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Protein kinase C regulates MARCKS cycling between the plasma membrane and lysosomes in fibroblasts

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MARCKS is a protein kinase C (PKC) substrate that is phosphorylated during neurosecretion, phagocyte activation and growth factor-dependent mitogenesis. MARCKS binds calcium/calmodulin and crosslinks F-actin, and both these activities are regulated by PKC-dependent phosphorylation. We present evidence here that PKC-dependent phosphorylation also regulates the cycling of MARCKS between the plasma membrane and Lamp-1-positive lysosomes. Immunofluorescence and immunoelectron microscopy, and subcellular fractionation, demonstrated that MARCKS was predominantly associated with the plasma membrane of resting fibroblasts. Activation of PKC resulted in MARCKS phosphorylation and its displacement from the plasma membrane to Lamp-1-positive lysosomes. MARCKS phosphorylation is required for its translocation to lysosomes since mutating either the serine residues phosphorylated by PKC (phos$^-$) or the PKC inhibitor staurosporine, prevented MARCKS phosphorylation, its release from the plasma membrane, and its subsequent association with lysosomes. In the presence of lysosomotropic agents or nocodazole, MARCKS accumulated on lysosomes and returned to the plasma membrane upon drug removal, further suggesting that the protein cycles between the plasma membrane and lysosomes. In contrast to wild-type MARCKS, the phos$^-$ mutant did not accumulate on lysosomes in cells treated with NH_4Cl, suggesting that basal phosphorylation of MARCKS promotes its constitutive cycling between these two compartments.

Key words: lysosomes/MARCKS/membrane trafficking/protein kinase C/signal transduction

Introduction

The myristoylated alanine-rich C kinase substrate, MARCKS, is a widely distributed protein kinase C (PKC) substrate which is phosphorylated during neurosecretion, growth factor-dependent mitogenesis and phagocyte activation. MARCKS binds calcium/calmodulin and crosslinks actin, and these activities are regulated by phosphorylation (for reviews see Aderem, 1992; Blackshear, 1993). MARCKS is an acidic protein that is unusually rich in alanine, glycine, proline and glutamic acid. The protein is a rod-shaped molecule containing three distinct domains: an N-terminal myristoylated domain that mediates binding to membranes, a highly conserved MH2 domain of unknown function, and a basic effector domain containing the PKC phosphorylation sites and the calmodulin- and actin-binding sites (for reviews see Aderem, 1992; Blackshear, 1993). The binding of MARCKS to calmodulin and to actin is regulated in a complex manner. First, MARCKS binds calmodulin only in the presence of calcium, and the phosphorylation of MARCKS prevents the binding of calmodulin to it (Graff *et al.*, 1989; McIlroy *et al.*, 1991). Second, MARCKS binds to the sides of actin filaments and crosslinks them, and the crosslinking activity is disrupted by both phosphorylation and calcium/calmodulin (Hartwig *et al.*, 1992).

MARCKS binds to membranes via an N-terminal, myristoylated membrane-binding domain and by ionic interaction of the basic effector domain with acidic phospholipids (George and Blackshear, 1992; Taniguchi and Manenti, 1993; Kim *et al.*, 1994). Membrane binding places the protein in close apposition to PKC and allows efficient phosphorylation (Rosen *et al.*, 1990). Upon phosphorylation MARCKS is released from the membrane and its subsequent dephosphorylation is accompanied by its reassociation with the membrane (Wang *et al.*, 1989; Thelen *et al.*, 1991). In macrophages, MARCKS has a punctate distribution, and many of the structures containing MARCKS are found at the substrate-adherent surface of pseudopodia and filopodia (Rosen *et al.*, 1990). Many of these structures also contain vinculin and talin, known components of focal contacts (Rosen *et al.*, 1990). Immunoelectron microscopy demonstrates the presence of MARCKS at points where actin filaments interact with the cytoplasmic surface of the plasma membrane in macrophages (L.Allen *et al.*, manuscript in preparation). The data suggest that MARCKS regulates actin–membrane interactions, and the structure of actin at the membrane (Aderem, 1992).

