA precipitate to form. CHO-K1 medium was removed from the cells by aspiration and replaced by the precipitated DNA which was carefully

dispersed dropwise over the cells and left for 20 min at 25°C. CHO-K1 medium (10 ml) was added and the cells returned to a humidified atmosphere containing 95% air and 5% CO_2 at 37°C for a 4 h incubation. The cells were then washed twice with PBS (7 ml each) prior to shocking the cells by addition of 5.5 ml 20% (v,v) sterile glycerol in CHO-K1 medium to the cells for 4 min at 25°C. The cells were washed first with 7 ml PBS then 5 ml PBS prior to the addition of 10 ml of CHO-K1 medium and incubation in a humidified atmosphere containing 95% air and 5% CO_2 at 37°C for 24 h. The cells were grown in selection media (CHO-K1 medium containing 600 μg G418 ml^{-1}) for 13 days and single colonies were picked and expanded and selected clones were analysed for MARCKS expression by Western blot analysis. Cell lines used as controls were produced by transfection and selection in an identical manner but with the pcDNA3 expression plasmid lacking a MARCKS cDNA insert (mock transfected).

xiii. Indirect immunofluorescence localization of MARCKS and confocal microscopy

CHO-K1 stable transfectants were seeded onto glass coverslips in 35-mm culture dishes at 40 000 cells per dish and incubated in a humidified atmosphere containing 95% air and 5% CO_2 at 37°C. After 48h, coverslips were transferred to a 6-well culture dish containing 3% (v/v) formaldehyde in PBS for 15 min at 25°C. The formaldehyde solution was removed and cells washed twice with 2 ml of PBS containing 5 mM ammonium chloride. Cells were then incubated at -20°C for 10 min in 3 ml PBS containing 0.05% (w/v) Triton X-100 then in 2 ml of PBS containing 1.0% BSA (PBS/BSA) for 15 min at

25°C prior to incubation for 1 h at 37°C with primary antibody (0.5 ml fresh PBS/BSA containing 10 μg/ml anti-MARCKS raised against GST-MARCKS fusion protein). After the incubation period, the cells were washed 3 times with 2 ml of PBS/BSA (15 min each) and incubated with 0.5 ml PBS/BSA containing 7.5 mg/ml FITC-goat anti-rabbit antibody for 45 min at 37°C. The cells were then washed twice with 2ml of PBS/BSA and once with distilled water prior to mounting coverslips on coverslides with 90% (v/v) glycerol containing 50 mM Tris-HCl (pH 9.0) and 2.5% (w/v) 1,4 diazobicyclo-[2.2.2]-octane. Indirect immunofluorescence localization of MARCKS was visualized using an Olympus microscope and 100 x magnification oil-immersion objective or by confocal microscopy using a Carl Zeiss Axiovert 100 laser scan microscope and 63 x magnification oil-immersion objective with 3.5 x enlargement (Dept. of Biology, Dalhousie University).

III. Inhibitors of actin polymerization and calmodulin binding enhance PKC-induced translocation of MARCKS in C6 glioma cells

A. Introduction

The localization and interactions of MARCKS at the membrane are undoubtedly essential to its function. In macrophages for example, many of the structures containing MARCKS are found at the substrate-adherent surface of pseudopodia and filopodia (Rosen *et al.*,1990). Immunoelectron microscopy shows MARCKS to be in clusters at points where actin filaments terminate at the plasma membrane (Aderem, 1992a) and MARCKS colocalizes with PKCα on phagosomes of macrophages, as well as in the transient adhesion zones known as podosomes (Rosen *et al.*, 1990; Allen and Aderem, 1995b). More recent investigations have indicated that interaction of MARCKS with membranes requires both a hydrophobic contribution from the N-terminal myristate moiety and electrostatic interaction between the effector domain and acidic phospholipids (Kim *et al.*, 1994a; Taniguchi and Manenti, 1993a; Kim *et al.*, 1994b; Nakaoka *et al.*,1995; Swierczynski and Blackshear, 1995). Phosphorylation of, or calmodulin binding to, the effector domain was shown to negatively affect the latter interaction. However, a clear relationship between MARCKS phosphorylation and its translocation has not been observed in several other studies (George and Blackshear, 1992; James and Olson, 1989; Byers *et al.*, 1993), suggesting that other cellular components such as the cytoskeleton might influence the membrane localization of MARCKS.

While the interactions of MARCKS with CaM, PKC, and membrane lipids have received considerable attention, relatively little is known about the effect of F-actin binding on the localization and properties of MARCKS. *In vitro*, MARCKS binds to the sides of actin filaments and cross-links them, and the effector domain is responsible for this cross-linking activity which is disrupted by both phosphorylation of MARCKS and by Ca^{2+}-CaM binding (Hartwig *et al.*,1992). Moreover, during phagocytosis, MARCKS colocalizes on the forming phagosome with F-actin, PKCα, vinculin, talin, and myosin I (Allen and Aderem, 1995b). These observations suggest a role for MARCKS as an integrator of PKC and Ca^{2+}-CaM signals in the regulation of the actin cytoskeleton and of actin-membrane interactions. It is possible that MARCKS could provide a reversible cross-link between membrane lipids and actin filaments or, conversely, F-actin might help tether MARCKS to the membrane, perhaps via additional PKC-sensitive actin binding proteins. MARCKS is phosphorylated during chemotaxis (Thelen *et al.*,1991) and phagocytosis (Allen and Aderem, 1995b), events which are accompanied by regulated rearrangement of the actin cytoskeleton. Thus, phosphorylation of MARCKS would promote the partitioning of MARCKS from the membrane and into the cytosol with concomitant regulation of actin-membrane interactions as well as actin structure at the membrane. Indeed, Ltk cells expressing phosphorylation-deficient MARCKS or dipalmitoylated MARCKS exhibit defective cell migration and cell spreading (Myat *et al.*,1994).

Although the physiological function of MARCKS has not been established, it is likely that competition for the effector domain is central to proposed roles for MARCKS

in Ca^{2+}/CaM and PKC-mediated membrane-cytoskeletal alterations accompanying cell migration and motility (Stumpo *et al.*, 1995; Rosen *et al.*, 1990) and vesicle trafficking (Allen and Aderem, 1995a,b).

B. Statement of Hypothesis

Given the multiplicity of interactions taking place at the effector domain together with its proposed role in mediating the myristoyl-electrostatic switch, it is likely that other components that interact with the effector domain such as CaM and F-actin also affect the membrane localization of MARCKS. The investigation presented in this section addresses these interactions using digitonin-permeabilized C6 glioma cells to assess membrane affinity of MARCKS resulting from a variety of conditions. Our results are consistent with a model in which F-actin and CaM may act to modulate MARCKS localization in response to PKC activation.

C. Results

i. Cytochalasin D causes complete cytoskeletal disruption in C6 glioma cells

Given the ability of MARCKS to cross-link F-actin (Hartwig *et al.*, 1992) and the co-localization of MARCKS with both vinculin and talin at focal adhesion sites (Rosen *et al.*, 1990), we examined whether the integrity of the actin cytoskeleton plays a role in the membrane association and behavior of MARCKS in response to activated PKC.

Many cells cultured on solid supports have an extensive F-actin cytoskeleton which can be selectively labelled with fluorescent phalloidin derivatives (Baldwin and Williams,

1992) and observed by fluorescence microscopy. C6 glioma cells were pretreated for 1 h with increasing concentrations of cytochalasin D (CD), an agent that selectively disrupts the networking of F-actin (Flanigan and Lin, 1980; Maclean-Fletcher and Pollard, 1980). Actin filaments were then visualized with fluorescein-phalloidin. CD pretreatment resulted in a dose-dependent gradual thinning and eventual loss of F-actin. The most complete cytoskeletal disruption was seen with 10 μM CD and, as illustrated in *Figure 5*, this was marked by the profound loss of F-actin seen spanning the cytoplasm of non-treated cells. As such, 10 μM CD was the concentration used to examine the effects of cytoskeletal disruption on MARCKS localization and behavior in C6 glioma cells.

ii. Cytochalasin D increases membrane association of MARCKS

We examined whether disruption of the F-actin cytoskeleton could affect the membrane-association of MARCKS and its localization in response to activated PKC. C6 glioma cells prelabelled with [^{3}H]myristic acid were pretreated with CD and then exposed to 200 nM βTPA (an activator of PKC) and/or 200 nM STS (a potent inhibitor of PKC). Cytosolic and membrane fractions were prepared by sequential extraction with digitonin and Triton X-100 and analyzed by fluorography *(Figure 6A)* and Western blotting *(Figure 6B)* with an anti-MARCKS polyclonal antibody. Under basal conditions approximately 51% of the total immunoreactive MARCKS was associated with the membrane fraction *(Table 1)*. CD treatment resulted in a significant increase in the proportion of immunoreactive MARCKS associated with the membrane fraction of C6 glioma cells (from 51% to 62%), with a reciprocal decrease in the cytosolic fraction. Treatment with

- Cytochalasin D

+ Cytochalasin D

Figure 5. **Fluorescence microscopy of F-actin in cytochalasin D-treated C6 glioma cells.** Cells incubated in the absence or presence of 10 μM cytochalasin D for 1 h were stained with fluorescein-phalloidin. Samples were observed with 100X oil emersion objective with 1.5x magnification and photographed with an Olympus fluorescence microscope. The micrographs shown are a set of typical results taken from a representative experiment.

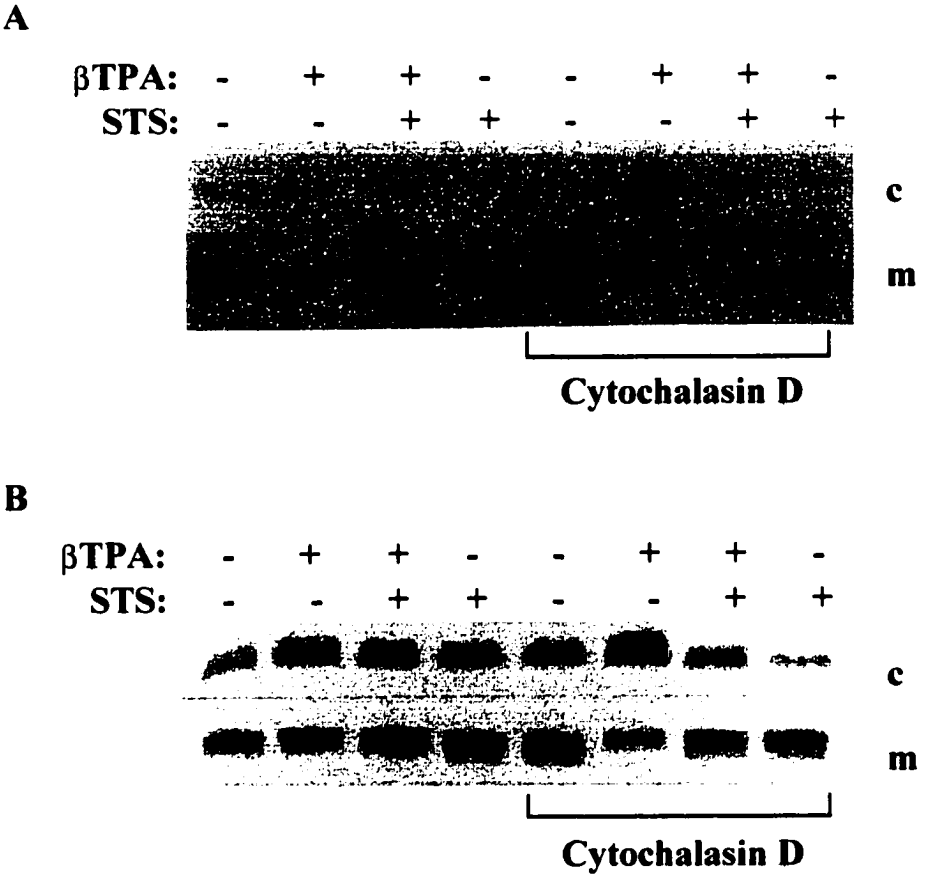


Figure 6. **The effect of βTPA and STS on translocation of MARCKS in control and cytochalasin D-treated C6 glioma cells.** Cytochalasin D (10 μM, 1h) pretreated and control cells were incubated with 200 nM βTPA and 200 nM STS (+/- as indicated) for 15 min. Soluble **(c)** and membrane **(m)** proteins were obtained by sequential extraction with digitonin followed by Triton X-100, separated by 10% SDS-PAGE, and subjected to either ***(panel A)*** fluorography for [^{3}H]myristoylated MARCKS, or ***(panel B)*** Western blot analysis for immunoreactive MARCKS using an anti-MARCKS antiserum.

Treatments			Membrane association (% of total)	
CD	βTPA	STS	Immunoreactive MARCKS	Immunoreactive PKCα
-	-	-	51±5	39±2
-	+	-	42±3a	87±3
-	+	+	51±6	87±2
-	-	+	64±5b	44±3
+	-	-	62±2c	44±4
+	+	-	35±1a	84±4
+	+	+	67±5	89±4
+	-	+	69±2	41±1

[a]Significantly (P<0.05) different from corresponding -βTPA control in Student *t*-test.
[b]Significantly (P<0.005) different from corresponding -STS control in Student *t*-test.
[c]Significantly (P<0.025) different from corresponding -CD control in Student *t*-test.

Table 1. **Effects of cytochalasin D, phorbol ester, and staurosporine on membrane association of MARCKS and PKCα in C6 glioma cells.** Cell cultures were treated as outlined in "Methods" with CD (10 μM for 1 h), βTPA (200 nM for 15 min) and/or STS (200 nM 15 min) as indicated prior to digitonin permeabilization. Proteins from soluble and particulate fractions were separated by 10% SDS-PAGE and analyzed by immunoblot analysis for MARCKS (see *Figure 6B* for representative experiment) and PKCα (see *Figure 7* for representative experiment). The amount of MARCKS or PKCα was determined by densitometric analysis and the membrane content expressed as a percentage of the total amount from that dish. The mean ± S.E.M. are shown for the membrane fractions from at least three independent experiments.

200 nM βTPA resulted in a modest redistribution of the membrane MARCKS to the cytosolic fraction (from 51% to 42%). The increased membrane association of MARCKS in the presence of CD actually enhanced its subsequent translocation to the cytosol upon activation of PKC: addition of 200 nM βTPA decreased the proportion of membrane MARCKS to 35% in CD pretreated cells (vs 42 % in βTPA-treated control cells). Thus, the amount of MARCKS translocated from membrane to cytosol by βTPA was almost three-fold greater in CD-treated than in untreated cells. Similar results were also observed when MARCKS was visualized by [^{3}H]myristate-labelling or when F-actin was disrupted with CB (not shown). From these observations, we conclude that disruption of F-actin in C6 cells increases the membrane affinity of MARCKS, yet renders it more sensitive to βTPA-induced translocation to the cytosol.

The non-specific PKC inhibitor STS also increased the membrane association of MARCKS to the level seen in CD-pretreated cells; however, unlike CD, STS antagonized rather than enhanced βTPA-induced translocation to the cytosol *(Figure 6, Table 1)*. Addition of both STS and CD to cells produced no greater membrane association of MARCKS than either reagent alone, but completely blocked any effect of βTPA on MARCKS translocation. In a separate experiment, 10 μM bis-indolylmaleimide (bIM) did not appreciably alter MARCKS localization (47% vs 49% membrane associated in BIM-treated vs untreated cells) *(Figure 7)*. As bIM is a much more specific inhibitor of PKC (Toullec *et al.*, 1991), which completely blocks PKC activity at this concentration in C6 cells (Van Iderstine *et al.*, 1996), these data suggest that CD and STS might act

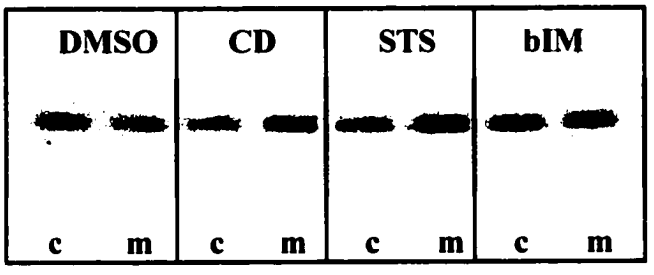


Figure 7. **The effect of bIM on MARCKS localization in C6 cells.** Confluent cell cultures were incubated with either cytochalasin D (CD, 10 μM) for 1 h, STS (200 nM) for 15 min, or bIM (10 μM) for 15 min. Soluble **(c)** and membrane **(m)** proteins were obtained by sequential extraction with digitonin followed by Triton X-100, separated by 10% SDS-PAGE, and Western blotted for the presence of MARCKS.

similarly to enhance membrane association of MARCKS in a PKC-independent manner. MARCKS was not found in the Triton X-100 insoluble fraction under any of experimental treatment conditions.

iii. Cytoskeletal integrity is not required for phosphorylation of MARCKS by activated PKC

In addition to being the site of interaction with F-actin, the basic domain of MARCKS contains the only serine residues phosphorylated by PKC (Graff *et al.*, 1989a). To determine which of the effects on membrane association of MARCKS might be due to changes in basal or βTPA-stimulated phosphorylation of the protein, we therefore assessed the effect of cytoskeletal disruption on the phosphorylation of MARCKS by activated PKC. C6 glioma cells were prelabelled with [^{32}P]P_i prior to CD pretreatment then treated with 200 nM βTPA and/or 200 nM STS. Cytosol and membrane fractions were prepared by sequential extraction with digitonin and Triton X-100 and MARCKS was partially purified from each fraction by heat treatment and analyzed by SDS-PAGE and autoradiography. MARCKS was identified as the 80 kDa heat-stable phosphorylated band on the basis of its selective heat stability (Blackshear *et al.*, 1986) and earlier 2D-PAGE analysis of proteins phosphorylated in response to βTPA in C6 cells (Byers *et al.*, 1993). Consistent with the latter study, βTPA treatment stimulated MARCKS phosphorylation in both soluble and membrane fractions, but CD had no effect on either the basal phosphorylation of MARCKS or the degree of stimulation produced by βTPA (phosphorylation of total cellular MARCKS was

increased 4.5 ± 0.7 and 4.5 ± 1.5 fold by βTPA in the absence and presence of 10 uM CD, respectively, in three experiments). STS completely blocked the βTPA-induced phosphorylation of MARCKS (not shown), but when added alone did not decrease basal levels of MARCKS phosphorylation below that of control cells *(Figure 8)*. These results indicate that cytoskeletal integrity is not required for phosphorylation of MARCKS by activated PKC, and that the influence of CD and STS on basal MARCKS distribution (and enhanced translocation by βTPA in the case of CD) is not due to additional changes in its phosphorylation state.

iv. PKC translocation is independent of cytoskeletal integrity

Since the activation of PKC results in the reversible translocation of MARCKS from the plasma membrane to the cytosol (Thelen *et al.*, 1991) and F-actin crosslinking activity of MARCKS is also regulated by PKC phosphorylation (Hartwig *et al.*, 1992), we examined whether the effects of cytoskeletal disruption on MARCKS localization could affect the cellular localization of PKCα, a prominent PKC isoform in C6 cells (Sproull *et al.*, 1995; Chen *et al.*, 1995) that has been implicated in the function of MARCKS (Sheu *et al.*, 1995; Kim *et al.*, 1994a). C6 glioma cells were pretreated with CD then treated with either 200 nM βTPA and/or 200 nM STS. Cytosolic and membrane fractions were prepared by sequential extraction with digitonin followed by Triton X-100 and analyzed by Western blotting with anti-PKCα polyclonal antibody. As shown in *Figure 9*, most of the PKCα immunoreactivity was found in the cytosolic fraction under basal conditions (39% in the membrane fraction; *Table 1*). As expected, treatment with

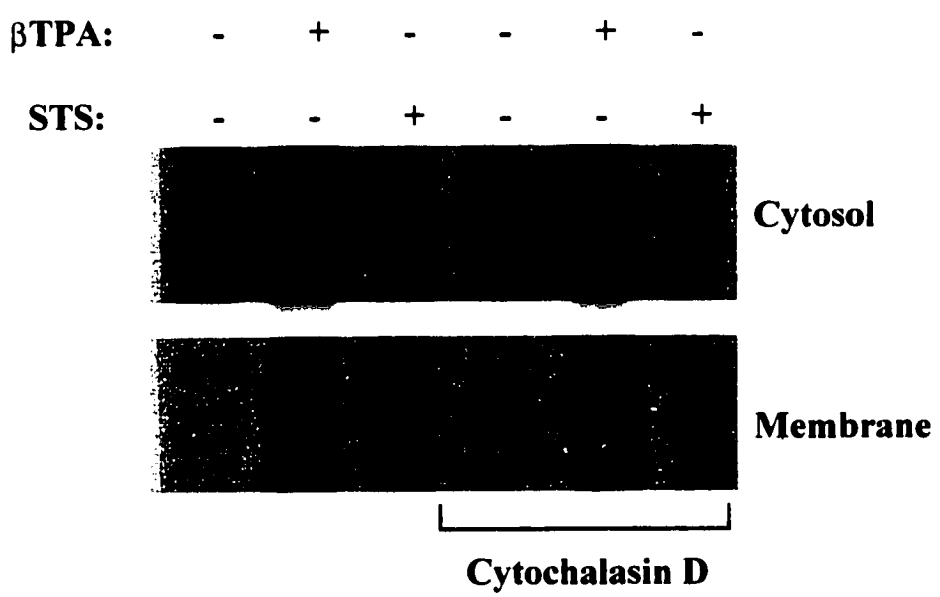


Figure 8. **Effects of βTPA and staurosporine on phosphorylation of MARCKS in control and cytochalasin D-treated C6 glioma cells.** Confluent C6 glioma cells were prelabeled with [^{32}P]P$_i$ for 3 hours and then exposed to either cytochalasin D (10 μM) or an equivalent volume of DMSO (0.1%) for 1 h. For the final 15 minutes of the incubation period, 200 nM βTPA or 200 nM STS (+/- as indicated) was added. Soluble and membrane proteins were obtained by sequential extraction with digitonin followed by Triton X-100. Samples were heated at 80°C for 10 min and heat-stable proteins were precipitated with trichloroacetic acid prior to separation by 10% SDS-PAGE and autoradiography.