MARCKS is also highly concentrated in presynaptic junctions and is phosphorylated when synaptosomes are depolarized (Wu *et al.*, 1982; Albert *et al.*, 1986; Wang *et al.*, 1989), suggesting a role in secretion or membrane recycling. This is supported by the observation that both tumor necrosis factor and bacterial lipopolysaccharide induce the synthesis of MARCKS and simultaneously prime macrophages and neutrophils for enhanced secretion of inflammatory mediators and cytokines (Aderem *et al.*, 1986, 1988; Thelen *et al.*, 1990). We now report that MARCKS cycles between the plasma membrane and Lamp-1-positive lysosomes in fibroblasts; phosphorylation of MARCKS by PKC shifts the equilibrium towards lysosomes, lysosomotropic agents and microtubule destabilizing agents trap the protein in this compartment, and down-regulation of PKC or inhibition of the kinase promotes MARCKS reassociation with the plasma membrane.

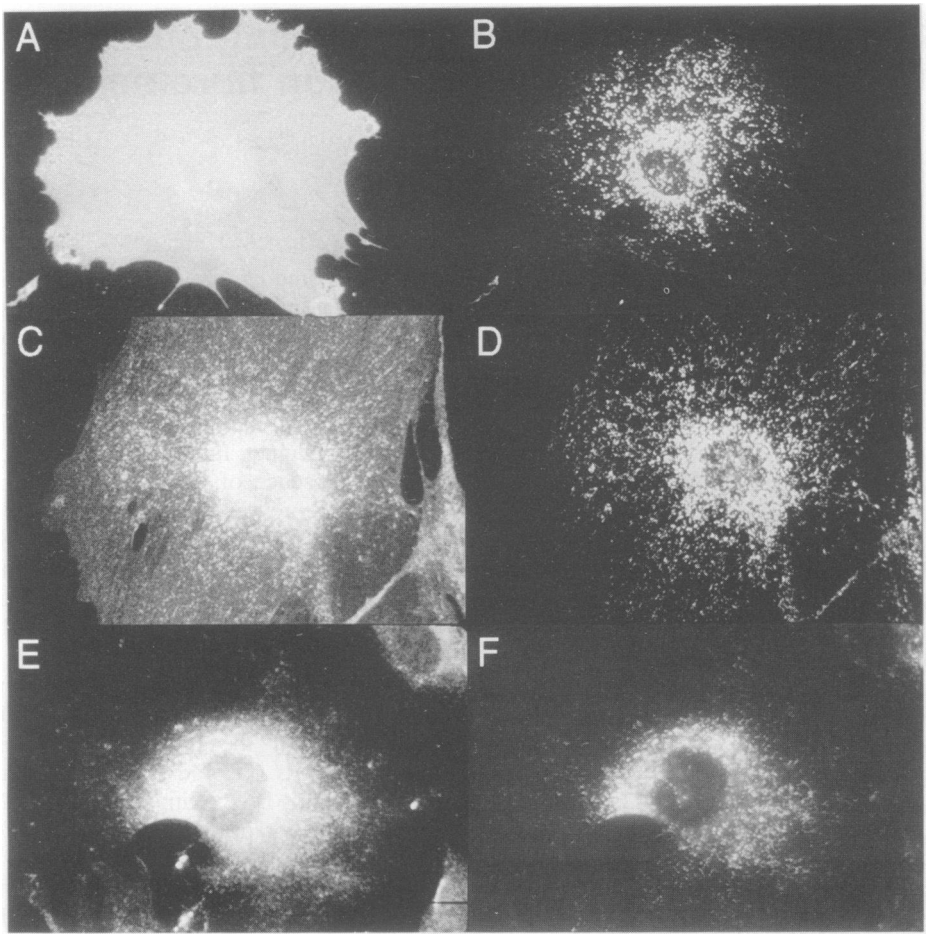

Fig. 1. Localization of MARCKS in control and PMA-treated MEFs using indirect immunofluorescence microscopy. MEFs were left untreated (**A** and **B**), or exposed to 200 nM PMA for 15 min (**C** and **D**) or 30 min (**E** and **F**). Fixed and permeabilized cells were double-stained for MARCKS (A, C and E) and Lamp-1 (B, D and F) as described in the Materials and methods. MARCKS was photographed using rhodamine optics and Lamp-1 was photographed using fluorescein optics.

Results

Activation of PKC promotes MARCKS translocation from the plasma membrane onto lysosomes

MARCKS was localized in mouse embryo fibroblasts (MEFs) using an affinity-purified polyclonal antibody which detects a single band in Western blots of whole cell lysates (Rosen *et al.*, 1990 and data not shown). In control MEFs, MARCKS was primarily distributed in a diffuse pattern close to the plasma membrane as judged by indirect immunofluorescence (Figure 1A) or confocal microscopy (Figure 2A), and was present in trace amounts on intracellular membranes (Figures 1A and 2B).

When MEFs were treated with phorbol esters there was a time-dependent appearance of MARCKS on Lamp-1-positive punctate structures, and a corresponding decrease in MARCKS at the plasma membrane (Figures 1 and 2). MARCKS was detected on Lamp-1-positive structures as early as 5 min after PMA addition (data not shown), and completely colocalized with this marker by 30 min (Figures 1E and F and 2E and F). At this time, MARCKS was essentially undetectable at the plasma membrane (Figures 1E and 2D). Similar results were obtained when PKC was activated by dioctanoyl-*sn*-glycerol instead of PMA (data not shown). Lamp-1 is a glycoprotein associated primarily with lysosomes and late endosomes, and is found in trace amounts on the plasma membrane and early endosomes (Green *et al.*, 1987; Howe *et al.*, 1988; Harter and Mellman, 1992). In MEFs there is very little co-localization of Lamp-1 and the cation-independent mannose-6-phosphate receptor (CIM6PR) (data not shown). Since the CIM6PR is a marker of the trans-Golgi network and late endosomes (Brown *et al.*, 1986; Geuze *et al.*, 1988), the data suggest that the majority of the Lamp-1-positive structures in MEFs are lysosomes. For these reasons we will refer to Lamp-1-positive structures as lysosomes, although a small portion of these may be endosomes. MARCKS staining was easily distinguished from markers of the medial-Golgi or the rough endoplasmic reticulum (data not shown).

The intracellular location of MARCKS was also examined in cryosections of MEFs using affinity-purified anti-MARCKS antibodies and secondary antibodies coupled to 10 nm gold particles. Similarly, lysosomes were identified using antibodies to Lamp-1 and a secondary antibody conjugated to 5 nm gold particles (Figure 3). In control MEFs anti-MARCKS gold particles were distributed all along the plasma membrane, including filopods (Figure 3, left panels) with very few gold particles associated with lysosomes or other structures. By contrast, the number of anti-MARCKS gold particles was precipitously decreased along the plasma membrane of cells treated with PMA for 15 min (Figure 3, right panels).