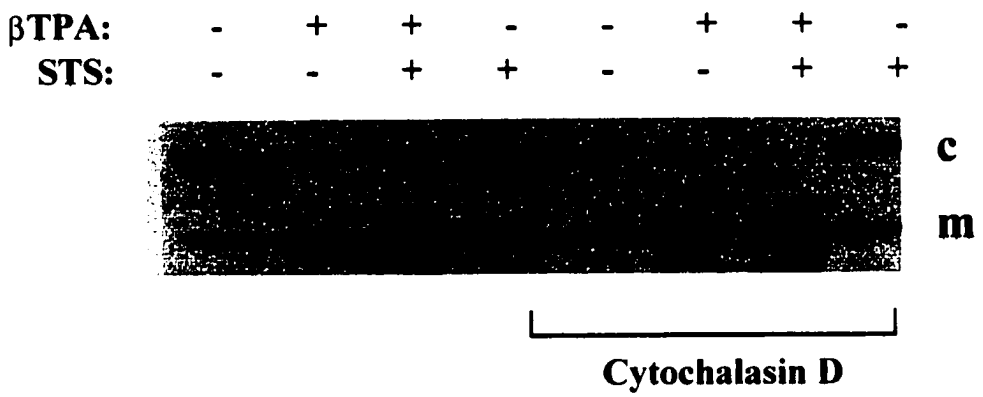


Figure 9. **The effect of βTPA and STS on the translocation of PKCα in control and cytochalasin D-treated C6 glioma cells.** Confluent cell cultures were incubated with either cytochalasin D (10 μM) or an equivalent volume of DMSO (0.1%) for a total period of one hour. For the final 15 minutes of the incubation period, the cell cultures were treated with 200 nM βTPA and 200 nM STS (+/- as indicated). Soluble and membrane proteins were obtained by sequential extraction with digitonin followed by Triton X-100, separated by 10% SDS-PAGE, and Western blotted for the presence of PKCα using a PKCα-specific antibody.

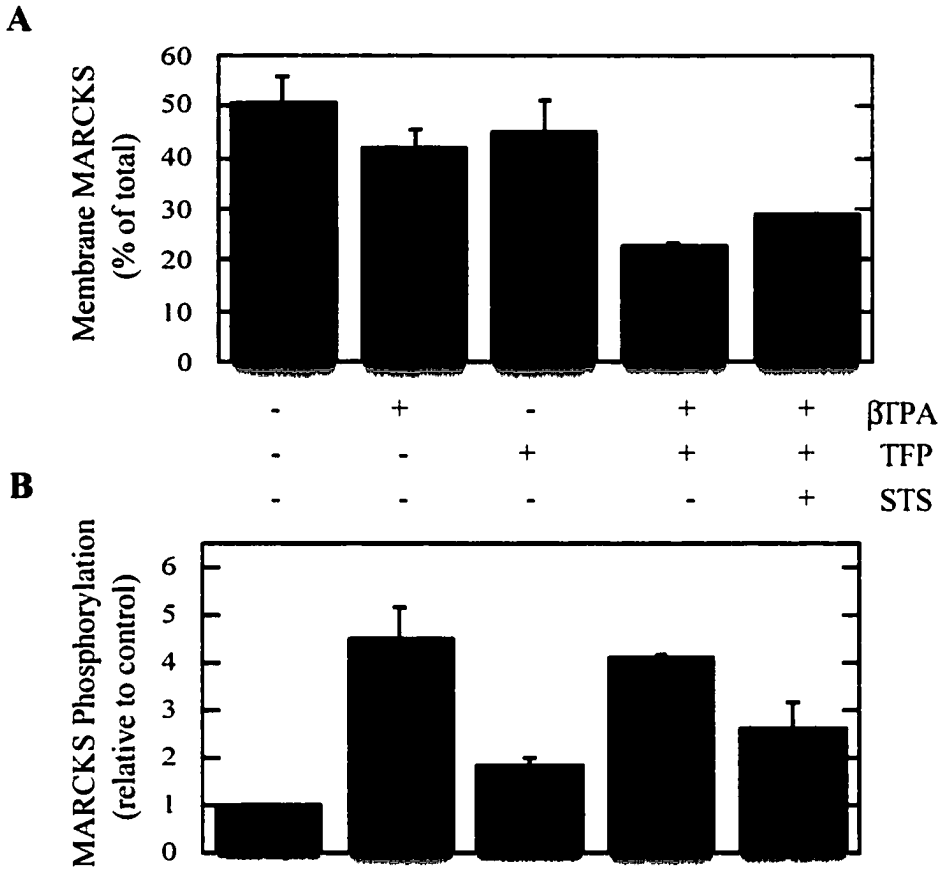


Figure 10. **Effects of trifluoperazine and βTPA on translocation and phosphorylation of MARCKS in C6 glioma cells.** *Panel A:* Confluent cells were treated with 200 nM βTPA, 200 nM STS or 50 μM TFP (+/- as indicated) for 15 min prior to protein extraction and quantification of membrane associated MARCKS by immunoblotting as described in Table 1. *Panel B:* cells were prelabelled with [^{32}P]Pi for 4 h and treated as above. Phosphorylated MARCKS was detected by SDS-PAGE and autogradiography and quantified by densitometry. Data is expressed relative to untreated control samples. The mean ± S.E.M. of three separate experiments is shown.

associated in the presence of both TFP and βTPA, vs 41% with βTPA alone (significantly different by Student-Newman-Keuls analysis, $P<0.05$). Similar results were observed with the more potent and specific CaM antagonist CMZ (Silver *et al.*, 1986), indicating that the effects of TFP are indeed due to inhibition of CaM binding *(Figure 11)*. TFP and CD added together had little effect on basal MARCKS distribution, and the response to βTPA under these conditions indicated that enhanced PKC-mediated translocation by TFP and CD are not additive *(Figure 12)*.

As with CD, the synergistic effect of TFP on βTPA-induced translocation of MARCKS was not accompanied by a comparable change in its phosphorylation state *(Figure 10B)*: although TFP alone slightly increased phosphorylation of MARCKS (1.8-fold), it had no further effect on the increase produced by βTPA. Interestingly, while STS effectively antagonized βTPA-induced phosphorylation and translocation of MARCKS *(Table 1)*, it only partially reversed these parameters when TFP was present *(Figure 10)*.

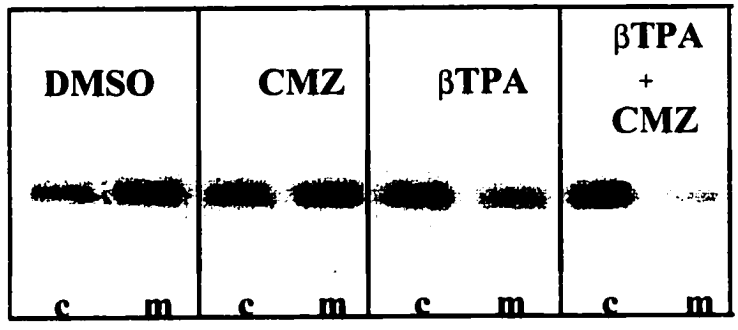


Figure 11. **The effect of calmidazolium on MARCKS localization in C6 cells.** Confluent cell cultures were incubated with βTPA (200 nM) and/or calmidazolium (CMZ, 1 μM), or DMSO alone for 15 min. Soluble **(c)** and membrane **(m)** proteins were obtained by sequential extraction with digitonin followed by Triton X-100, separated by 10% SDS-PAGE, and Western blotted for the presence of MARCKS.

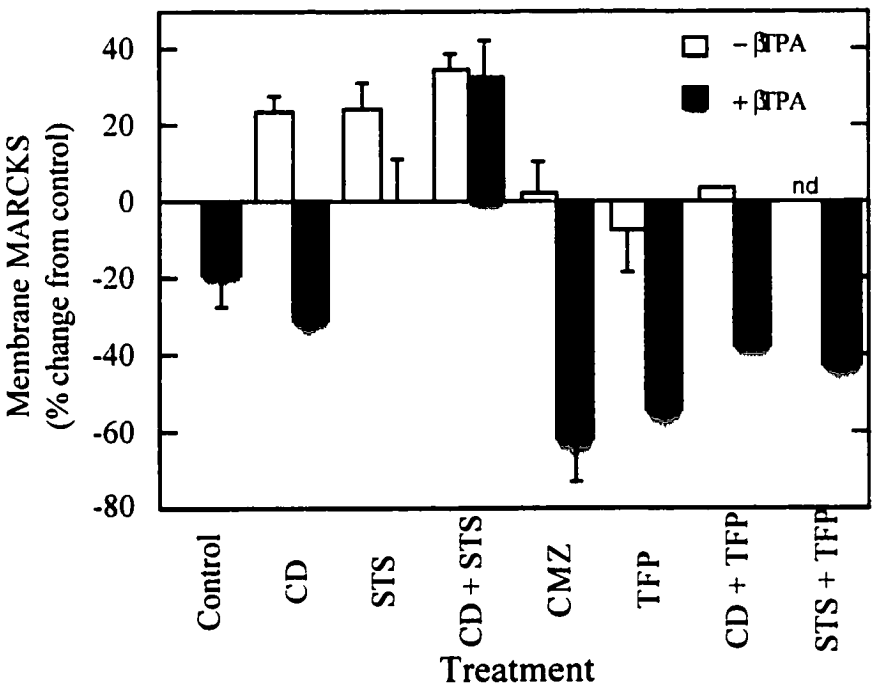


Figure 12. **Individual and combined influence of inhibitors of PKC activity, cytoskeletal assembly, and CaM binding on membrane association of MARCKS in the presence and absence of βTPA.** C6 glioma cells were pretreated 1 h with 10 μM CD, and/or 15 min with 200 nM STS, 1 μM CMZ, or 50 μM TFP prior to extraction and quantification of membrane associated MARCKS by immunoblotting. βTPA (200 nM) was also present (solid bars) or absent (white bars) during the final 15 min incubation as indicated. Data are from several independent experiments and show the percent change in membrane MARCKS (mean ± S.E.M or range, n = 2-8) in the treatment groups relative to untreated cells (control) from that experiment; 51 ± 3% of total MARCKS was membrane associated under control conditions, nd; not determined.

D. Discussion

For this study, we have used a permeabilization protocol that had been previously optimized to selectively extract cytosolic and membrane-bound proteins (Douglas *et al.*, 1997). Under the basal conditions established for C6 glioma cells, MARCKS was approximately equally distributed between the soluble digitonin and membrane fractions. Acute exposure of the cells to the phorbol ester βTPA, a known activator of PKC, produced only a modest translocation of MARCKS to the cytosol despite a four-fold increase in phosphorylation. One of the most important results obtained from this investigation is that inhibition of either CaM binding or actin polymerization can potentiate the PKC-induced translocation of MARCKS, apparently without affecting further the level of MARCKS phosphorylation. This observation may resolve apparent discrepancies in the litererature about the relationship between MARCKS phosphorylation and translocation.

It is generally accepted that phosphorylation by PKC is the dominant mechanism causing MARCKS redistribution from membrane to cytosol. In macrophages and neutrophils as well as in intact synaptosomes, activation of PKC is concomitant with translocation of MARCKS to the cytosol (Rosen *et al.*, 1990; Thelen *et al.*, 1991; Wang *et al.*, 1989). In these cell types, the translocation of MARCKS can be explained by the myristoyl-electrostatic switch model in so far as the binding of MARCKS to membranes is regulated by phosphorylation. In this study, the observation that the bulk of phosphorylated MARCKS was found to reside within the cytosolic fraction agrees with the myristoyl-electrostatic switch model. *In vitro* studies with both synaptosomal

membranes (Wang *et al.*, 1989) and purified MARCKS bound to lipid vesicles (Nakaoda *et al.*, 1995; Taniguchi and Manenti, 1993a; Kim *et al.*, 1994a) have demonstrated that phosphorylation by PKC markedly reduces the affinity of MARCKS for membranes.

The myristoyl-electrostatic switch does not explain why some of the newly phosphorylated MARCKS remained associated with the membrane fraction. While inhibition of the electrostatic interaction of the effector domain with membranes by phosphorylation should be sufficient to trigger the translocation of the protein, other studies would seem to suggest a more complex relationship. In some cell types increased phosphorylation of MARCKS is not synonymous with its translocation (James and Olson, 1989; Byers *et al.*, 1993), and the two events can be temporally dissociated in C6 glioma cells (Byers *et al.*, 1993). In light of the modest translocation observed here, it is tempting to speculate that, in C6 glioma cells at least, cytosolic MARCKS in addition to membrane MARCKS may serve as a substrate for activated PKC. A similar observation has been previously made with myocytes (James and Olson, 1989), where both membrane-bound and soluble forms of MARCKS are phosphorylated to similar extents in response to phorbol ester, while the subcellular distribution of MARCKS is unaffected.

The cycle of membrane attachment and detachment of MARCKS has been suggested to provide a mechanism for the formation of a reversible crossbridge between the actin-cytoskeleton and the plasma membrane (Hartwig *et al.*, 1992). The central basic domain of MARCKS is a site of interaction with both the plasma membrane (Swierczynski and Blackshear, 1995) and F-actin (Hartwig *et al.*, 1992). There were

several possible outcomes anticipated prior to this study. First, interaction of MARCKS with F-actin might not interfere with the membrane association of MARCKS, then cytoskeletal disruption should have no effect on the basal distribution of MARCKS. Second, F-actin might compete with acidic lipids in the membrane for binding to the effector domain of MARCKS, then cytoskeletal disruption should increase the membrane association of MARCKS. Third, F-actin might help to tether MARCKS to membranes, then cytoskeletal disruption should decrease the membrane association of MARCKS. The data presented here contradicts the hypothesis that MARCKS acts as a functional link between the plasma membrane and the actin cytoskeleton. Instead, these data demonstrate that the interaction of MARCKS with F-actin prevents interaction of MARCKS with the plasma membrane, possibly by virtue of the known association of F-actin with the central basic domain of MARCKS (Hartwig *et al.*, 1992). As it has been reported that G-actin does not interact with MARCKS (Hartwig *et al.*, 1992), one explanation for this observation may be that disruption of F-actin liberates the effector domain of MARCKS for increased interaction with acidic lipids in the membrane. The increased electrostatic interaction of the effector domain with acidic lipids would provide an important energetic contribution to the affinity of membrane interaction already provided by the myristoyl group (Kim *et al.*, 1994a; Kim *et al.*, 1994b). F-actin is known to compete with both CaM binding and PKC phosphorylation for this domain (Hartwig *et al.*, 1992), but direct evidence that F-actin and membrane binding are competitive has not been previously reported. In any case, our results suggest that F-actin does not restrict MARCKS solubilization by tethering it to the membrane or Triton X-100-

resistant cytoskeletal fractions under basal conditions.

Disruption of the F-actin cytoskeleton rendered the membrane pool of MARCKS more susceptible to solubilization following βTPA-induced PKC activation. While the role of the cytoskeleton in mediating signaling through PKC remains unclear, at least one study has shown that cytochalasins abrogate PKC translocation in neutrophils (Obel, 1992). By contrast, cytoskeletal integrity does not appear to be essential for either βTPA-stimulated translocation of PKCα to the membrane or resulting phosphorylation of MARCKS in C6 cells. Cytochalasin D also had no effect on bombesin-stimulated MARCKS phosphorylation in Swiss 3T3 fibroblasts, whereas phosphorylation of the focal adhesion site-associated tyrosine kinase p125FAK was completely blocked (Sinnett-Smith *et al.*, 1993), suggesting that the effect of actin filament disruption on PKC activity is substrate specific.

How is it that disruption of F-actin potentiated phorbol ester-induced translocation of MARCKS without affecting further the phosphorylation of MARCKS? Phosphorylation by PKC has been shown to reverse the F-actin crosslinking activity of MARCKS but not the ability of MARCKS to bind F-actin (Hartwig *et al.*, 1992). Two separate models were proposed in that study to explain this observation. In one model, MARCKS has one F-actin binding site and can dimerize; phosphorylation induces dissociation of MARCKs and thus disruption of F-actin crosslinking. In the second model, MARCKS has two binding sites for F-actin and only one is modulated by PKC, namely the effector domain. Both of these models consider that in order for the binding of F-actin to the effector domain to be modulatd by PKC dependent phosphorylation, the

effector domain is in its dephosphorylated state and therefore most likely membrane-bound. Neither of these models are consistent with the present observation that F-actin does not tether MARCKS to the membrane or Triton X-100-resistant cytoskeletal fractions under basal conditions. The observation that cytoskeletal disruption increased the membrane association of MARCKS in C6 cells suggests that at least some of the F-actin-bound pool of MARCKS is soluble or at least extractable into the soluble phase by the digitonin permeabilization procedure used in this investigation. The model presented in *Figure 13* is a modification of the previous models in that the pool of MARCKS involved in F-actin crosslinking is cytosolic. In this model, whether MARCKS has one F-actin binding site or two is irrelevant; it is only necessary that one binding site is the effector domain. Activation of PKC results in the phosphorylation of both membrane-bound MARCKS and F-actin-bound MARCKS (which is more soluble). Phosphorylation of the membrane-bound pool would result in translocation whereas phosphorylation of the latter pool would not lead to apparent translocation but instead a local release of actin from the effector domain and hence PKC-dependent alterations in cytoskeletal plasticity.

Two phosphorylatable pools of MARCKS, of which only one is translocatable, might explain the modest translocation of MARCKS seen in response to βTPA, despite a four-fold increase in phosphorylation. Furthermore, liberating the effector domain for increased membrane binding by disruption of F-actin with cytochalasin D would increase the translocatable pool of MARCKS in response to activated PKC without further affecting the phosphorylation.

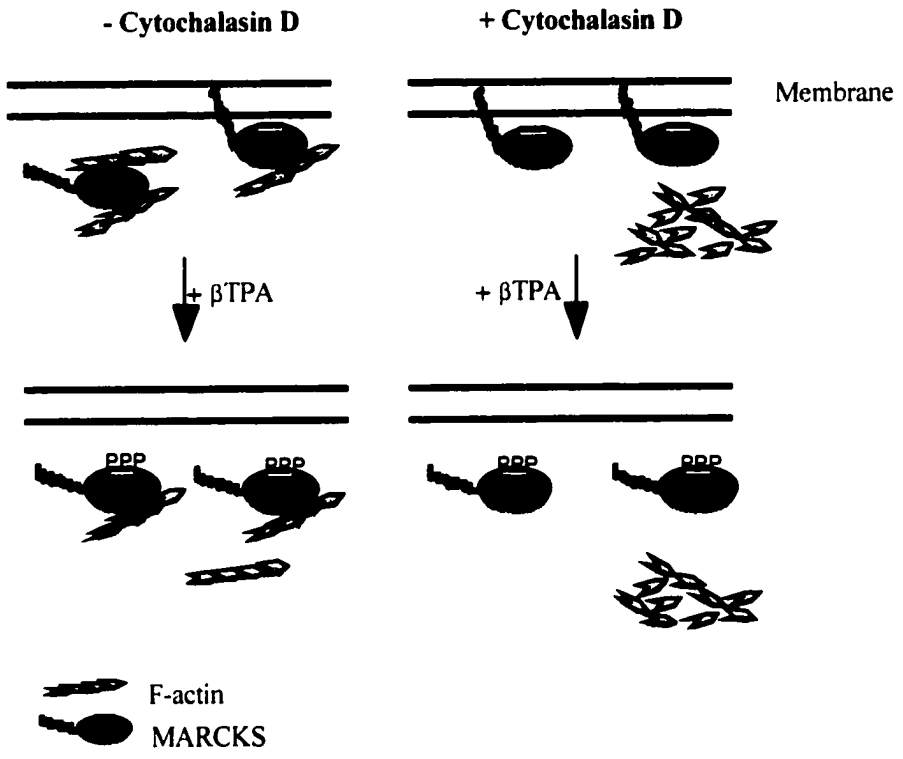


Figure 13. **Model indicating a mechanism by which cytochalasin D might potentiate βTPA induced release from the membrane without affecting further phosphorylation of MARCKS.** At rest, dephosphorylated MARCKS is associated with the cytoplasmic face of the membrane (i.e. not extractable by digitonin) or is involved in F-actin cross-linking (i.e. extractable by digitonin). Both of these pools of MARCKS compete for phosphorylation by PKC but only phosphorylation of the former can increase the amount of MARCKS extracted by digitonin. Disruption of F-actin with cytochalasin D increases the association of MARCKS with membranes thus increasing the translocatable pool of MARCKS not the amount of MARCKS phosphorylated in response to βTPA.

Unlike cytochalasin D treatment, inhibition of endogenous CaM with TFP or calmidazolium alone had little effect on the distribution of MARCKS between soluble and membrane fractions. As other studies have shown that saturating Ca^{2+}/CaM levels do inhibit MARCKS interaction with phospholipids (Swiercynski and Blackshear, 1995; Kim *et al.*, 1994b), our results might suggest either that basal CaM binding was minimal under the permeabilization conditions used (ie. low Ca^{2+}), or that Ca^{2+}/CaM binding does not influence membrane affinity in a more physiological cell model. However, it is also possible that the increase in phosphorylated MARCKS (1.8 fold) in the presence of TFP was sufficient to counter a slight increase in membrane affinity upon removal of CaM. Indeed, several reports indicate that Ca^{2+}/CaM binding can inhibit phosphorylation of MARCKS by PKC (Graff *et al.*, 1989a; Zhao *et al.*, 1991; Verghese *et al.*, 1994; Chakravarthy *et al.*, 1995a), and this inhibition has recently been shown to be Ca^{2+}-dependent in C6 cells (Chakravarthy *et al.*, 1995b).

In addition, this investigation has provided evidence for at least two distinct effects of STS on MARCKS localization in C6 cells. First, STS can inhibit βTPA-stimulated phosphorylation and translocation of MARCKS. This is likely due to direct inhibition of PKC activity, as indicated here and in previous studies with this cell type (Van Iderstine *et al.*, 1996; Thompson *et al.*, 1993). In most cell types, PKC is primarily present in a cytoplasmic state which can be solubilized without detergents (reviewed in Niedel and Blackshear, 1986). Phorbol ester binding and PKC activation are associated with a redistribution of PKC to the particulate fraction (Kraft and Anderson, 1983). Although STS blocked both phosphorylation and translocation of MARCKS in response

to βTPA, it had no affect on the translocation of PKCα to the membrane in either CD-treated or untreated cells. The latter observation is not surprising, as STS, a microbial alkaloid and potent inhibitor of PKC (IC_{50} of 2.7 nM), mediates its effects on PKC activity by binding to the catalytic domain at the ATP-binding site (Tamaoki *et al.*, 1986), while the N-terminal regulatory domain is responsible for βTPA/DAG, Ca^{2+}, and PS-dependent interaction with the membrane bilayer.

Finally, the sensitivity of MARCKS phosphorylation and membrane localization in response to STS may be influenced by other factors: TFP appears to render MARCKS phosphorylation and translocation less sensitive to inhibition by STS. Second, STS appears to have PKC-independent effects on MARCKS distribution, as this inhibitor (but not the more specific PKC inhibitor BIM) increased membrane associaton of MARCKS without altering its basal phosphorylation. This could reflect a PKC-independent disruption of the cytoskeleton by STS: previous studies have noted that this inhibitor induces reorganization of actin filaments in 3T3 cells (Hedberg *et al.*, 1990) and alteration of C6 glioma cell morphology (Thompson *et al.*, 1993; Kronfeld *et al.*, 1995).

In conclusion, CaM and F-actin exhibit distinct effects on membrane association of MARCKS and both act to modulate PKC-mediated changes in its subcellular distribution. The influence of CaM and F-actin appear to be at least partially independent of changes in the phosphorylation state of MARCKS. These data are consistent with previous evidence in C6 cells (Chakravarthy *et al.*, 1995b) showing that MARCKS is poised to respond to and coordinate temporally distinct Ca^{2+}- and PKC-mediated signals as part of

a physiological role in regulating membrane-cytoskeletal interactions.

IV. MARCKS is phosphorylated and translocated by a phorbol ester-insensitive and calcium-independent PKC-like kinase in C6 glioma cells

A. Introduction

Molecular cloning and biochemical studies have revealed the presence of a number of PKC members that can be divided into distinct classes based on differences in their structures and biochemical requirements for their activation (Nishizuka, 1992). The first group, the conventional PKCs (cPKCα, βI, βII, and γ), require phosphatidylserine and DAG (or phorbol ester) in the presence of Ca^{2+} for their activation (Nishizuka, 1992), whereas the second group, the novel PKCs (nPKCδ, ε, η(λ), θ, and perhaps the recently indentified PKCμ isoform), require phospholipid and DAG (or phorbol ester) but are Ca^{2+}-independent (Ono *et al.*, 1988; Osada *et al.*, 1990; Osada *et al.*, 1992; Johannes *et al.*, 1994; Johannes *et al.*, 1995). The third group has been classified as atypical PKCs (aPKCζ and λ); while this subgroup still requires PS for their activation, these isoforms do not require DAG, phorbol ester, or Ca^{2+} for their activation.

Although the phosphorylation of MARCKS has been extensively used to monitor the activation of PKC in intact cells, very little is known about the specificity of MARCKS as a substrate for various members of the PKC family. Recently, MARCKS has been shown to be efficiently phosphorylated *in vitro* by cPKC and nPKC isoforms including the newly identified nPKCμ (Dieterich *et al.*,1996), but not by aPKCζ (Herget

et al., 1995; Fujise *et al.*, 1994) or aPKCλ (Uberall *et al.*, 1997). MARCKS has also been reported to be phosphorylated by proline-directed kinases (Schonwaber *et al.*, 1996; Taniguchi *et al.*, 1994). However, which kinases phosphorylate MARCKS in intact cells and the functional consequences of phosphorylation remain unknown.

Rat C6 glioma cells provide a good system to examine the regulation of MARCKS phosphorylation and cellular localization. As seen in the previous section (Section III), MARCKS is abundant in this cell line and acute exposure of these cells to the phorbol ester βTPA results in its phosphorylation and partial translocation to the cytosol. In addition, C6 cells have been shown previously (Chen, 1993), as well as in this study, to host at least one isoform from each of the cPKC, nPKC and aPKC subtypes.

B. Statement of Hypothesis

The permeabilized C6 glioma cell system used in the previous investigation was initially developed to examine the interactions of MARCKS in a physiological setting in which the environment could be carefully controlled. Specifically, the hypothesis was that we could establish the specificity of factors influencing phosphorylation and release of membrane bound MARCKS by exogenous PKC isoforms after removal of endogenous cellular PKC, at least after downregulation. As will become evident in the following results, this experimental system has provided evidence for an alternative phorbol ester- and Ca^{2+}-independent mode of regulation of MARCKS phosphorylation and translocation which may operate in addition to the usual βTPA-stimulated

phosphorylation and translocation of MARCKS by cPKC and nPKC isoforms.

C. Results

i. ATP stimulates solubilization of membrane associated MARCKS in permeabilized C6 glioma cells

As noted in the previous section *(Section III)*, approximately 50% of either myristoylated or immunoreactive MARCKS remains associated with the membrane fraction of unstimulated glioma cells following cytosol (c1) depletion by digitonin permeabilization. Further incubation of permeabilized cytosol-depleted cells for 15 min with digitonin-free K-Hepes buffer prior to Triton X-100 extraction resulted in selective release of additional MARCKS from the membrane fraction relative to other [^{3}H]myristoylated proteins (*Figure 14*, c2 vs. m). This enrichment of MARCKS in fraction c2 suggested either weak association with the membrane and/or continuing release of a membrane-bound pool of MARCKS following permeabilization. The former possibility is unlikely since additional MARCKS was not released from the membrane by a second incubation of permeabilized cells with digitonin-free K-Hepes (not shown).

Addition of ATP during subsequent incubation of permeabilized C6 cells with K-Hepes buffer significantly enhanced the release of MARCKS from the membrane fraction: densitometric analysis of experiments such as that shown in *Figure 15A* for immunoreactive MARCKS revealed that ATP increased MARCKS in the c2 fraction by 1.6-2 fold, with a concomitant decrease in the remaining membrane fraction *(Figure 15B)*. Similar results were observed for ^{3}H-labelled MARCKS (not shown). The ATP-

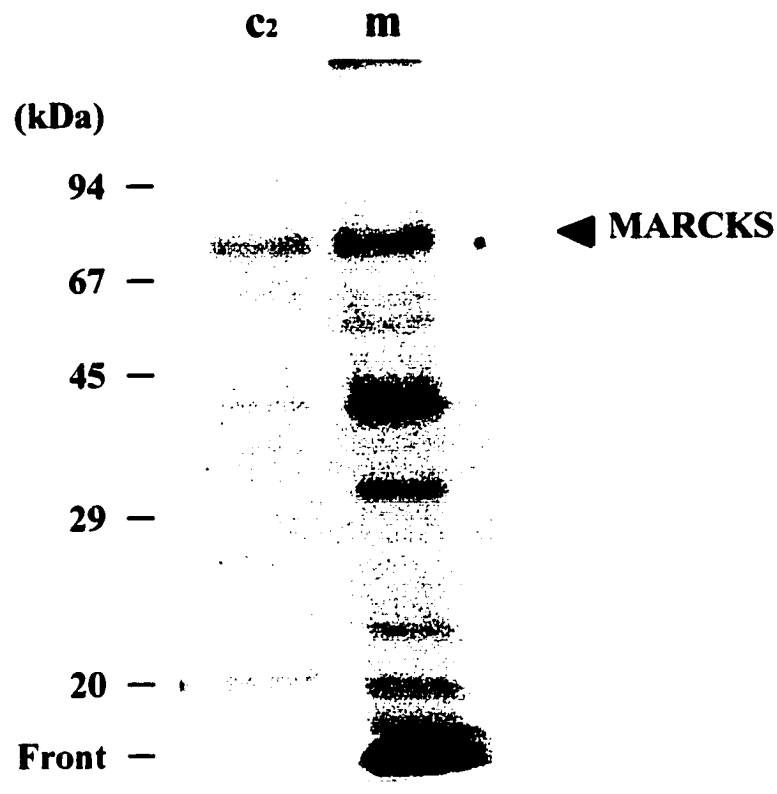
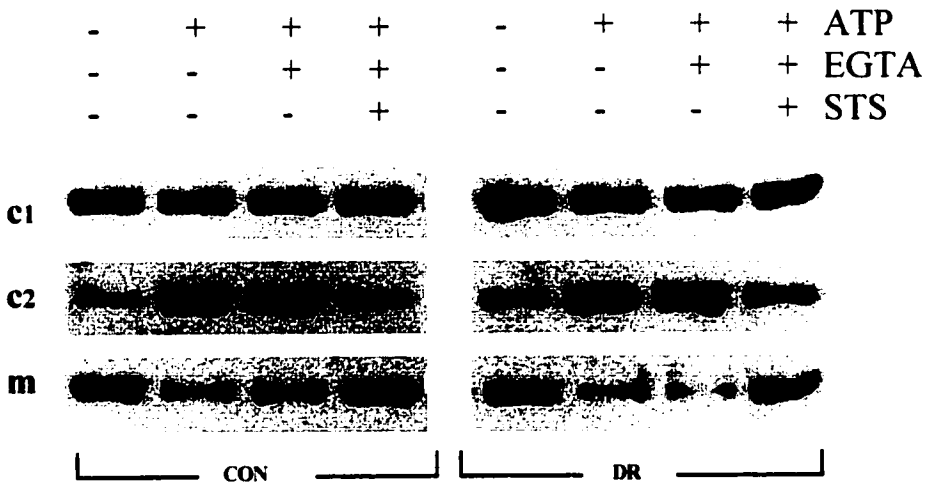


Figure 14. **Specific release of MARCKS relative to other [^{3}H]myristoylated proteins from membranes in permeabilized glioma cells.** Cells labelled for 18 h with 50 μCi/ml [^{3}H]myristate were washed then incubated with 1 ml of K-Hepes buffer containing 120 μg ml^{-1} digitonin for 10 min. The cytosol-depleted cells were then sequentially incubated with 1 ml of digitonin-free K-Hepes buffer containing 1 μM free calcium **(c2)** and 1% Triton X-100 **(m)**. The proteins in each fraction were precipitated with acetone, and equivalent volumes of each fraction (corresponding to 10 and 35 μg protein, respectively, to reflect subcellular distribution) were fractionated by SDS-PAGE and visualized by fluorography. These data were obtained from work done in our lab by Dr. H.-S. Fink (Douglas *et al.*, 1997).

Figure 15. **Effects of staurosporine and EGTA on ATP-stimulated translocation of MARCKS from membranes of control and downregulated C6 glioma cells.** Control **(CON)** and downregulated **(DR)** cells were washed and sequentially extracted with 1 ml of K-Hepes buffer containing: 120 μg ml^{-1} digitonin and 200 nM staurosporine (+/- STS as indicated) **(c1)**, 0.6 mM ATP **(c2)**, and 1% Triton X-100 **(m)**. In some cases, 1 mM EGTA (+/- as indicated) was included in all of the extraction buffers. Proteins from each fraction were separated by 10% SDS-PAGE and subjected to Western blot analysis using anti-MARCKS antiserum and ECL detection as outlined in "Methods" *(panel A)*. Immunoreactive MARCKS was quantified by densitometry and expressed as the amount released into **c2** as a percentage of total in **c2** plus **m** fractions for both control (white bars) and downregulated (black bars) cells *(panel B)*. The data represent the mean ± SEM for three independent experiments.

A


- + + + - + + + ATP
- - + + - - + + EGTA
- - - + - - - + STS
c1
c2
m
CON
DR

B

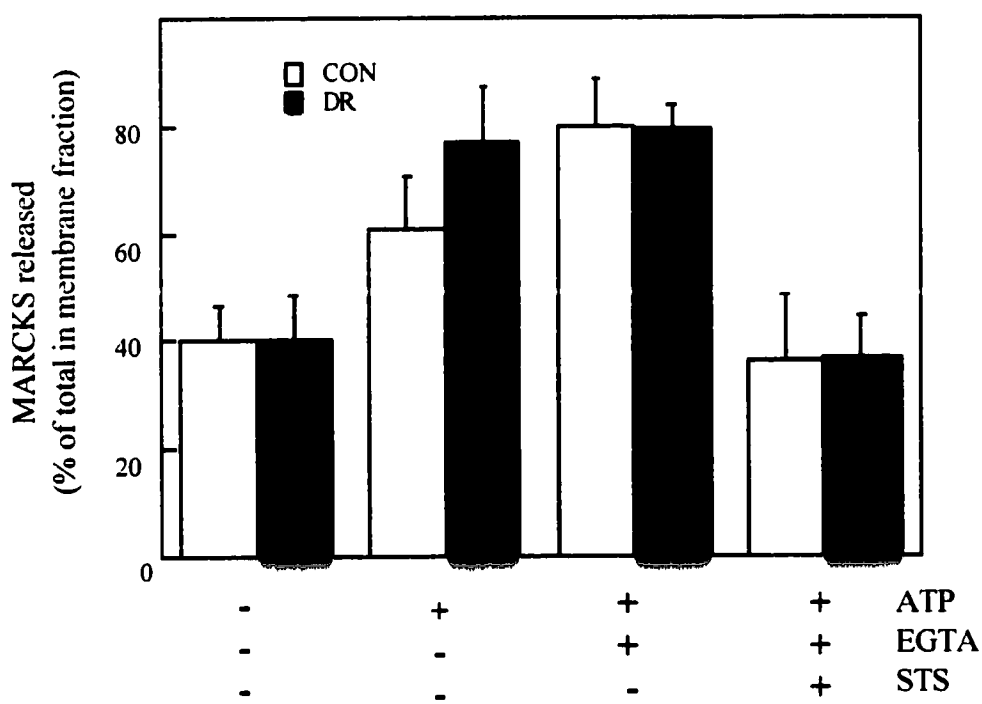

MARCKS released
(% of total in membrane fraction)
CON
DR
80
60
40
20
0
- + + + ATP
- - + + EGTA
- - - + STS

Figure 15

stimulated release of MARCKS from membranes was not affected by buffers containing 1 mM EGTA, indicating that calcium is not required. Inclusion of STS during digitonin permeabilization inhibited the stimulatory effect of ATP on subsequent MARCKS release in c2. The amount of MARCKS in the c1 fraction did not change as a result of any of the pretreatments. As in the previous investigation, MARCKS was not found in the Triton X-100 insoluble fraction.