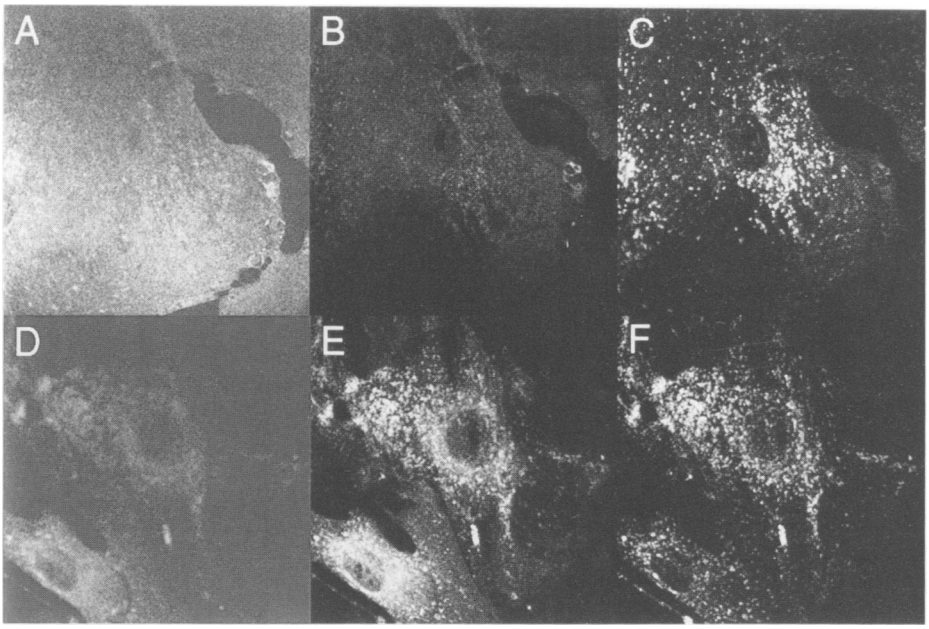

Fig. 2. Localization of MARCKS in MEFs using confocal microscopy. Control MEFs (A–C) or cells treated with 200 nM PMA for 30 min (D–F) were fixed and permeabilized, and double-stained for MARCKS and Lamp-1. MARCKS and Lamp-1 were detected using secondary antibodies conjugated to Texas Red and fluorescein, respectively. (**A**) An optical section, 0.2 μm thick, showing MARCKS in the plasma membrane of control MEFs. (**B** and **C**) An optical section, 0.2 μm thick, through the center of the same cell showing that Lamp-1-positive lysosomes (C) contain only traces of MARCKS (B). (**D**) An optical section, 0.2 μm thick, at the plasma membrane of a cell treated with PMA for 30 min. Note that the plasma membrane is depleted of MARCKS. (**E** and **F**) An optical section, 0.2 μm thick, through the center of the same cell as shown in (D). This section contains numerous lysosomes that stain positively for both MARCKS (E) and Lamp-1 (F).

This decrease was accompanied by a large increase in the number of MARCKS gold particles associated with Lamp-1-positive structures and other small vesicles (Figure 3, right panels). Quantification of the gold particle distribution demonstrated that the vast majority of MARCKS (~84%) were associated with the plasma membrane of resting MEFs, ~12% were associated with lysosomes and ~2% were associated with small vesicles (Table I). Fifteen minutes after PMA was added only ~17% of the anti-MARCKS gold particles remained associated with the plasma membrane, ~55% were associated with lysosomes and ~15% were associated with small vesicles (Table I). The distribution of Lamp-1 was not affected by PMA (Table I). A similar enrichment of MARCKS on lysosomes was apparent when the data were normalized as gold particles/μm membrane (Table I).

Subcellular fractionation of MEFs using differential centrifugation also demonstrated that MARCKS was associated with a plasma membrane-enriched membrane fraction in resting cells (Table II), and translocated to a dense lysosome-enriched membrane fraction within 60 min of PMA addition (Table II). Immunoblotting experiments demonstrated that the total amount of MARCKS in MEFs is the same in control and PMA-treated cells (data not shown).

MARCKS phosphorylation is required for its translocation from the plasma membrane onto lysosomes

MARCKS was poorly phosphorylated in resting MEFs, and highly phosphorylated in cells treated with PMA (Figure 5A), as may be expected for a PKC substrate. Inhibition of PKC by staurosporine blocked the incorporation of $^{32}P_i$ into MARCKS (Figure 5A) as well as its redistribution from the plasma membrane onto lysosomes (Figure 4E and F). In addition, PKC-dependent redistribution of MARCKS was completely reversible. For example, when PMA-treated cells were exposed to staurosporine, MARCKS rapidly returned from lysosomes to the plasma membrane (Figure 4I and J). Similarly, prolonged exposure of MEFs to PMA, which results in the down-regulation of PKC (Nishizuka, 1992), caused the redistribution of MARCKS from lysosomes to the plasma membrane (Figure 4G and H).

Further clues to the mechanism by which PKC might stimulate MARCKS relocation were gained from fractionation of MEFs labeled with [^{3}H]myristic acid or [^{32}P]orthophosphate (Figure 5B). In control MEFs >90% of MARCKS was associated with the membrane fraction (Figure 5B, upper panel) and very little MARCKS was phosphorylated (Figure 5B, lower panel). Activation of PKC for 15 min resulted in the release of ~50% of MARCKS from the membrane into the cytosol (Figure 5B, upper panel), and this soluble protein was highly phosphorylated (Figure 5B, lower panel). Longer exposure of the cells to diacyl-*sn*-glycerols or phorbol esters (>30 min) was accompanied by the reassociation of MARCKS with the membrane fraction (data not shown). At this time point MARCKS colocalized with Lamp-1 by fluorescence microscopy (Figure 1E and F), and fractionated with lysosomes by differential centrifugation (Table II), suggesting that MARCKS reassociated with lysosomal membranes. Taken together, these data imply that MARCKS cycles between the plasma membrane and lysosomes, that phosphorylation shifts the equilibrium towards the lysosomal compartment, and that MARCKS transport may involve a soluble intermediate.