Surprisingly, ATP-dependent release of MARCKS from cytosol-depleted membrane fractions was not affected when cells were preincubated overnight with 2 μM βTPA (*Figure 15A*, DR), conditions previously shown to downregulate conventional and novel PKC isoforms in C6 cells (Chen, 1993; Chen *et al.*, 1995). These results suggest the presence of a downregulation-resistant kinase involved in the release of MARCKS from C6 membranes.

ii. The long-term effects of βTPA on the expression of PKC isoforms in C6 glioma cells

To ensure complete downregulation of PKCs in membrane fractions after permeabilization and removal of the cytosolic (c1) fraction, expression of cPKCα, nPKCδ, nPKCμ and aPKCζ were analyzed by Western blot analysis *(Figure 16)*. After 18 h exposure to 2 μM βTPA, membrane associated cPKCα and nPKCδ were decreased by 91±4% and 97±1%, respectively (mean ± S.E.M. of three independent experiments); similar results were noted for cytosolic PKC. As noted previously (Chen *et al.*, 1995), nPKCε is barely detectable in C6 cells and was absent altogether in downregulated cells

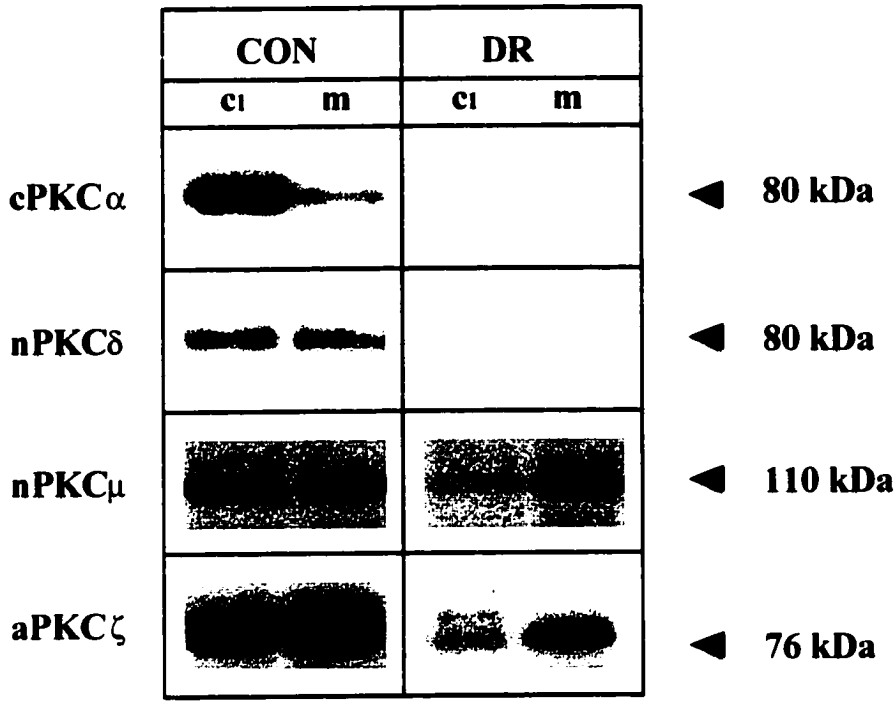


Figure 16. **Downregulation of PKC isoforms in C6 glioma cells in response to βTPA.** Control **(CON)** and downregulated **(DR)** cells were sequentially extracted with 1 ml K-Hepes buffer containing 120 μg ml^{-1} digitonin (**cl**), then with 1% Triton X-100 (**m**). Immunoblots of **cl** and **m** fractions were carried out as described in "Methods" to show the relative levels of cPKCα, nPKCδ, nPKCμ and aPKCζ.

(not shown). The expression of membrane nPKCμ was unaffected by the downregulating conditions while soluble nPKCμ decreased by 89±5% (mean ± range of two independent experiments). By contrast, the expression of cytosolic and membrane aPKCζ was relatively unaffected by the downregulating conditions; the disappearance of an upper band may in fact be due to cross reaction of the anti-PKCζ antibody with cPKCα (Batlle *et al.*, 1994; Allen *et al.*, 1994).

Thus, ATP stimulated release of soluble MARCKS from membranes in permeabilized C6 cells appears to be due to a mechanism which (i) does not involve calcium, (ii) is inhibited by STS, and (iii) remains intact following downregulation of conventional and novel PKC isoforms.

iii. Downregulation abolishes βTPA-stimulated phosphorylation of MARCKS in intact C6 cells

It has been shown previously that phosphorylation of MARCKS can result from the activation of PKC by short-term exposure to βTPA (Wu *et al.*, 1982; Rozengurt *et al.*, 1983; Rodriguez-Pena and Rozengurt, 1985; Blackshear *et al.*, 1985; Blackshear *et al.*, 1986; James and Olson, 1989). *Figure 17* shows the effect of treatment with 200 nM βTPA for 15 min at 37°C on the basal level of MARCKS phosphorylation in control and downregulated C6 cells. Activation of PKCs by βTPA (+/- as indicated) resulted in a 4-fold stimulation of MARCKS phosphorylation seen in the cytosol of control cells (CON, c1). By contrast, 200 nM βTPA had no acute effect on the phosphorylation of

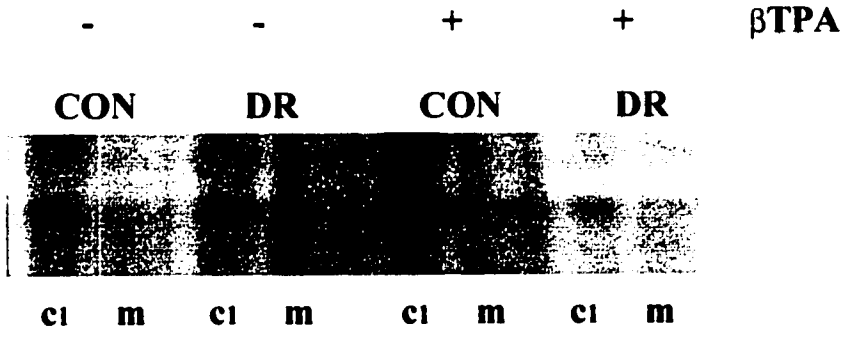


Figure 17. **Effects of downregulation on βTPA-stimulated phosphorylation of MARCKS in C6 glioma cells.** Control **(CON)** and downregulated **(DR)** C6 cells were prelabelled with [^{32}P]P_i for a total period of 3 hours and then exposed to βTPA (200 nM) for 15 min (+/- as indicated). Soluble **(cl)** and membrane **(m)** proteins were obtained by sequential extraction with digitonin followed by Triton X-100. Samples were heated at 80°C for 10 min and heat-stable proteins were precipitated with trichloroacetic acid prior to separation by 10% SDS-PAGE and autoradiography.

MARCKS in downregulated (DR) cells, confirming the downregulation of βTPA-stimulable PKC isoforms. In addition, it is worth noting that basal levels of MARCKS phosphorylation were maintained in PKC downregulated cells implicating a kinase which is not susceptible to downregulation by chronic exposure to βTPA in maintaining the phosphorylation status of MARCKS *in vivo*.

iv. ATP-dependent release of MARCKS from the membrane fraction is accompanied by MARCKS phosphorylation

While several studies have shown that phosphorylation of MARCKS by PKC results in displacement of MARCKS from the membrane, other work has shown that additional factors may regulate the membrane affinity of MARCKS in C6 cells (refer to *Section III.D*). To further explore the possibility of a causal relationship between MARCKS phosphorylation and the ATP-dependent translocation of MARCKS from the membrane fraction, permeabilized cytosol-depleted C6 cells were incubated for up to 25 min with digitonin-free K-Hepes buffer containing [γ-^{32}P]ATP *(Figure 18)*. There was a time dependent increase in the amount of phosphorylated MARCKS released into the c2 fraction of control cells; levels were only slightly lower in cells downregulated by βTPA treatment, indicating that the rate of phosphorylation is similar under both conditions. Under both control and downregulating conditions, less than 10% of the ^{32}P-labelled MARCKS remained associated with the membrane fraction at all time points (not shown), suggesting that the decreased membrane affinity seen here is likely due to

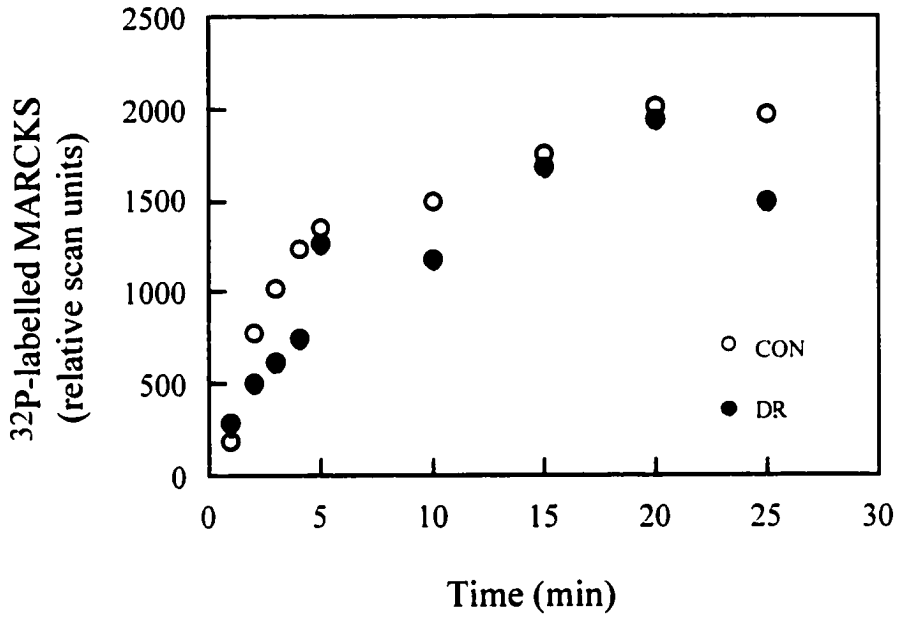


Figure. 18. **Time course of ATP-stimulated MARCKS phosphorylation in permeabilized C6 glioma cells.** Cytosol was removed from control **(CON)** and downregulated **(DR)** cells by extraction with 1 ml of K-Hepes buffer containing 120 μg ml^{-1} digitonin for 10 min. The remaining membrane fraction was then incubated with 1 ml of K-Hepes containing 20 μCi [γ-^{32}P]ATP (0.6 mM) for the times indicated. All buffers contained 1 mM EGTA. The c2 fraction was removed and heat-stable proteins were separated by 10% SDS-PAGE and visualized by autoradiography. ^{32}P-labelled MARCKS in the c2 fraction was quantified by densitometry and the results expressed as the integrated intensity in arbitrary scan units.

increased phosphorylation.

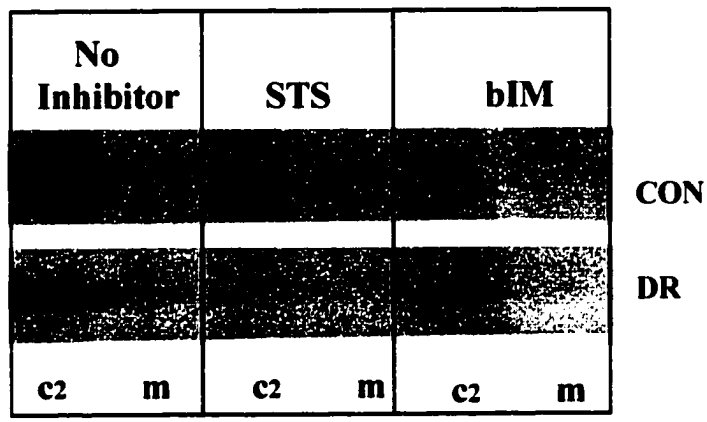
v. Inhibition of ATP-dependent phosphorylation and release of MARCKS from permeabilized cells implicate a PKC-like kinase

Inhibitors were used to explore the nature of the membrane-bound kinase responsible for phosphorylation and translocation of MARCKS and a representative experiment is shown in *Figure 19A.* Both STS (a relatively non-specific inhibitor of PKC) and bIM (a more selective PKC inhibitor) added to the digitonin permeabilization buffer substantially (> 80%) inhibited subsequent phosphorylation of MARCKS in the c2 fraction of both control and downregulated cells *(Figure 19B),* clearly implicating a PKC-like kinase. The concentration of bIM required to achieve half-maximal activity was found to be greater than 100 nM *(Figure 20)* as determined by nonlinear regression. To discriminate among PKC isozymes, the ability of the PKC-selective inhibitor Gö6976 to inhibit the ATP-dependent phosphorylation and release of MARCKS was also evaluated. Unlike STS and bIM, Gö6976 was not an effective inhibitor in downregulated cells *(Figure 21A)* even at concentrations as high as 5 μM). By contrast Gö6976 (100 nM) inhibited phorbol ester-induced phosphorylation of MARCKS *in vivo (Figure 21B).*

As MARCKS is known to be phosphorylated *in vitro* by PKC on serine residues within the central calmodulin-binding domain, we evaluated the ability of various synthetic peptides from different regions of the human MARCKS protein (Harlan *et al.*, 1991) to inhibit MARCKS phosphorylation in membranes from permeabilized cells. A

Figure. 19. **Inhibition of MARCKS phosphorylation in permeabilized C6 cells by staurosporine and bis-indolylmaleimide.** In ***Panel A***, control **(CON)** and downregulated **(DR)** cell cultures were permeabilized with 120 μg ml^{-1} digitonin in 1 ml of K-Hepes buffer containing either no inhibitor, 200 nM staurosporine **(STS)**, or 10 μM *bis*-indolylmaleimide I **(bIM)**, then incubated 15 min with 1 ml of K-Hepes containing 20 μCi [γ-^{32}P]ATP (0.6 mM). This fraction **(c2)** was removed prior to extraction of the remaining membrane fraction **(m)** with 1% Triton X-100. All buffers contained 1 mM EGTA. Heat-stable proteins were separated by SDS-PAGE and visualized by autoradiography. In ***Panel B***, ^{32}P-labelled MARCKS in the c2 fraction was quantified by densitometry and the results for control (white bars) and downregulated (black bars) cells expressed as the integrated intensity relative to control cells that received no inhibitor (arbitrarily set at 1). The data represent the mean ± range of two independent experiments.

A


No Inhibitor
STS
bIM
CON
DR
c2
m
c2
m
c2
m

B

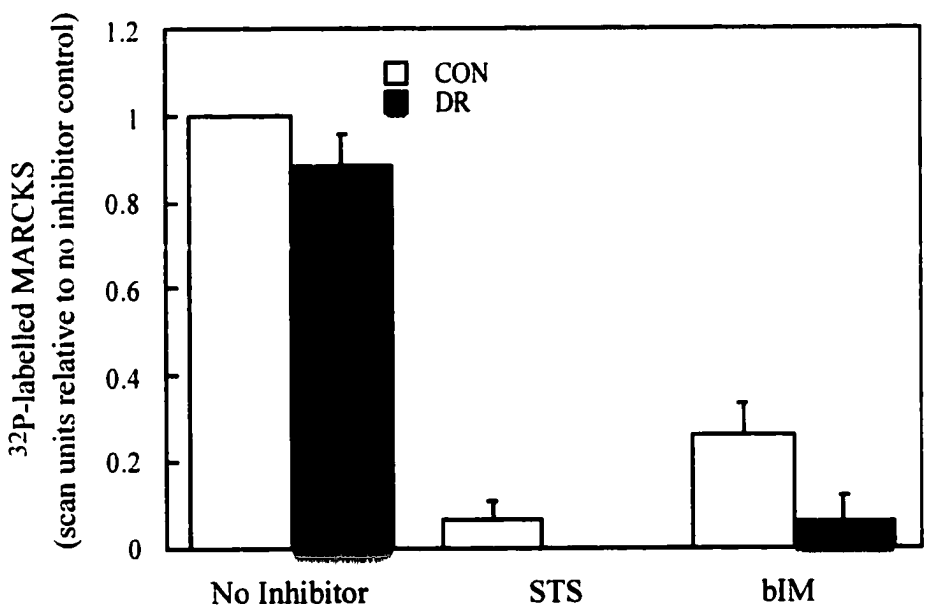

32P-labelled MARCKS
(scan units relative to no inhibitor control)
1.2
1
0.8
0.6
0.4
0.2
0
CON
DR
No Inhibitor
STS
bIM

Figure. 19

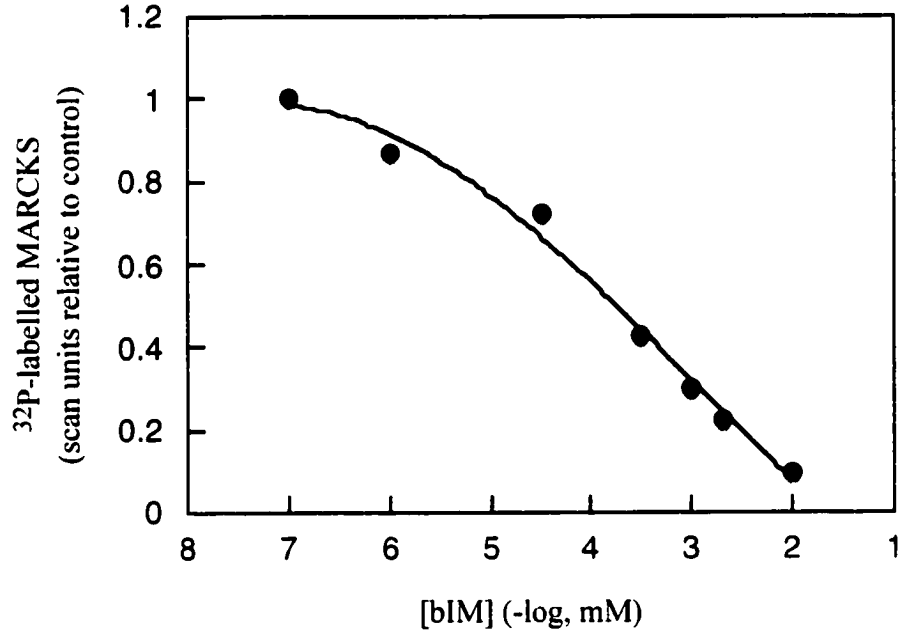


Figure. 20. **Dose-response of the inhibition of MARCKS phosphorylation in permeabilized C6 cells by *bis*-indolylmaleimide I.** Cytosol was removed from downregulated cells by extraction with 1 ml of K-Hepes buffer containing 120 μg ml^{-1} digitonin and the indicated concentrations of bis-indolylmaleimide I (bIM) for 10 min. The remaining membrane fraction was then incubated with 1 ml of K-Hepes containing 20 μCi [γ-^{32}P]ATP (0.6 mM) and the indicated concentrations of bIM for 15 minutes. All buffers contained 1 mM EGTA. The supernatant (c2-fraction) was removed and its heat-stable proteins separated by 10% SDS-PAGE and visualized by autoradiography. ^{32}P-labelled MARCKS in the c2 fraction was quantified by densitometry and the results expressed as the integrated intensity in arbitrary scan units relative to cells that received no inhibitor (set at 1).

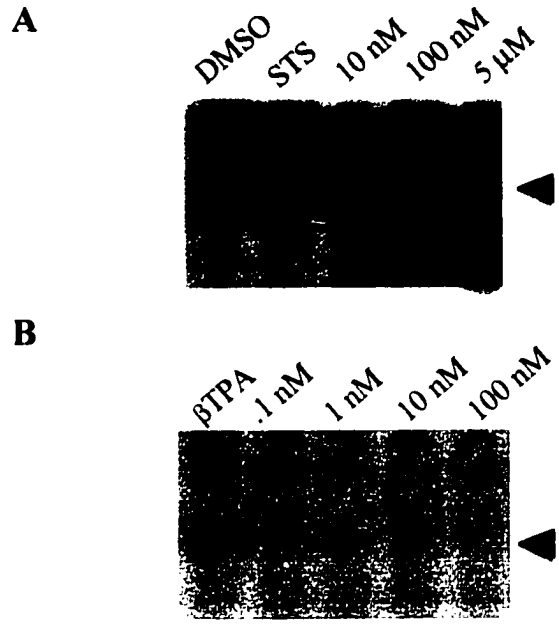


Figure. 21. **The effect of Gö6976 on MARCKS phosphorylation in permeabilized and intact C6 cells.** Cytosol was removed from downregulated cells by extraction with 1 ml of K-Hepes buffer containing 120 μg ml^{-1} digitonin and no inhibitor **(DMSO)**, 200 nM STS **(STS)**, or 10 nM, 100 nM and 5 μM Gö6976 (as indicated). The remaining membrane fraction was then incubated with 1 ml of K-Hepes containing 20 μCi [γ-^{32}P]ATP (0.6 mM) and the indicated concentrations of Gö6976 for 15 min. All buffers contained 1 mM EGTA. The supernatant (c2-fraction) was removed and its heat-stable proteins separated by 10% SDS-PAGE and visualized by autoradiography ***(Panel A)***. For comparison, MARCKS was also labelled in intact control cells preincubated 4 h with 20 μCi/ml [^{32}P]P_i and treated with 200 nM **βTPA** plus 0.1, 1, 10 or 100 nM Gö6976 (as indicated) for 15 min prior to extraction with buffer containing 1% Triton X-100. Heat-stable proteins were separated by 10 % SDS-PAGE and visualized by autoradiography ***(Panel B)***. Bands corresponding to ^{32}P-labelled MARCKS are indicated by arrowheads.

representative experiment for control cells is shown in *Figure 22A* and the results of two separate experiments were quantified by densitometric scanning and are presented in *Figure 22B.* Only the peptide from the calmodulin-binding domain of MARCKS inhibited phosphorylation and translocation of MARCKS in both control and downregulated cells. This peptide contains two serine residues (corresponding to S152 and S156 of rat MARCKS (Erusalimsky *et al.*, 1991) phosphorylated by PKC. In another experiment (not shown), inhibition of MARCKS phosphorylation by the calmodulin-binding domain peptide was also found to be dose-dependent and essentially complete at concentrations as high as 120 μM of peptide.

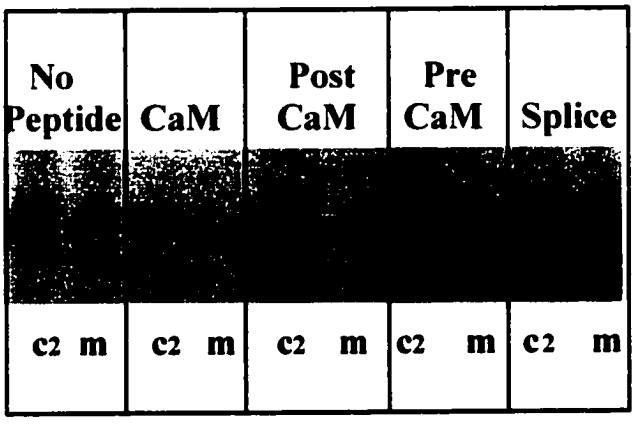
vi. MARCKS phosphorylation occurs at the same sites in intact and permeabilized C6 cells

Phosphoamino acid analysis was carried out with ^{32}P-labelled MARCKS obtained either from permeabilized downregulated cells incubated with [^{32}P]ATP *(Figure 23, lane 1)*, or from [^{32}P]Pi-prelabelled intact cells stimulated with βTPA *(Figure 23, lane 2)*. In both cases, MARCKS was found to be phosphorylated on serine residues exclusively.

Limited proteolytic mapping was used to further characterize MARCKS phosphorylation by the β-TPA-insensitive, Ca^{2+}-independent, membrane-associated kinase. MARCKS was labelled *in vitro* by incubation of membrane fractions from control or downregulated cells with [^{32}P]ATP, then partially digested with *S. aureus* V8 protease

Figure 22. **Inhibition of MARCKS phosphorylation in C6 cell membranes by a calmodulin-binding domain MARCKS peptide.** Cytosol depleted permeabilized membranes from control and downregulated cells were prepared and incubated 15 min with 20 μCi [γ-^{32}P]ATP (0.6 mM) in the presence of 60 μM of each of the following synthetic peptides derived from human MARCKS: **CaM** (KKKKRFSFKKSFKL), residues 153-166 from the calmodulin-binding domain; **Post CaM** (KNKKEAGEGGE), residues 173-183; **Pre CaM** (SPKAEDGATPSPS), residues 135-147; and **Splice** (SSPSKANGQENG), residues 26-37. ***Panel A*** shows an autoradiogram of heat-stable proteins from the resulting **c2** and **m** fractions of control cells separated by SDS-PAGE and visualized by autoradiography. ^{32}P-labelled MARCKS in the c2 fraction was quantified by densitometry and the results for control (**CON**: white bars) and downregulated (**DR**: black bars) cells expressed as the integrated intensity relative to their respective controlled counterparts that received no peptide (arbitrarily set at 1) (***Panel B***). The data represent the mean ± range for two independent experiments.

A


No Peptide
CaM
Post CaM
Pre CaM
Splice
c2 m
c2 m
c2 m
c2 m
c2 m

B

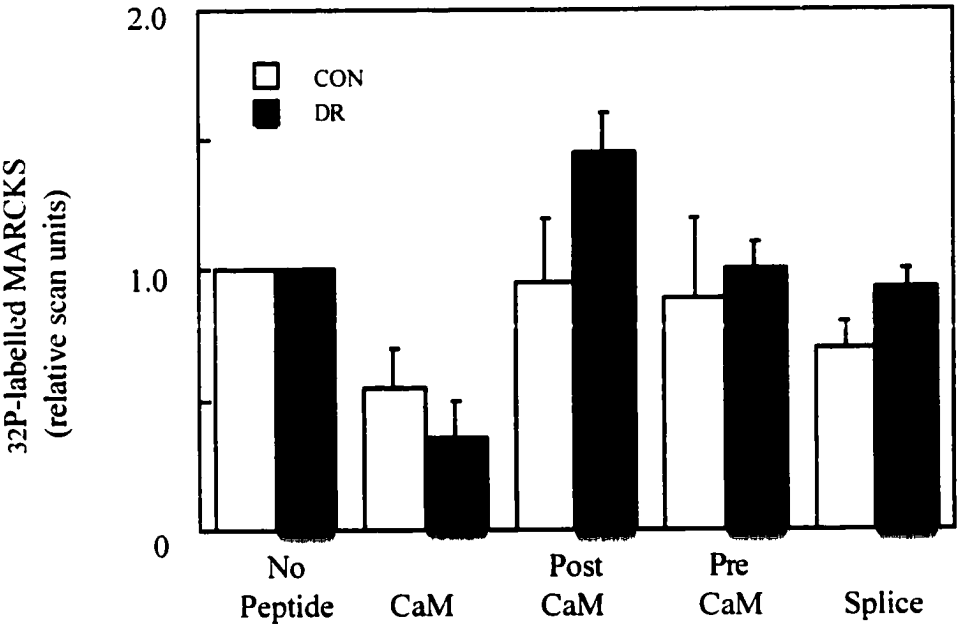

32P-labelled MARCKS (relative scan units)
2.0
1.0
0
CON
DR
No Peptide
CaM
Post CaM
Pre CaM
Splice

Figure 22

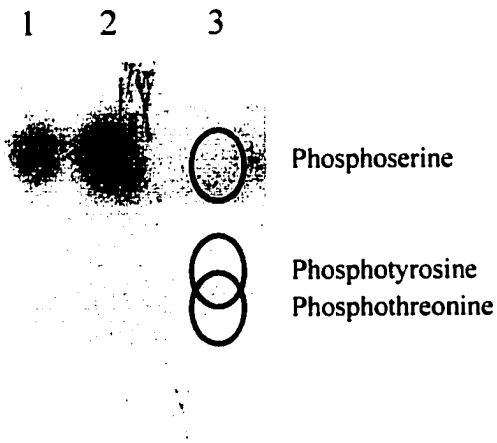


Figure. 23. **Phosphoamino acid analysis of *in vivo* and *in vitro* phosphorylated MARCKS.** ^{32}P-labelled MARCKS was obtained from the membrane fractions of digitonin permeabilized downregulated cells incubated with [γ-^{32}P]ATP to obtain the c2 fraction **(lane 1)**. For comparison, MARCKS was also labelled in intact cells preincubated 4 h with 20 μCi/ml [^{32}P]P_i and treated with 200 nM βTPA for 15 min prior to extraction with buffer containing 1% Triton X-100 **(lane 2).** Heat-stable ^{32}P-labelled proteins were separated by SDS-PAGE and bands corresponding to 80 kDa ^{32}P-labelled MARCKS were recovered from dried gels and subjected to acid hydrolysis as outlined in "Methods". Hydrolyzed amino acids were separated by thin layer electrophoresis and phosphoamino acids observed by autoradiography. Authentic phosphoamino acids were electrophoresed in parallel and their relative migration as revealed by ninhydrin staining is shown in **lane 3**.

and separated on a 15% acrylamide gel *(Figure 24)*. The labelled protein fragments observed (ie. the 13 kDa fragment noted in an earlier study (Yamamoto *et al.*, 1995) were identical to those obtained from MARCKS phosphorylated *in vivo* by βTPA treatment of cells prelabelled with [^{32}P]P_i.

In order to further explore the phosphorylation of MARCKS by the downregulated permeabilized cells, MARCKS was labelled *in vitro* by incubation of membrane fractions of downregulated cells with [^{32}P]ATP, then digested to completion by incubation for 18h with 2 μg TPCK-trypsin and the resulting phosphopeptides were then subjected to 2-dimensional electrophoresis *(Figure 25, panel A)*. The 2-dimensional phosphopeptide pattern was identical to that obtained from MARCKS phosphorylated *in vivo* by βTPA treatment of intact cells prelabelled with [^{32}P]P_i *(Figure 25, panel B)*.

D. Discussion

The increasing number of identified PKC isoforms has led to their classification as cPKC, nPKC or aPKC. The diversity of the PKC family and the fact that multiple PKC isotypes are expressed in a single cell (Makowske *et al.*, 1988; Olivier and Parker, 1994) prompted us to investigate the phosphorylation of the major *in vivo* substrate MARCKS in permeabilized C6 glioma cells. MARCKS is an excellent *in vitro* substrate for cPKC and nPKC isoforms (Fujise *et al.*, 1994; Herget *et al.*, 1995; Sheu *et al.*, 1995; Mahoney *et al.*, 1995) and proline-directed kinases (Yamamoto *et al.*, 1995; Taniguchi *et al.*, 1994). However, at least two studies show that MARCKS is not phosphorylated

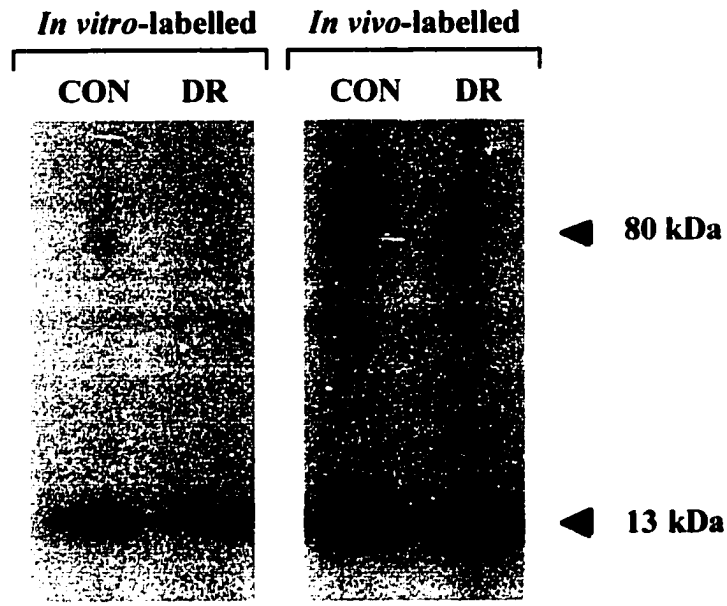


Figure 24. **Comparison of *in vivo* and *in vitro* phosphorylated MARCKS by limited *S. aureus* V8 proteolytic digestion.** ^{32}P-labelled MARCKS was obtained from membranes of permeabilized control **(CON)** or downregulated **(DR)** cells incubated with [γ-^{32}P]ATP to obtain the c2 fraction **(*in vitro* labelled).** For comparison, MARCKS was also labelled in intact cells preincubated 4 h with 20 μCi/ml [^{32}P]P_i and treated with 200 nM βTPA for 15 min prior to extraction with buffer containing 1% Triton X-100 **(*in vivo* labelled)**. *In vitro*-labelled MARCKS from heat-stable c2 fractions and *in vivo*-labelled MARCKS from total cell lysates which had been heat-treated were visualized on dried gels by autoradiography, excised and rehydrated as described in "Methods". The gel pieces were then mounted onto a 15% acrylamide gel in the presence of of *Staphylococcus aureus* V8 protease in sample loading buffer and electrophoresed. The gels were dried and phosphopeptides visualized by autoradiography (results shown are representative of five independent experiments).

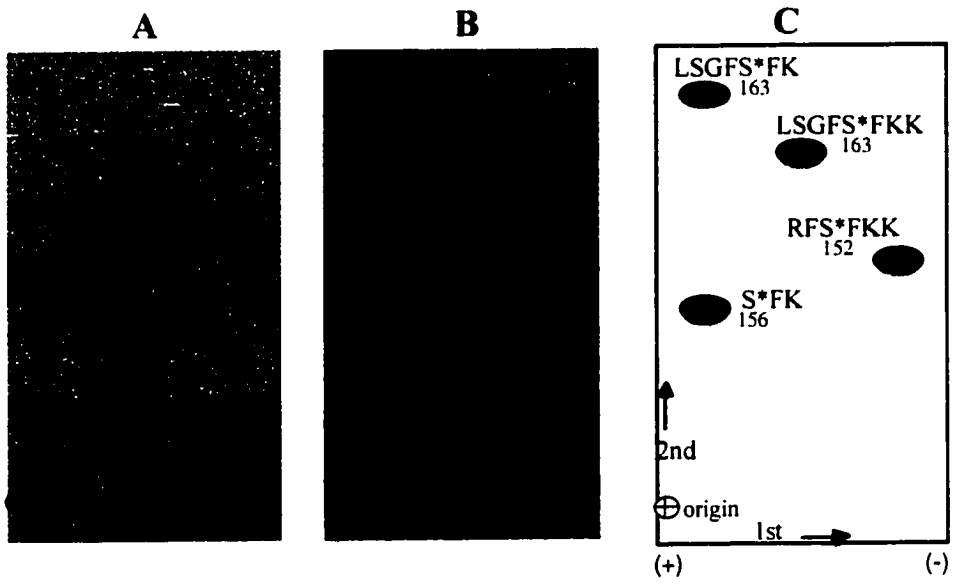


Figure 25. **Two dimensional tryptic-phosphopeptide map of ^{32}P-labelled MARCKS from downregulated permeabilized C6 cells.** MARCKS was phosphorylated *in vitro* by incubation of downregulated permeabilized cells with [γ-^{32}P]ATP to obtain the c2 fraction ***(Panel A)***. For comparison, MARCKS was also labelled in intact cells preincubated 4 h with 20 μCi/ml [^{32}P]P*i* and treated with 200 nM βTPA for 15 min prior to extraction with buffer containing 1% Triton X-100 ***(Panel B)***. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose; the phosphorylated MARCKS band was digested *in situ* on the nitrocellulose by TPCK-trypsin. Tryptic peptides were resolved by thin-layer electrophoresis at pH 3.5 in the first dimension and by thin-layer chromatography in the second dimension as described in "Methods" and phosphopeptides were observed by autoradiography. ***Panel C*** shows the phosphorylated serines **(S*)** as determined by sequencing (Herget *et al.*, 1995).

by PKCζ, although the effector domain peptide is a good substrate for this atypical PKC isoform (Fujise *et al.*, 1994; Herget *et al.*, 1995; Sheu *et al.*, 1995; Mahoney *et al.*, 1995). The permeabilized C6 glioma cell system used in the studies presented here and in Section III led to the unexpected discovery that MARCKS is released in an ATP-stimulated manner after permeabilization and cytosol depletion, and that the membrane-bound kinase responsible is independent of Ca^{2+} and neither stimulated nor downregulated by phorbol ester.

Several pieces of information implicate a PKC-like kinase in the phosphorylation and release of MARCKS from C6 cell membranes. First, phosphorylation and release of MARCKS are inhibited by the PKC inhibitors STS and bIM. Moreover, specific inhibition by a peptide based on the effector domain of MARCKS and containing two serine residues known to be phosphorylated by PKC (Fujise *et al.*, 1994; Herget *et al.*, 1995), but not by other serine-containing MARCKS peptides, was observed. Furthermore, phosphoamino acid analysis and phosphopeptide mapping approaches have confirmed that phosphorylation occurs on only serine residues and at the same sites those phosphorylated by βTPA-stimulated PKC *in vivo*. This rules out phosphorylation by proline-directed kinases such as cdc2, which phosphorylate MARCKS (on both serine and threonine residues) at different sites (Yamamoto *et al.*, 1995).

As in previous studies (Chen, 1993; Chen *et al.*, 1995; Sproull *et al.*, 1995) we have found that C6 glioma cells are heterogeneous with respect to PKC isoforms. This cell line expresses cPKCα, nPKCδ, and aPKCζ. In addition, C6 glioma cells also express

the relatively newly identified novel PKC isoform, nPKCμ. Overnight pretreatment of cells with 2 μM βTPA did not have an appreciable effect on the extent, rate or sites of MARCKS phosphorylation. Downregulating conditions were shown previously (Chen *et al.*, 1995) and in this study *(Figure 16)* to decrease cPKCα and nPKCδ (but not aPKCζ or nPKCμ) isoforms in the membrane by >90%. Downregulation of PKCμ was apparent after even acute exposure to phorbol ester, but this downregulation was only observed as the depletion of cytosolic PKCμ while the membrane PKCμ remained virtually unchanged. By contrast, Johannes *et al.* (1995) have observed a late optimum and persisting activation of PKCμ by phorbol ester treatment of cells but complete resistance of PKCμ to βTPA-induced proteolytic degradation and downregulation of kinase activity. These downregulating conditions did not have an appreciable effect on the cellular levels of aPKCζ. That aPKCζ is resistant to phorbol ester-induced activation and downregulation has been demonstrated previously (Liyanage *et al.*, 1992; McGlynn *et al.*, 1992; Nakanishi and Exton, 1992) and has been attributed to its lack of a functional C1 domain.

Among the candidates for membrane MARCKS phosphorylation and release observed in this study, aPKCζ cannot be ruled out as it is clearly resistant to downregulation by βTPA. MARCKS has been shown to be a poor substrate for aPKCζ but a synthetic MARCKS-peptide of 25 amino acids, which resembles the PKC phosphorylation sites of MARCKS was as good a substrate for aPKCζ as it was for other cPKC and nPKC isoforms (Herget *et al.*, 1995). It is possible that fully activated

PKCζ can phosphorylate MARCKS under appropriate activation conditions and permeabilized glioma cells may provide a more suitable environment for MARCKS kinase activity than assays using recombinant PKCζ and purified MARCKS. Another possible candidate is nPKCμ, which has recently been shown to phosphorylate MARCKS and its effector domain peptide (Dieterich *et al.*, 1996). Within that study, nPKCμ was shown to phosphorylate the MARCKS peptide at only one out of four potential PKC phosphorylation sites (Dieterich *et al.*, 1996). This differs significantly from the 2-dimensional tryptic phosphopeptide pattern obtained in this investigation which is reminiscent of the MARCKS peptide phosphorylation pattern obtained with other PKC subtypes where serines 152, 156 and 163 were all phosphorylated (Herget *et al.*, 1995).