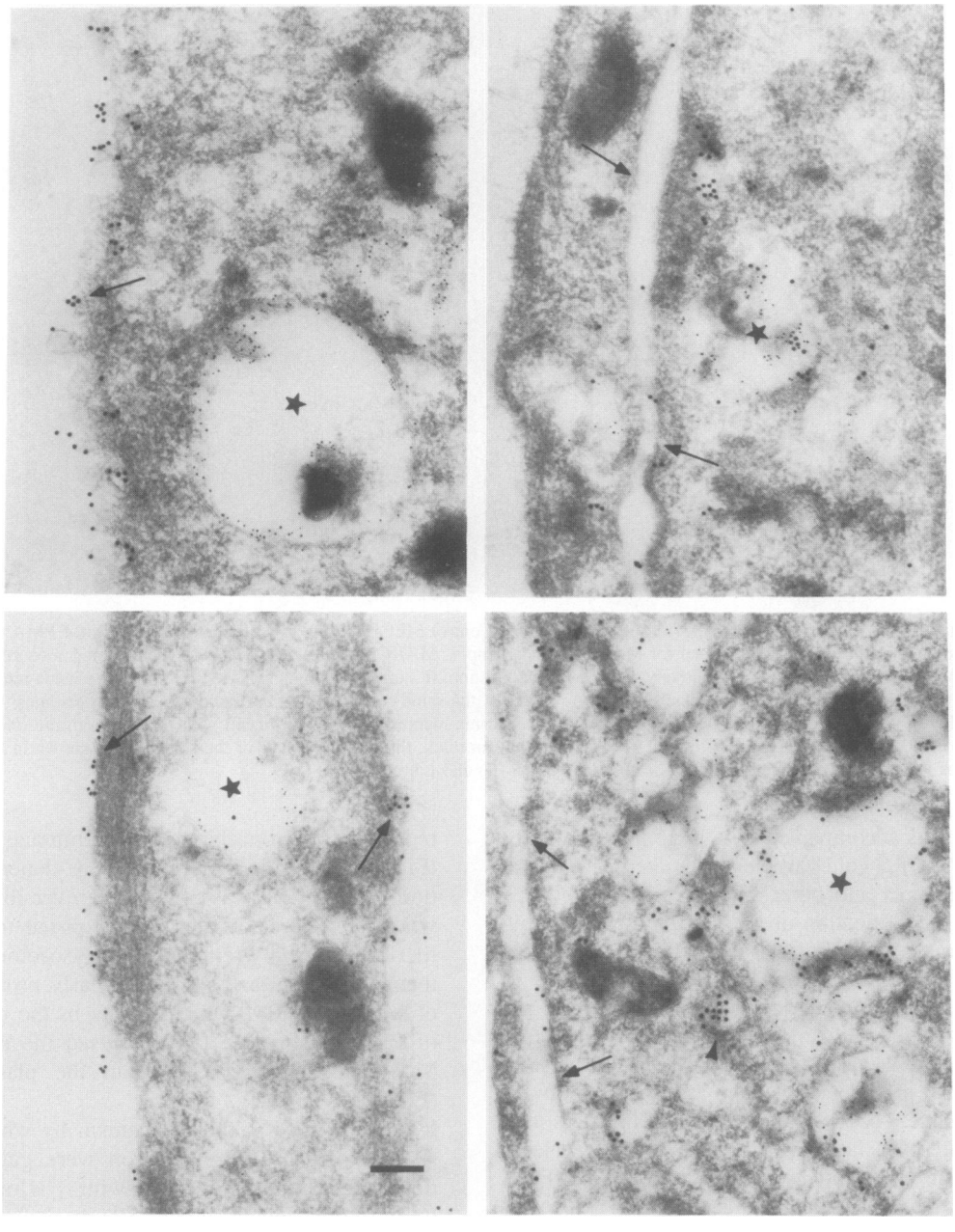

Fig. 3. Immunogold labeling of MARCKS and Lamp-1 in cryosections of control and PMA-treated MEFs. Cells were untreated (left panels) or exposed to 200 nM PMA for 15 min (right panels) prior to processing for EM. MARCKS and Lamp-1 were detected in cryosections as described in the Materials and methods. Gold particles of 10 nm indicate MARCKS and 5 nm gold particles indicate Lamp-1. Arrows indicate the plasma membrane. Stars indicate lysosomes. Arrowheads indicate Lamp-1-negative small vesicles. The bar is 0.2 μm.