To further distinguish between aPKCζ and nPKCμ, various inhibitors known to suppress PKC activity were tested for their capacity to inhibit the phosphorylation and translocation of MARCKS by the kinase in downregulated C6 membranes. The indolocarbazole Gö6976 is a more specific inhibitor of PKC than staurosporine (Gschwendt *et al.*, 1995) and displays isoform selectivity (Zang *et al.*, 1994; Gschwendt *et al.*, 1995; Martiny-Baron *et al.*, 1993). The order of selective inhibition by Gö6976 in terms of potency is cPKC (IC50s in nM range) > nPKCs and aPKCs (IC50s in μM range). There is an exception with nPKCμ, which is equally well inhibited by Gö6976 as cPKCs (IC50 of 20 nM) (Gschwendt *et al.*, 1996). By contrast, Gö6976 has been shown to be an ineffective inhibitor of aPKCζ. The observation that Gö6976 was ineffective in inhibiting the phosphorylation of MARCKS by the kinase in downregulated C6 membranes supports a role for membrane-bound aPKCζ. However, the finding that bIM

was an effective inhibitor in the nM range is inconsistant with the involvement of aPKCζ (IC_{50} of 5 μM) (Gschwendt *et al.*, 1996).

Further implication for a role for aPKCζ comes from the observation that phosphorylation and solubilization of MARCKS (i) was not dependent on the presence of Ca^{2+}, which would be required for cPKC isoforms; and (ii) could not be stimulated by phorbol ester, which would be required for both cPKC and nPKC isoforms (including nPKCμ).

For successful *in vivo* phosphorylation the activated PKC isotype has to possess not only the ability to phosphorylate MARCKS, but both kinase and MARCKS have to be co-localized within the cell. It is probable that phosphorylation of MARCKS which induces its translocation to the cytosol (Herget and Rozengurt, 1994b) is performed by membrane-bound PKC isoforms (Fujise *et al.*, 1994; Herget *et al.*, 1995; Sheu *et al.*, 1995; Mahoney *et al.*, 1995). aPKCζ, at least *in vitro*, exhibits a constitutive protein kinase activity (Liyanage *et al.*, 1992; McGlynn *et al.*, 1992; Nakanishi and Exton, 1992) and it is tempting to speculate that the lack of a functional C1 region with respect to DAG or phorbol ester binding obviates the need for activation. However, a physiological regulator of this kinase remains to be elucidated.

To our knowledge, this is the first study to show that kinases other than typical cPKC and nPKC isoforms can influence the membrane association of MARCKS. A cycle of MARCKS phosphorylation-dephosphorylation appears to be essential to its function: directed mutagenesis and PKC inhibitors disrupt cycling of MARCKS between the plasma membrane and lysosomes in fibroblasts, presumably related to membrane-

cytoskeletal alterations involved in endosomal transport (Allen and Aderem, 1995b). Rapid stimulation of MARCKS phosphorylation upon agonist-induced DAG formation and PKC activation suggests that MARCKS is poised to dynamically respond to extracellular signals leading to membrane cytoskeletal rearrangement (Aderem, 1995). The observations made from this investigation could be related to a necessity for a low (perhaps constitutive?) level of MARCKS cycling to maintain membrane plasticity in the absence of overt PKC activation. Alternatively, they could reveal the presence of a parallel signalling pathway that converges at MARCKS.

V. Mutational analysis of serines 152 and 156 of rat MARCKS demonstrate specific roles in targeting MARCKS to distinct cellular locations

A. Introduction

The effector domain of MARCKS represents an extremely basic motif within a highly acidic molecule. In the rat protein for example, the effector domain contains a cluster of 13 basic and no acidic residues between amino acids 145 and 169. As discussed previously, phosphorylation by PKC within this domain is thought to weaken its electrostatic interaction with acidic phospholipids. However, there are conflicting results regarding the binding specificity toward specific phospholipids. Kim *et al.* (1994b) showed that phosphatidylserine (PS), phosphatidylglycerol (PG), and phosphatidylinositol (PI) were effective; whereas Nakaoka *et al.* (1993) demonstrated that MARCKS is a specific PS-binding protein in the absence of Ca^{2+}. Binding of MARCKS to PS was shown to inhibit the PKM-catalyzed phosphorylation of this protein (Taniguchi and Manenti, 1993a). On the other hand, Seki *et al.* (1996) demonstrated that although interaction of MARCKS with acidic phospholipids lacks specificity when determined by binding assays, the phospholipids exert distinct effects on the phosphorylation of MARCKS by PKC. Preincubation of MARCKS with PS or PG enhanced phosphorylation; whereas PA, PI, PIP, or PIP_2 inhibited phosphorylation of this substrate.

In addition to the myristoyl-electrostatic switch model for the interaction of

MARCKS with membranes, there has been the suggestion that the binding of MARCKS to biological membranes may involve protein-protein interactions. For example, in macrophages MARCKS has a punctate distribution resembling focal contacts, the transient adhesion complexes that form during locomotion (Rosen *et al.*, 1990). During phagocytosis in macrophages, MARCKS colocalizes with F-actin, PKCα, talin and myosin I under the forming phagosome (Allen and Aderem, 1995). Furthermore, in fibroblasts, MARCKS is translocated from the plasma membrane to lysosomes upon phosphorylation (Allen and Aderem, 1995), suggesting that MARCKS has recognition elements for both types of membranes. Thus, the rather non-specific hydrophobic and electrostatic interactions of MARCKS with membranes might increase effective concentrations of MARCKS at the membrane and facilitate association with other proteins at the membrane.

Another factor that might influence the targetting of MARCKS to selected membranes are the specific serines within the effector domain. Site analysis revealed that only three of the five potential serine residues (S152, S156 and S163) within this domain are actually phosphorylated (Amess *et al.*, 1992; Heemskerk *et al.*, 1993; Brooks *et al.*, 1991b) while S160 and S167 are not (Brooks *et al.*, 1991b). Moreover, the extent of phosphorylation was found to be S163>S156>S152 regardless of the PKC isoform used (Brooks *et al.*, 1991b). In a separate study, Herget and coworkers (1995) were able to demonstrate through kinetic analysis that the three serine residues were sequentially phosphorylated in the order S156>S163>S152 rather than being simultaneously phosphorylated. At least one study has shown that not all three phosphorylation events are required to decrease membrane affinity. Through the use of

various deletion mutations of rat MARCKS fused to glutathione S-transferase, the minimum PS-binding region of MARCKS and the regulatory phosphorylation sites were determined (Nakaoka *et al.*, 1995). The results of that study strongly suggested that rat MARCKS binds PS molecules through residues 127-156, with residues 152-156 (SFKKS) being particularly important. Replacement of serine at both residues S156 and S163 with alanine did not affect regulation of the binding of MARCKS to PS through PKC-catalyzed phosphorylation, but substitution of S152 with alanine clearly affected this regulation. Therefore, phosphorylation of serine at residue 152 appears to be sufficient for affecting the binding to PS.

B. Statement of Hypothesis

Given that phosphorylation of MARCKS by PKC appears to regulate the cycling of MARCKS among multiple membrane compartments as well as between the plasma membrane and the cytosol, one hypothesis is that the phosphorylation of individual serine residues might serve to target MARCKS to various cellular locations. To examine this, site-directed mutants of rat MARCKS in which serines known to be phosphorylated by PKC were replaced with alanines were generated and stably expressed in CHO-K1 cells to examine the cellular localization of MARCKS and its response to activated PKC.

C. Results

i. Site-Directed mutagenesis and Stable Transfection in CHO-K1 cells.

Through deletion mutant analysis, the minimum acidic binding region of rat MARCKS is known to be confined within residues 124-156 (Nakaoka *et al.*, 1995). Since phosphorylation of MARCKS within this region is also known to decrease its affinity with membranes, mutagenesis of serines 152 and 156 to alanines was done to explore the possible role of these residues in targeting and PKC-dependent modulation of MARCKS dissociation from membranes *in vivo*. The cDNA encoding wild-type rat MARCKS (Wt) was used as a template to generate the serine to alanine substitutions at residues 152 and 156 and the mutations were confirmed by dideoxy sequencing *(Figure 26).*

Transfection of CHO-K1 cells with rat MARCKS cDNA (Wt) and the S152A and S156A mutant cDNAs led to the production of a series of cell lines stably expressing various levels of MARCKS as determined by Western blot analysis *(Figure 27, lanes 2-11,).* No immunoreactive MARCKS was detected in mock transfected cells *(lane 1)* and labelled MARCKS was not observed in [^{3}H]myristate-labelled CHO-K1 cells (not shown). For further experiments, cell lines expressing roughly equal levels of MARCKS were used *(lanes 2, 5, and 9 for Wt, S152A and S156A expressors, respectively).*

Figure 26. **Dideoxy sequencing of rat MARCKS cDNA.** Wild-type rat MARCKS cDNA (Wt) was used as a template to generate two separate cDNAs encoding serine to alanine mutations at residues 152 and 156 (S152A and S156A, respectively). Sequencing was performed on the non-coding strand and the A to C substitutions resulting in serine to alanine mutations are indicated by the arrowheads.

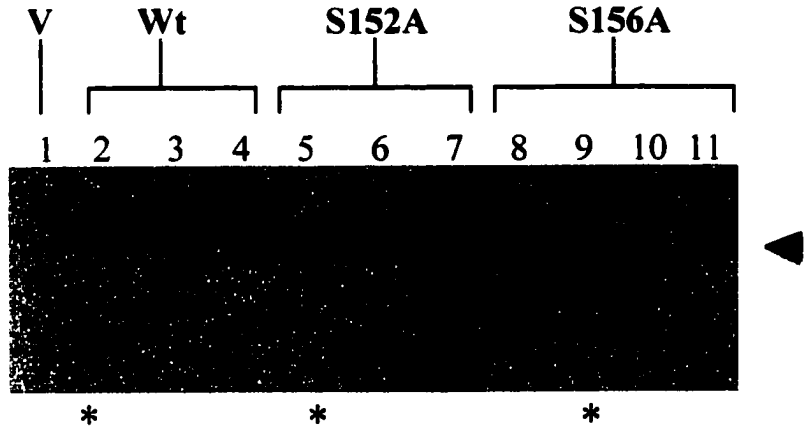


Figure 27. **Expression of MARCKS in CHO-K1 clones.** Multiple CHO-K1 clones stably transfected with vector (V, *lane 1*) or vector containing wild-type rat MARCKS (Wt, *lanes 2-4*), MARCKS with serine residue 152 to alanine mutation (S152A, *lanes 5-7*), or MARCKS with serine residue 156 to alanine mutation (S156A, *lanes 8-11*) were evaluated by Western blot analysis for MARCKS expression. Expression of 80 kDa MARCKS is indicated by the arrowhead. Clones used for primary studies herein are indicated by an asterix (*).

ii. Phosphorylation of Wt and S152A (but not S156A) MARCKS is increased by treatment of intact cells with phorbol ester.

To study the interaction of MARCKS with membranes in each of the cell lines, a permeabilization procedure similar to that used for C6 glioma cells was established. Conditions required to selectively extract cytosolic and membrane-bound proteins were optimized in CHO-K1 cells. Incubation with digitonin *(Figure 28)* resulted in time- and concentration-dependent release of lactate dehydrogenase (a cytosolic marker) into the medium. Incubation of CHO-K1 cells with 10 μg ml^{-1} digitonin for 10 min released approximately 80% of total lactate dehydrogenase activity and was chosen for further studies.

Before examining the effects of phorbol ester on the phosphorylation and cellular localization of MARCKS in the stably transfected cell lines, it was necessary to determine which PKC isoforms were present in CHO-K1 cells. Thorough screening of CHO-K1 cells for PKC isoforms (α, βI, βII, δ, γ, ζ, μ, ε, η and θ) by Western blot analysis revealed that this cell line expresses cPKCα, nPKCμ, and aPKCζ *(Figure 29)*. Treatment with 200 nM βTPA for 15 min resulted in considerable loss of PKCα and PKCμ from the cytosolic fraction, indicating activation. aPKCζ was unaffected by phorbol ester treatment. Of these isoforms, both aPKCα and nPKCμ have been shown to be phorbol ester-responsive and capable of phosphorylating MARCKS *in vitro*.

To determine whether or not phorbol ester could induce phosphorylation of MARCKS in the stably transfected cell lines, MARCKS was labelled *in vivo* with ^{32}Pi and partially purified by heat treatment prior to analysis by SDS-PAGE and autoradiography *(Figure 30)*. As in previous investigations, MARCKS was identified

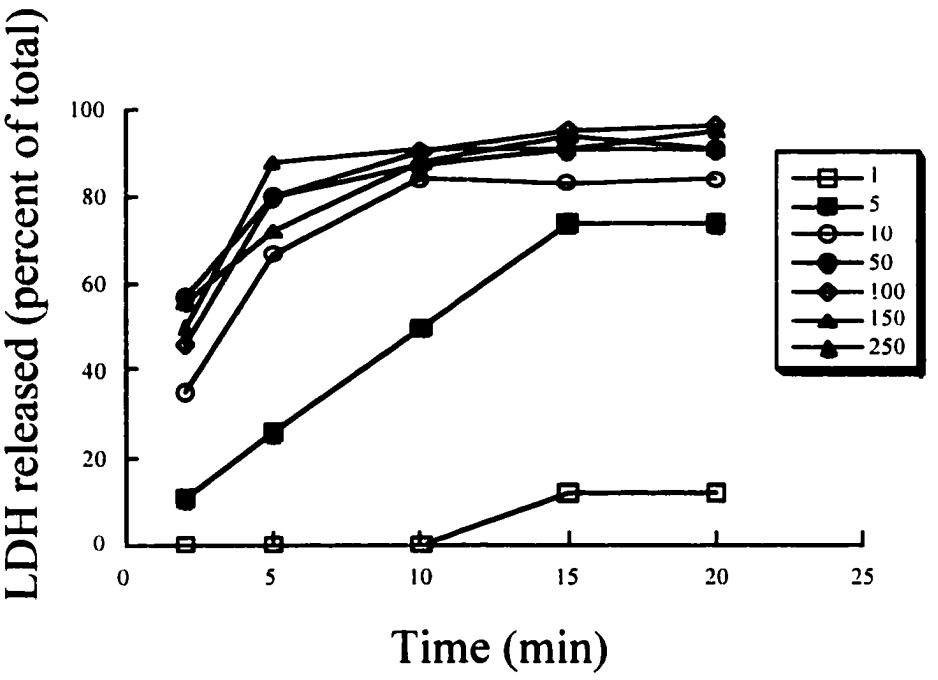


Figure 28. **Release of lactate dehydrogenase from CHO-K1 cells by digitonin permeabilization.** CHO-K1 cells grown for 3 days in 35 mm dishes were washed with Na-Hepes buffer (see Methods) and incubated at 25°C with the indicated concentrations of digitonin (μg ml^{-1}) in 1 ml K-Hepes buffer for the times shown. Dishes were then incubated with K-Hepes buffer containing Triton X-100 and lactate dehydrogenase activity was measured in both fractions; total activity was constant in all samples. Data are expressed as percent of total activity released by digitonin.

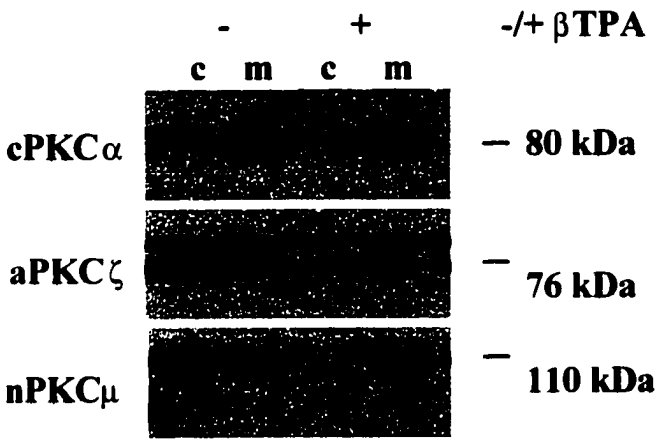


Figure 29. **Distribution of PKC isoforms in CHO-K1 cells in response to βTPA.** Non-transfected CHO-K1 cells were pretreated with βTPA and then sequentially extracted with 1 ml K-Hepes buffer containing 10 μg ml^{-1} digitonin (**c**) or 1% Triton X-100 (**m**). Equal protein from each fraction was separated by 10% SDS-PAGE and immunoblots were performed as described in the "Methods" to show the relative levels of cPKCα, nPKCμ and aPKCζ.

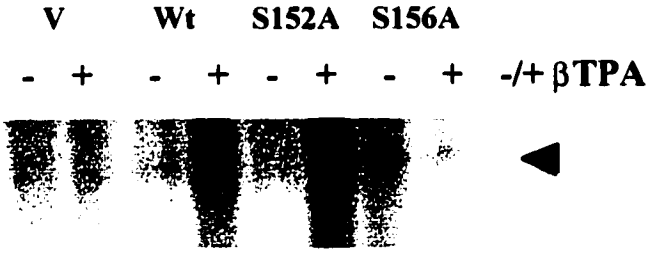


Figure 30. **Effects of βTPA on phosphorylation of MARCKS in CHO-K1 stable transfectants.** Cells were prelabelled with [^{32}P]P_i for a total period of 3 hours and then exposed to βTPA (200 nM) for 15 min (+/- as indicated). Proteins were obtained by extraction with Triton X-100. Samples containing equivalent amounts (30 μg) of protein were heated at 80°C for 10 min and heat-stable proteins were precipitated with trichloroacetic acid prior to separation by 10% SDS-PAGE and autoradiography. The migration of ^{32}P-labelled MARCKS is indicated by the arrowhead.

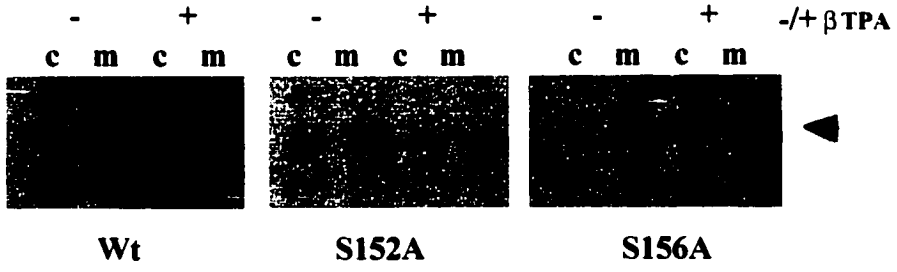
as the 80 kDa phosphorylated band on the basis of its selective heat stability. Although levels of MARCKS phosphorylation were less than seen with C6 cells and difficult to quantify, βTPA treatment stimulated phosphorylation of Wt and S152A MARCKS but had no affect on the basal phosphorylation of S156A MARCKS.

Surprisingly, the increased phosphorylation seen for Wt and S152A MARCKS was not accompanied by increased solubilization with digitonin (a representative experiment is shown in *Figure 31A*). Under basal conditions, 62 ± 9% of Wt, 67 ± 6% S152A and 97 ± 6% S156A MARCKS remained associated with the membrane fraction after digitonin permeabilization *(Figure 31B)*. In all cases, the membrane association of MARCKS was not significantly affected by phorbol ester treatment.

iii. Wt and S152A MARCKS but not S156A exhibit altered membrane localization in response to phorbol ester.

In some cases, MARCKS has been shown to interact with multiple membranous compartments within the cell. Confocal immunolocalization microscopy was used to further explore the cellular localization of MARCKS in the CHO-K1 stable transfectants *(Figure 32)* under basal conditions *(panels A, C, and E)* and following the stimulation of intact cells with phorbol ester *(panels B, D, and F)*. Under basal conditions, Wt MARCKS appeared to be predominantly associated with the plasma membrane as seen by the uniform staining around the periphery of the cells. Only slight diffuse staining could be seen in the cytoplasm of these cells *(Figure 32A)*. Acute exposure of intact cells to phorbol ester resulted in the redistribution of Wt MARCKS from the plasma membrane to punctate structures within the cytoplasm *(Figure 32B)*.

A



B

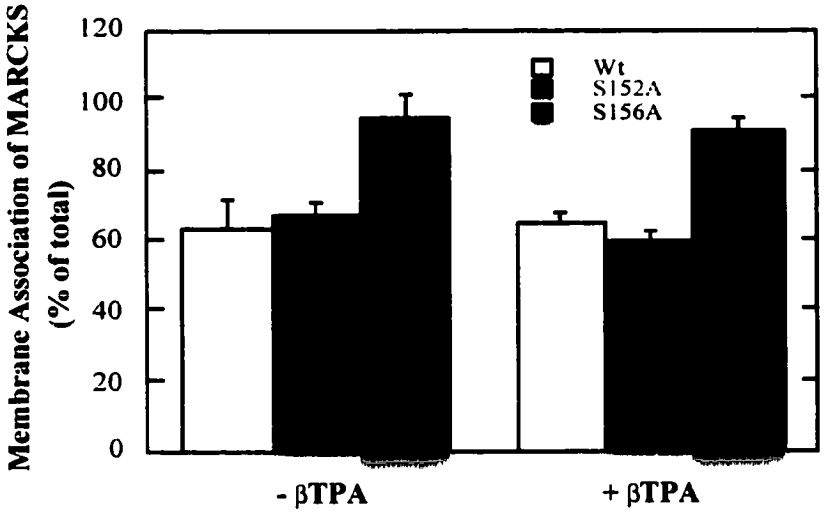
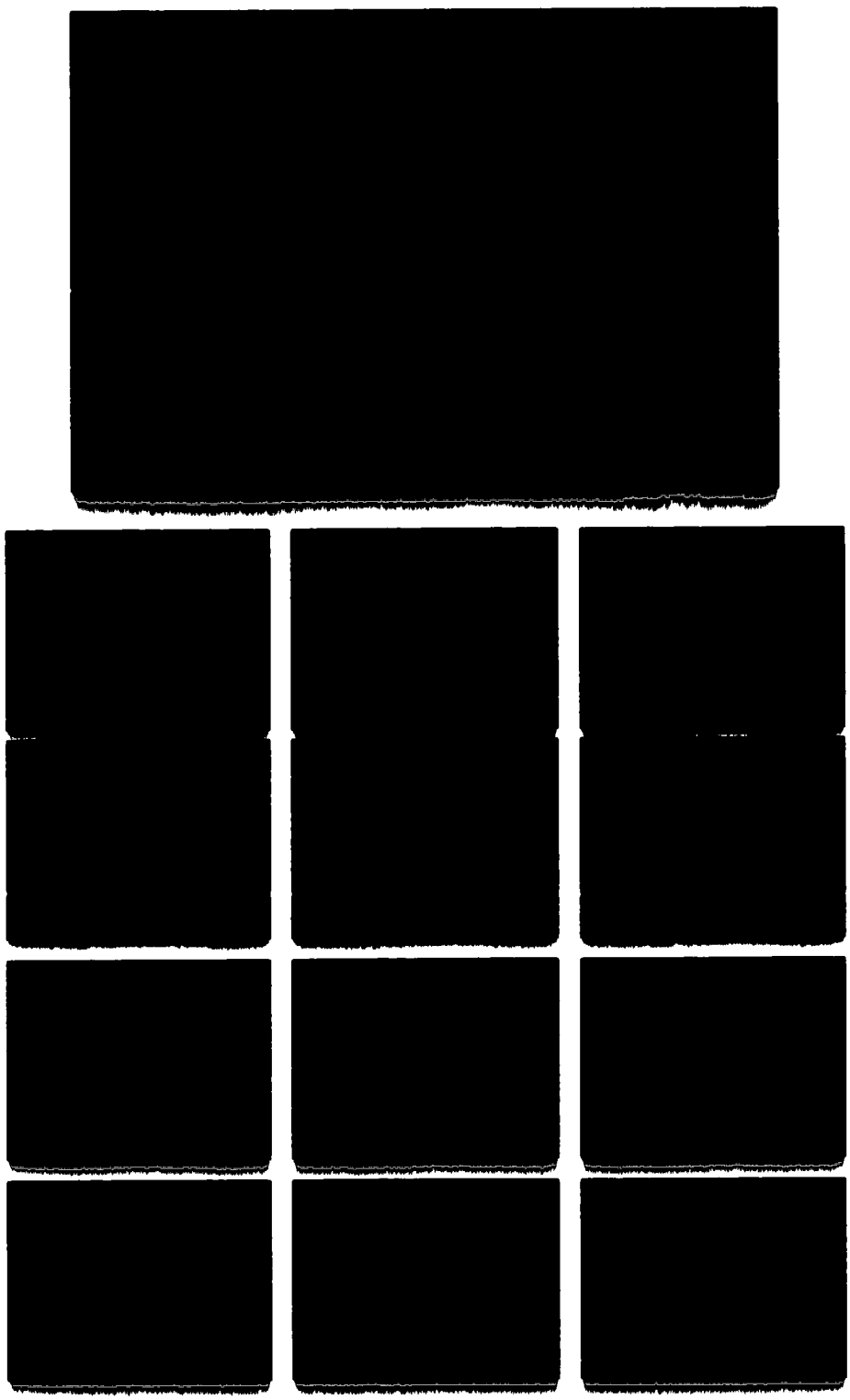


Figure 31. **Distribution of MARCKS in CHO-K1 stable transfectants in response to βTPA.** CHO-K1 cells stably overexpressing Wt, S152A, and S156A MARCKS were pretreated (15 min) with βTPA and then sequentially extracted with 1 ml K-Hepes buffer containing 10 μg ml^{-1} digitonin (**c**), and 1% Triton X-100 (**m**). Samples containing 10 μg protein from each fraction were separated by 10% SDS-PAGE and immunoblots were carried out as described in the "Methods" to show the relative levels of MARCKS *(Panel A)*. Immunoreactive MARCKS was quantified by densitometry and expressed as the amount in the membrane fraction as a percentage of total *(Panel B)*. The data represent the mean ± SEM for three independent experiments.

Figure 32. **Fluorescence immunolocalization and confocal microscopy of MARCKS in CHO-K1 stable transfectants.** CHO-K1 cells stably expressing Wt MARCKS **(A and B)**, S152A MARCKS **(C and D)** and S156A MARCKS **(E and F)** were cultured in CHO-K1 medium without G418 for 48 h prior to addition of 200 nM βTPA **(B, D and F)**. Control cells received DMSO **(A, C and E)**. After treatment with βTPA for 15 min, cells were fixed and MARCKS was immunolocalized by confocal microscopy as outlined in "Methods". *Bar* 10 μm. *Color panels* represent the three-dimensional image of all focal planes stacked in series. *Black and white panels* represent horizontal confocal planes 1 μm thick beginning with the substrate adherent surface of the cells.

A



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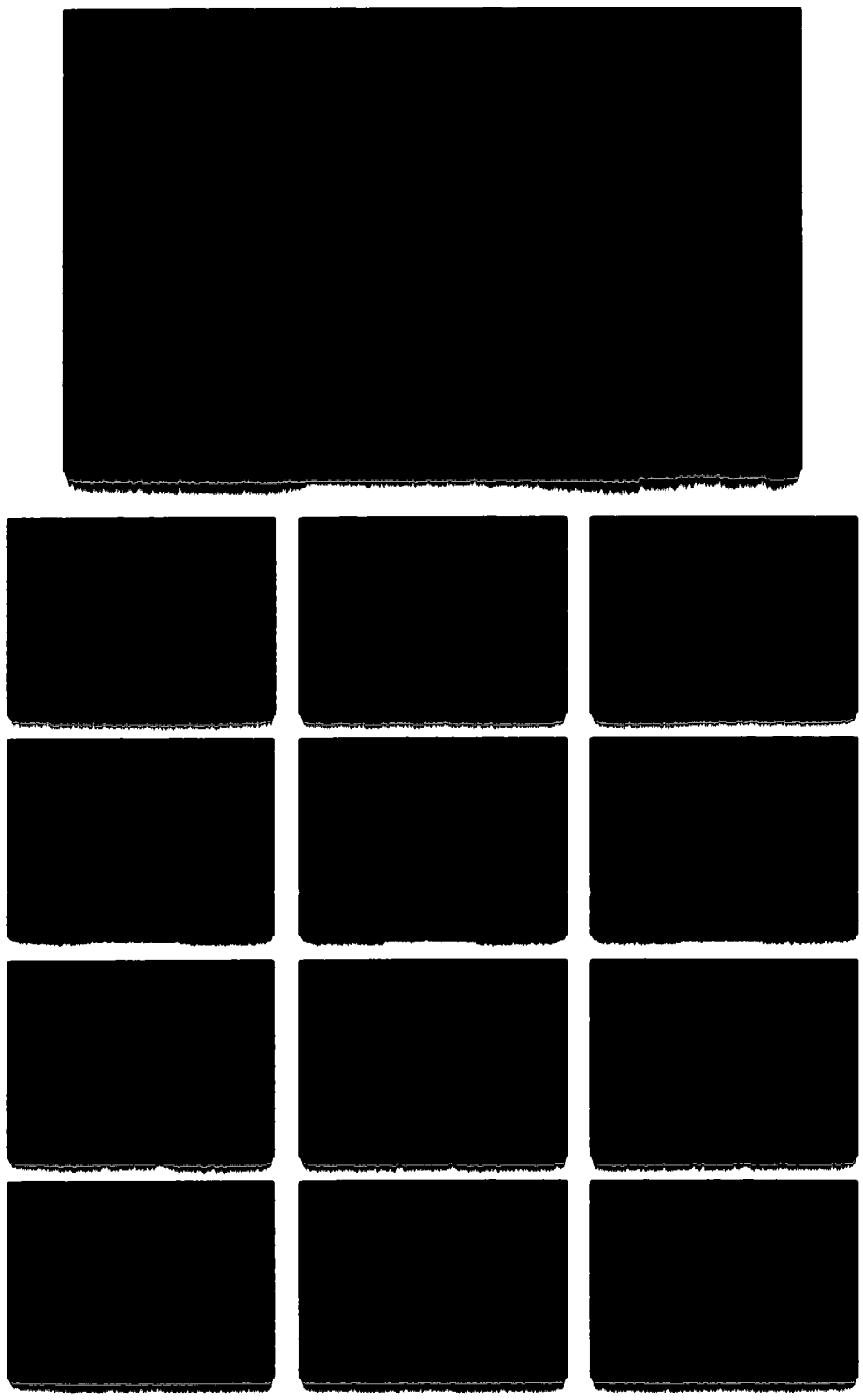
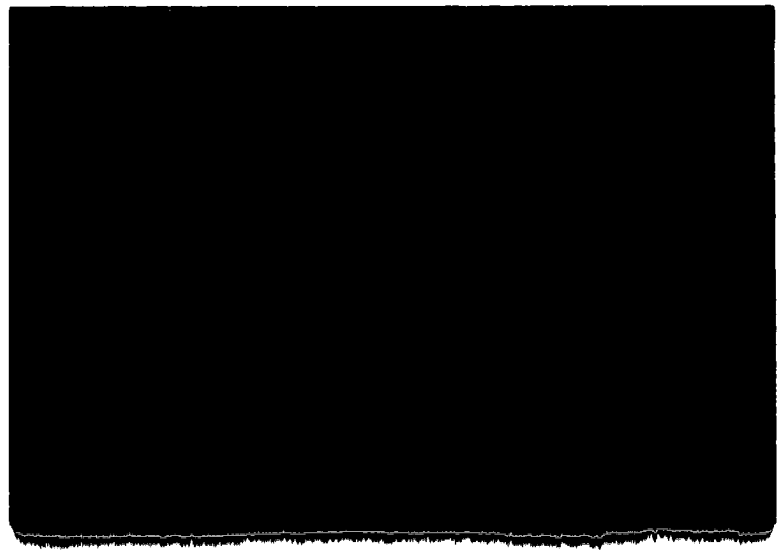
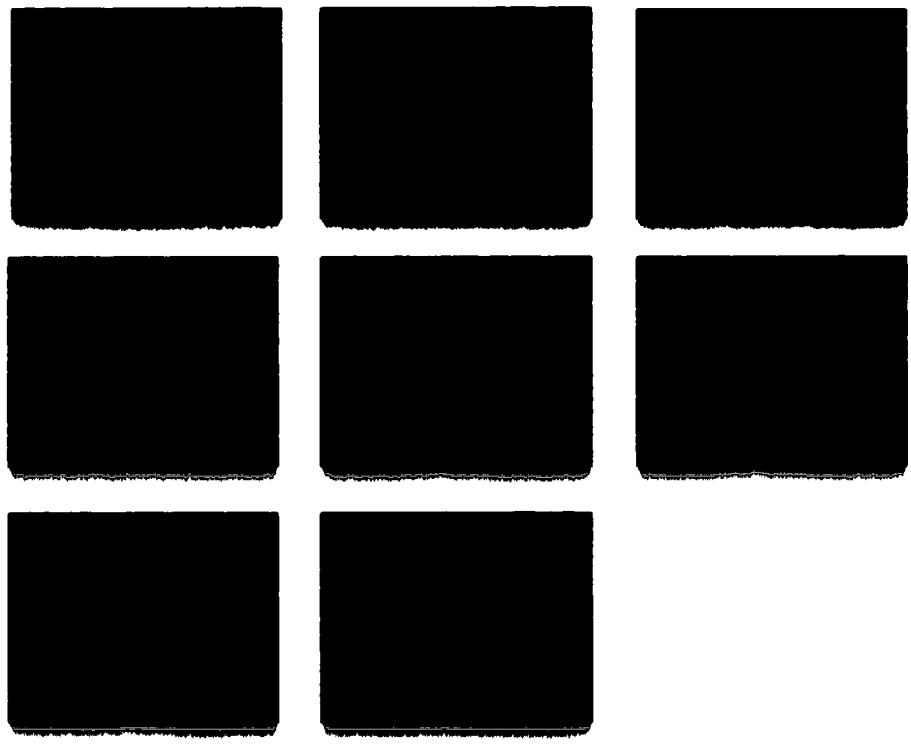
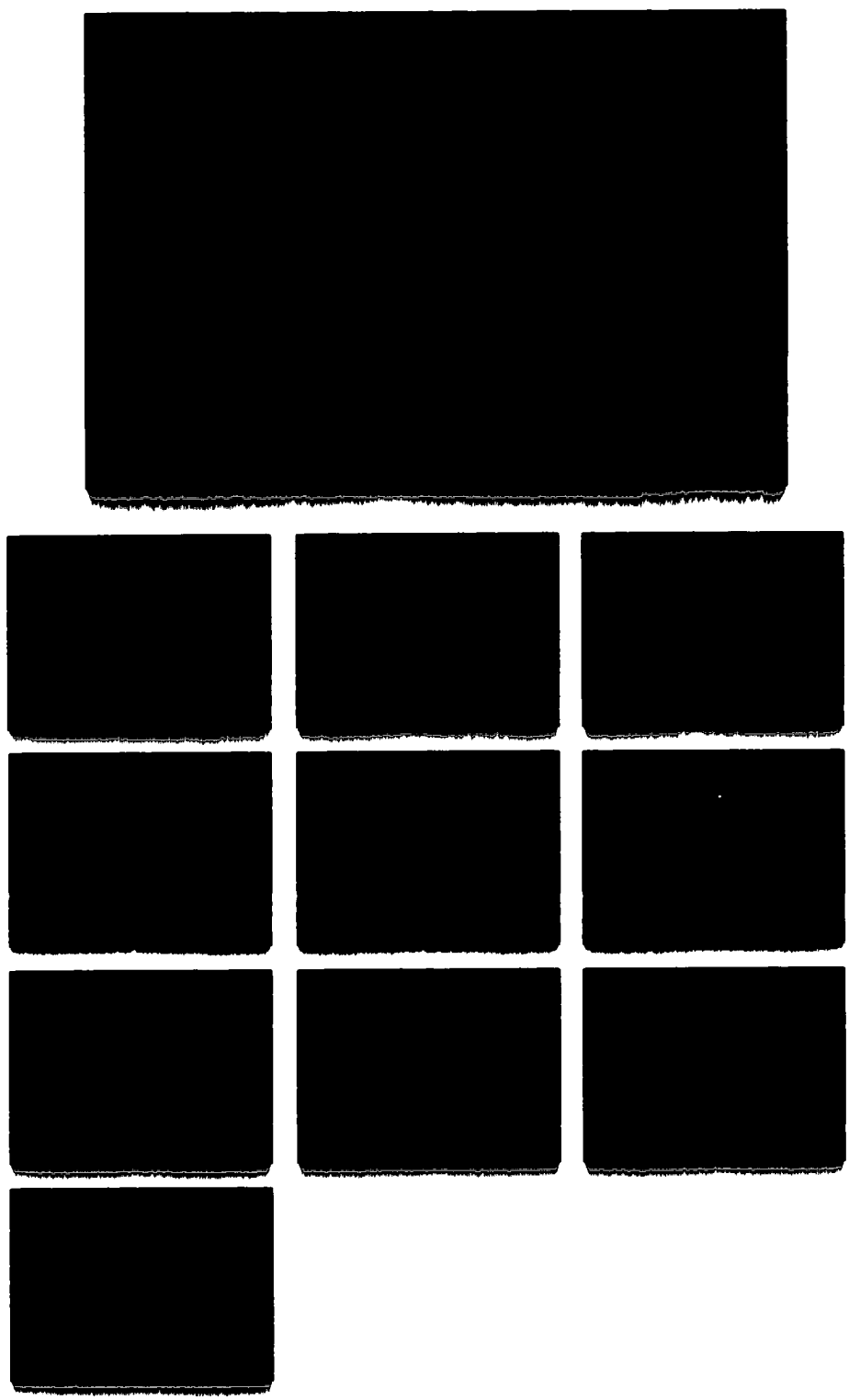