Ammonium chloride treatment or microtubule depolymerization traps MARCKS on lysosomes

We perturbed membrane trafficking with NH_4Cl to gain additional insights into the intracellular pathway taken by MARCKS. Lysosomotropic agents cause the vacuolation of lysosomes and increase their internal pH (Mellman *et al.*, 1986). In the presence of lysosomotropic agents MARCKS was gradually depleted from the plasma membrane and accumulated on large vacuolated lysosomes (Figure 6). Twenty-four hours after addition of the drug, MARCKS was completely redistributed to large vacuolated lysosomes in the center of the cell (Figure 6E–G). The colocalization of MARCKS and Lamp-1 was confirmed using confocal microscopy (data not shown). Similar data were obtained for cells treated with chloroquine (data not shown). When NH_4Cl-treated cells were returned to normal medium, MARCKS gradually reappeared in the plasma membrane with a half-time of ~2 h (Figure 6H and I) and this was unaffected by protein synthesis inhibitors (data not shown). Interestingly, MARCKS returned to the plasma membrane before lysosomes regained their normal size and distribution (Figure 6H and J). On the other hand, MARCKS did not accumulate on lysosomes swollen by hypertonic sucrose (data not shown), suggesting that prevention of acidification, and not expansion of the lysosomal compartment *per se*, was responsible for MARCKS accumulation on these structures in NH_4Cl-treated cells.

Over the time course of NH_4Cl treatment there was no noticeable increase in MARCKS phosphorylation relative

Table I. Effect of PMA on the intracellular distribution of MARCKS in MEFs

Treatment		MARCKS		Lamp-1	
		Percent	Gold (μm^{-1})	Percent	Gold (μm^{-1})
Control	plasma membrane	84 ± 11	16.2	3 ± 3	0.4
	lysosomes	12 ± 10	4.2	96 ± 4	47.2
	small vesicles	2 ± 2	7.4	NA	NA
	other	2 ± 2	NA	1 ± 2	NA
PMA	plasma membrane	17 ± 9	1.2	1 ± 2	0.1
	lysosomes	55 ± 18	18.5	96 ± 4	48.9
	small vesicles	15 ± 14	13.1	NA	NA
	other	13 ± 7	NA	3 ± 3	NA

Immunogold particles were counted in cryosections of control MEFs (n = 16) or cells treated with 200 nM PMA for 15 min (n = 18). A total of 2179 and 1074 gold particles were counted for MARCKS in control and PMA-treated cells, respectively. Similarly, 2337 and 2443 gold particles were counted for Lamp-1 in control and PMA-treated cells. Lysosomes were defined as vesicles containing anti-Lamp-1 gold particles. All remaining vesicles were Lamp-1-negative. 'Other' refers to intracellular gold particles that were not associated with defined membraneous structures. Gold particle distribution is expressed as the percentage of total gold particles counted ± standard deviation, and as the number of gold particles per micrometer of each type of membrane. NA, not applicable.

to control cells (Figure 5C), and fractionation of cells labeled with [^{3}H]myristic acid showed that ~90% of MARCKS was membrane-bound during NH_4Cl treatment (data not shown). However, the basal level of phosphorylation observed in control cells not treated with PMA, as well as in cells treated with NH_4Cl (Figure 5C), was due to PKC, since peptide mapping revealed that the phosphate was incorporated into serines 152, 156 and 163 of the effector domain (data not shown). This observation, as well as the slow time course of MARCKS accumulation on lysosomes in the presence of NH_4Cl, implies that MARCKS fluxes through lysosomes at steady state and that lysosomotropic agents trap MARCKS on lysosomes.