C





D



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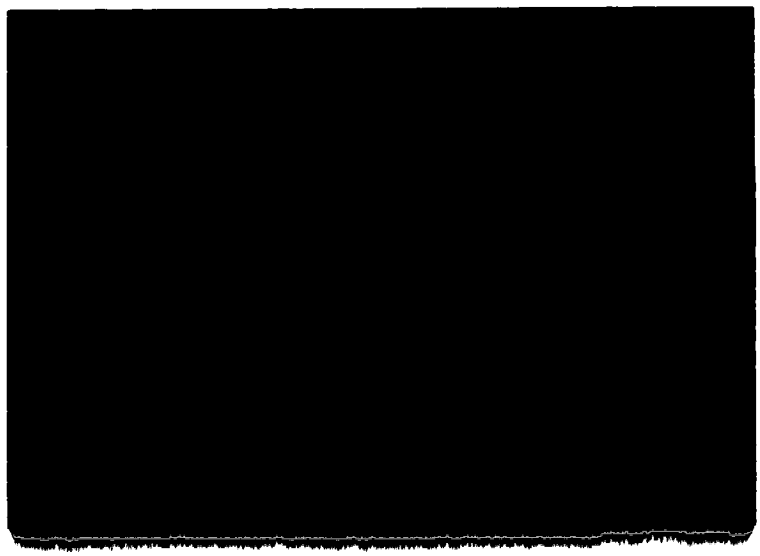
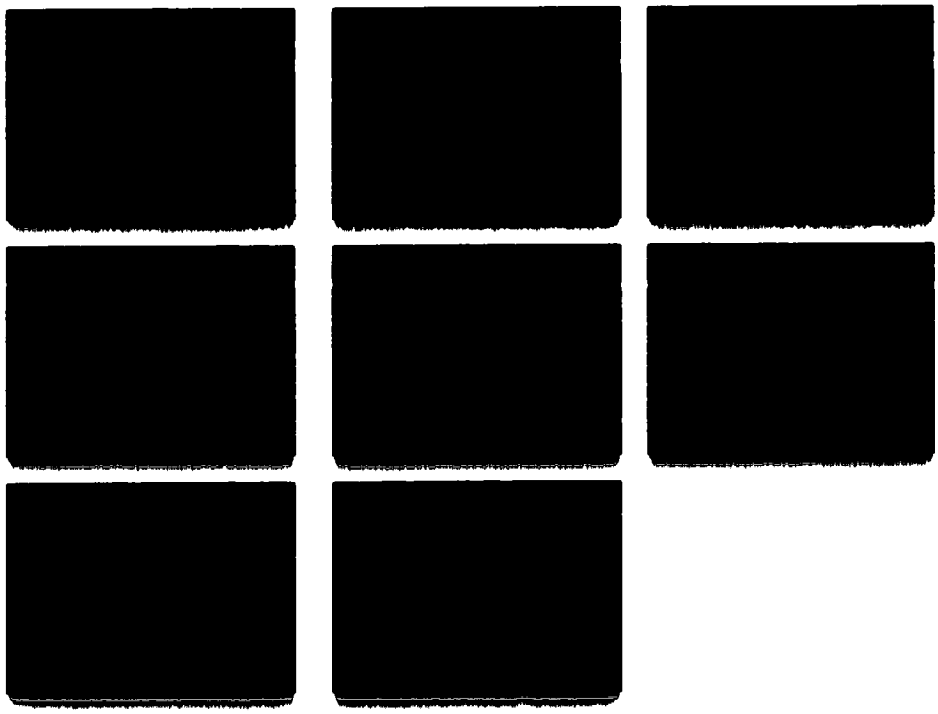
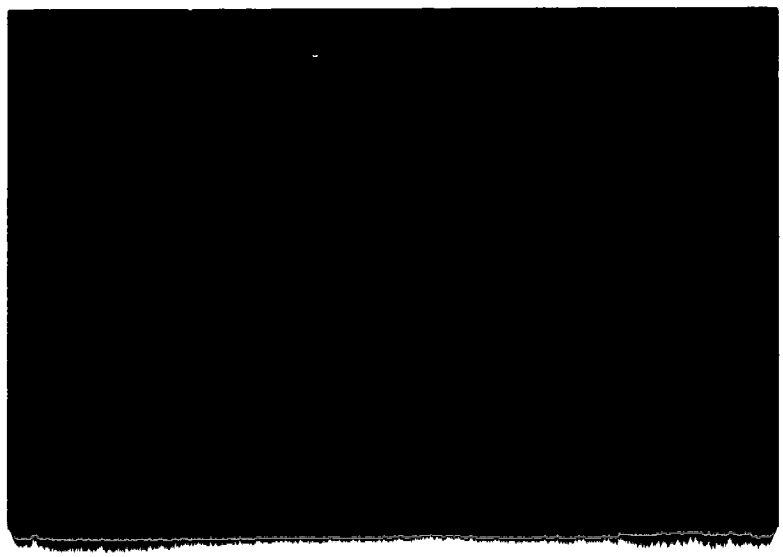
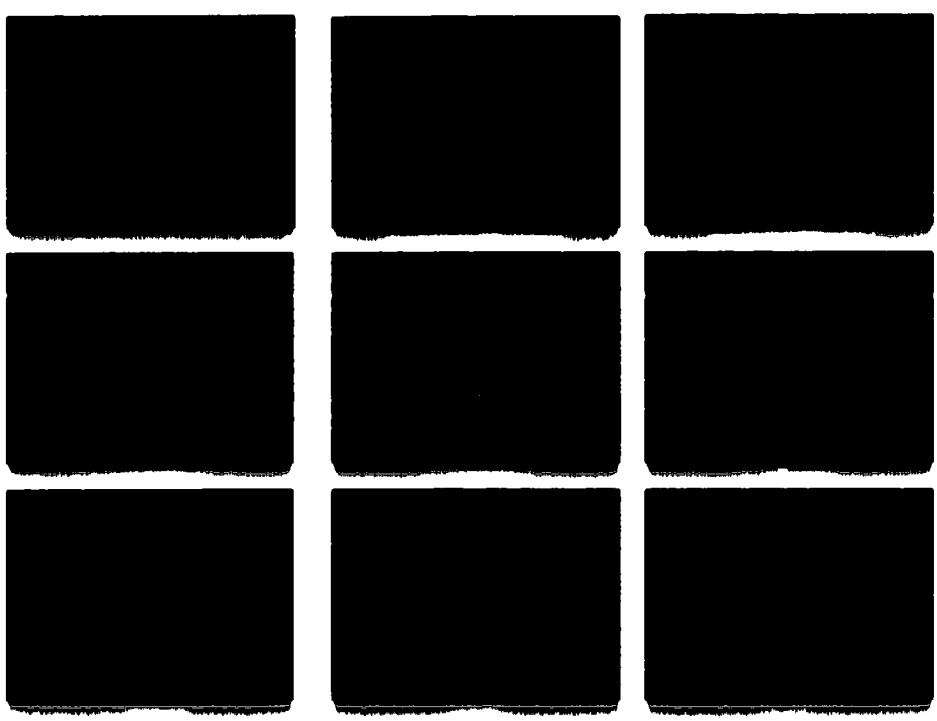




F





The visual contrast between cytoplasmic staining and plasma membrane staining is enhanced by the observation of a nuclear ghost in cells which have been treated with phorbol ester.

Like Wt MARCKS, S152A MARCKS was predominantly associated with the plasma membrane with only diffuse staining in the cytoplasm *(Figure 32C)*. However, cells expressing S152A MARCKS were more rounded and MARCKS was also found as a distinct ring around small but extensive bleb-like membrane protrusions which were not observed in cells expressing Wt MARCKS. Phorbol ester treatment resulted in only a slight reduction of immunoreactive MARCKS at the plasma membrane as visualized by a punctate staining pattern rather than uniform staining around the plasma membrane *(Figure 32D)*. In addition, phorbol ester treatment resulted in a distinct localization of immunoreactive MARCKS at one pole of the nuclear surface, a staining pattern reminiscent of Golgi-specific staining with lectins in CHO-K1 cells (Ridgway, 1992). Finally, S156A MARCKS was found to be associated with cytoplasmic punctate structures under both basal conditions and following phorbol ester treatment *(Figure 32E,F)* suggesting that S156 may be essential for the preferential localization of MARCKS at the plasma membrane.

D. Discussion

The experiments presented in this section were designed to address the possibility that individual serine residues within the effector domain of MARCKS may have distinct effects on the modulation of MARCKS membrane association by PKC. To do this, the effects of serine to alanine replacement at residues 152 and 156 on the

membrane association of the protein in intact cells were examined. Three interesting results emerged from this study. First, phorbol ester stimulates phosphorylation and redistribution of wild-type MARCKS to intracellular membranous compartment distinct from the plasma membrane without altering its overall membrane affinity. Second, S156 appears to be required for the association of MARCKS with the plasma membrane and subsequent phorbol ester-induced phosphorylation and release from the plasma membrane. Finally, S152 is not essential for phorbol ester-induced release of MARCKS from the plasma membrane but may play a role in targetting MARCKS to specific intracellular domains.

Several previous experiments in cell-free systems have suggested that the membrane association of the positively charged effector domain is mediated by electrostatic interactions. For example, the affinity of a positively charged, wild-type MARCKS effector domain peptide for synthetic lipid vesicles increased sigmoidally with the percentage of negatively charged PS in the vesicle (Kim *et al.*, 1994b). Binding to the vesicles decreased markedly when the peptide was either phosphorylated by PKC or the four serines were mutated to aspartic acids, mimicking phosphorylation (Kim *et al.*, 1994b). Similarly, murine MARCKS expressed in a baculovirus system (Wang *et al.*, 1989), MARCKS purified from bovine brain (Kim *et al.*, 1994b), and bacterially expressed MARCKS-glutathione S-transferase fusion proteins (Apel *et al.*, 1991) exhibited high affinity for negatively charged lipids which was disrupted by PKC dependent phosphorylation of MARCKS.

In this investigation, the simple and rapid cell permeabilization protocol which allowed for measurement of small changes in membrane association of MARCKS in C6

glioma cells (see previous sections and Douglas *et al.*, 1997) was adapted for CHO-K1 cells stably expressing Wt, S152A and S156A MARCKS. Phorbol ester stimulated the phosphorylation of both Wt MARCKS and S152A MARCKS but in both cases the increased phosphorylation was not accompanied by increased cytosolic translocation as reflected by digitonin extraction followed by Western blot analysis. While this result is consistent with other observations that translocation need not occur upon increased MARCKS phosphorylation (James and Olson, 1989; Byers *et al.*, 1993), confocal immunolocalization microscopy clearly indicated that phorbol ester-induced phosphorylation resulted in the displacement of MARCKS from the plasma membrane for both Wt and S152A MARCKS.

It has been previously reported that PKC activation promotes the movement of MARCKS from the plasma membrane to lysosomes in fibroblasts (Allen and Aderem, 1995), indicating that phosphorylation can direct the targeting of MARCKS to alternate membrane compartments. Two factors could contribute to the present observation that phorbol ester induced MARCKS phosphorylation and redistribution to intracellular punctate structures, even though phosphorylation normally solubilizes MARCKS from membranes. First, perhaps all three serines in the effector domain must be phosphorylated to prevent MARCKS association (or reassociation) with membranes (Kim *et al.*, 1994a). It is not clear from this study if Wt MARCKS was phosphorylated stoichiometrically in CHO cells. Second, the lipid and/or protein composition of the intracellular membrane compartment responsible for the cytoplasmic punctate staining pattern following phorbol ester treatment may differ somewhat from the bulk plasma membrane in that it may have MARCKS-targeting determinants.

Cells expressing S152A MARCKS differed from those expressing Wt MARCKS in that the cells were more rounded and exhibited blebs protuding from the plasma membrane. Under basal conditions, the S152A mutant localized to the plasma membrane but was also localized to a distinct ring surrounding the bleb-like protrusions around the plasma membrane. This observation is reminiscent of a recent study in which the expression of MARCKS containing a mutation which abrogates the myristoyl-electrostatic switch in fibroblasts prevented cell spreading by arresting the cells during an early stage of spreading as characterized by profuse membrane blebbing (Myat *et al.*, 1997). Membrane blebbing is a morphological transition to membrane ruffling and the formation of lamellae which preceeds cell spreading and flattening. Phorbol ester treatment abolished the rounded and blebbed morphology, suggesting that the blebbing seen under basal conditions was not a consequence of toxicity due to the expression of this mutant. Furthermore, phorbol ester treatment displaced some S152A MARCKS from the membrane and directed this mutant from the plasma membrane to a distinct pole of the nucleus; a staining pattern reminiscent of the Golgi apparatus. This observation further reinforces the hypothesis that serines within the effector domain play distinct roles in PKC-modulated targeting of MARCKS to various cellular locations.

PKC phosphorylation assays using a GST-MARCKS mutant with S152 and S156 changed to alanines showed that phosphorylation of S152 and S156 is not a pre-requisite for phosphorylation of S163 (Herget *et al.*, 1995). By contrast, the inability of S156A to be phosphorylated by phorbol ester suggests that, at least in CHO-K1 cells, phosphorylation of S156 may preceed phosphorylation at other sites thus supporting

the observation that MARCKS is sequentially phosphorylated in the order S156>S163>S152 by activated PKC (Herget *et al.*, 1995). It is also worth noting that the inability of phorbol ester to increase the phosphorylation of S156A MARCKS may also be in part be due to the low level of this mutant associated with the plasma membrane where PKC is known to be activated (reviewed in Bell and Burns, 1991). Nonetheless, the distinct cellular localization of this mutant suggests a role for S156 in the localization of MARCKS at the plasma membrane and in mediating PKC-dependent targetting of MARCKS to specialized membranes.

VI Summary and Conclusions

In several cell types, phosphorylation of MARCKS by PKC appears to result in the dissociation of the protein from membranes. However, due to the complexity of the interactions occurring at the effector domain, it is likely that other factors, in addition to hydrophobic and electrostatic interactions, regulate the membrane association of MARCKS. The work presented in this thesis has provided evidence for regulation of MARCKS membrane association by the effector domain on at least three different levels.

First, these studies have shown that CaM and F-actin have distinct effects on MARCKS localization which appear to be independent of the phosphorylation state of MARCKS. Interaction of MARCKS with F-actin might provide PKC with an alternate site for substrate phosphorylation distinct from that available when the protein is associated with the plasma membrane; phosphorylation of MARCKS at this locale could result in the local release of F-actin necessary for PKC-mediated alterations in actin plasticity. It would be worth examining, perhaps by fluorescent/immunofluorescent confocal imaging, whether MARCKS and F-actin colocalize with PKC in C6 cells.

Second, the serendipitous discovery that ATP stimulated the release of MARCKS from permeabilized C6 membranes in a Ca^{2+}-and phorbol ester-independent manner provides evidence for the first time that a PKC other than conventional or novel PKCs could phosphorylate MARCKS *in vivo*. Such an activity could be related to maintaining basal equilibrium with opposing phosphatases to ensure that MARCKS is poised to

dynamically respond to extracellular signals that lead to membrane-cytoskeletal alterations. That MARCKS can serve as functional substrate for all subclasses of PKC *in vivo* (perhaps even by recruiting PKCs to various cellular locations) hints that MARCKS may be involved in mediating several diverse signalling pathways, each culminating in distinct cellular responses. The use of specific antibodies or antisense mRNAs might prove useful in positively identifying the suspect kinase. Furthermore, a definitive physiological role for this kinase awaits the identification of physiological activators.

Finally these data also demonstrate that the serine residues within the effector domain may have distinct roles in targeting MARCKS to various cellular locations under basal conditions and following stimulation by activated PKC. Specifically, S156 of the rat sequence appears to be important for the regulation of MARCKS redistribution by PKC. The observation that phorbol ester redistributes MARCKS to punctate structures within the cytosol of CHO cells could account for the modest translocation of MARCKS to the cytosol seen in C6 cells in response to phorbol ester. This may also explain why in this study and in previous studies (Byers et al., 1993) that MARCKS phosphorylation and translocation were spatially and temporally dissociated in C6 cells: phosphorylation of MARCKS by PKC can cause the redistribution of MARCKS to cellular structures not susceptible to release by digitonin permeabilization. Two dimensional peptide mapping (hampered in CHO cells by the low level of MARCKS phosphorylation) might determine if the targeting of MARCKS to various cellular locations in response to PKC activation is the direct consequence of (sequential) phosphorylation of serines within the effector domain.

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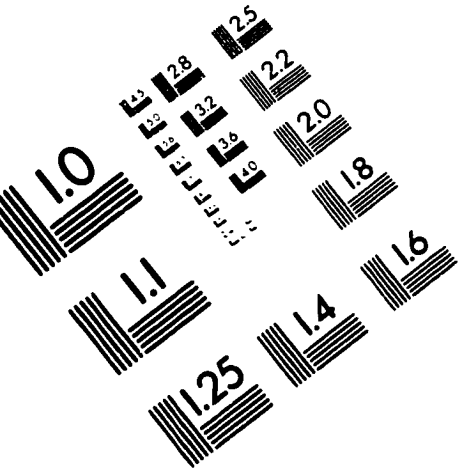
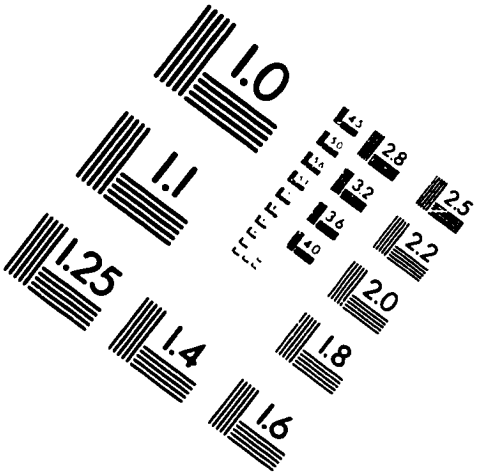
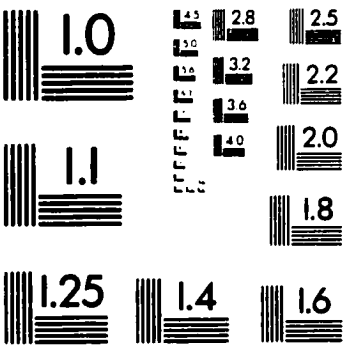
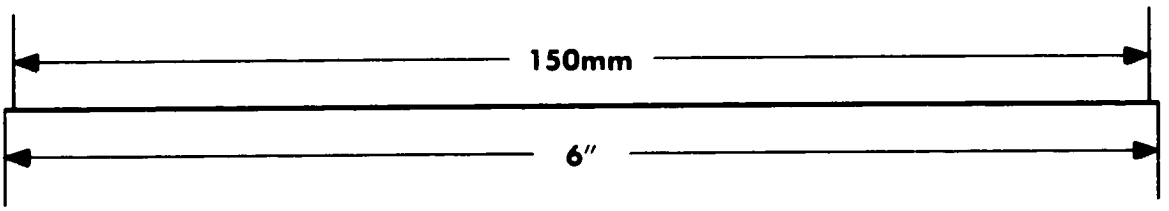
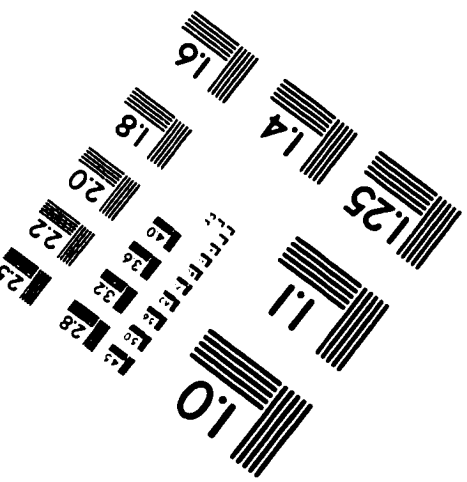
IMAGE EVALUATION
TEST TARGET (QA-3)

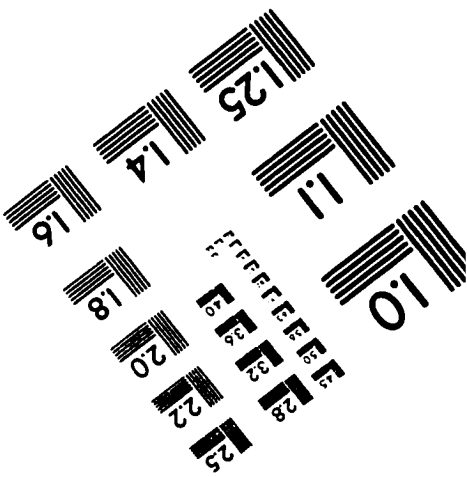












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989