MARCKS also accumulated on Lamp-1-positive structures when microtubules were depolymerized (Figure 7A and B), and returned to the plasma membrane after removal of the drug (Figure 7C and D). The distribution of MARCKS in cells treated with NH_4Cl plus nocodazole (Figure 7E and F) was similar to that of cells treated with NH_4Cl alone (Figure 6C–F). However, depolymerization of microtubules prevented MARCKS recycling to the plasma membrane upon NH_4Cl wash-out (compare Figure 6H with Figure 7G). Nocodazole treatment did not increase MARCKS phosphorylation over basal levels, nor did it promote the release of MARCKS from the membrane fraction in to the cytosol (data not shown). These data imply that transport of MARCKS from lysosomes to the plasma membrane requires intact microtubules, whereas transport in the opposite direction does not.

Mutant MARCKS proteins lacking PKC phosphorylation sites are trapped in the plasma membrane

Further support for the hypothesis that MARCKS phosphorylation is required for its translocation from the plasma membrane to lysosomes was obtained using site-directed mutagenesis. We have described elsewhere the stable expression of various murine MARCKS cDNAs in mouse Ltk$^-$ fibroblasts which lack endogenous MARCKS (J.Seykora *et al.*, submitted). S7B is a clone expressing wild-type MARCKS, and Phos20 is a clone in which the serines which are phosphorylated by PKC have been mutated to alanine residues (J.Seykora *et al.*, submitted). When PKC was activated by PMA, MARCKS was highly phosphorylated in S7B, whereas the Phos20 protein was not (Figure 8), confirming the absence of PKC phosphorylation sites in this mutant. The trace amount of phosphate incorporated into Phos20 MARCKS (Figure 8), occurs on sites outside of the effector domain and is likely due to a proline-dependent kinase (Taniguchi *et al.*, 1994). [^{3}H]Myristic acid labeling (Figure 8) and Western blotting (data not shown) demonstrated that S7B and Phos20 contain comparable amounts of MARCKS.

The intracellular location of wild-type MARCKS in S7B was very similar to that of endogenous MARCKS in MEFs (Figure 9). In control cells MARCKS was distributed throughout the plasma membrane (Figure 9A). Upon activation of PKC (Figure 9C and D), or in the presence of NH_4Cl (Figure 9E and F), MARCKS was translocated from the plasma membrane onto lysosomes where it colocalized with Lamp-1. NH_4Cl induced the association of MARCKS with lysosomes in S7B more rapidly than in MEFs (half-time of 90 min versus ~8 h).

Like the wild-type protein, Phos20 MARCKS was located in the plasma membrane, including the ruffling edges (Figure 10A). In contrast to wild-type MARCKS, the non-phosphorylatable protein [phos$^-$] remained in the plasma membrane of cells treated with PMA (Figure 10C and D), consistent with the hypothesis that phosphorylation is required for MARCKS translocation to lysosomes. All staining patterns for S7B and Phos20 were confirmed using confocal microscopy (data not shown). Interestingly, phos$^-$ MARCKS did not accumulate on lysosomes in NH_4Cl-treated cells (Figure 10E and F), further suggesting that basal levels of phosphorylation shuttle MARCKS 'constitutively' through lysosomes, and that lysosomotropic agents trap the protein in this compartment.

Discussion

We have shown that MARCKS can cycle between the plasma membrane and lysosomes in fibroblasts, and that PKC-dependent phosphorylation shifts the equilibrium towards the lysosomal compartment. MARCKS resides primarily in the plasma membrane of resting MEFs (Figures 1–3 and Table II), and PKC activation results in MARCKS phosphorylation and its rapid redistribution onto lysosomes (Figures 1–3 and Table II). When MARCKS phosphorylation is prevented, either by mutation of the PKC phosphorylation sites (Figure 10), or by inhibition of the kinase with staurosporine (Figure 4), the protein remains in the plasma membrane. This is also supported by the observation that PKC down-regulation is accompanied by the redistribution of MARCKS from lysosomes to the plasma membrane (Figure 4).

Further evidence that MARCKS cycles between the plasma membrane and lysosomes was obtained using lysosomotropic agents and microtubule destabilizing drugs. MARCKS accumulates on lysosomes in cells treated with NH_4Cl or nocodazole, and returns to the plasma

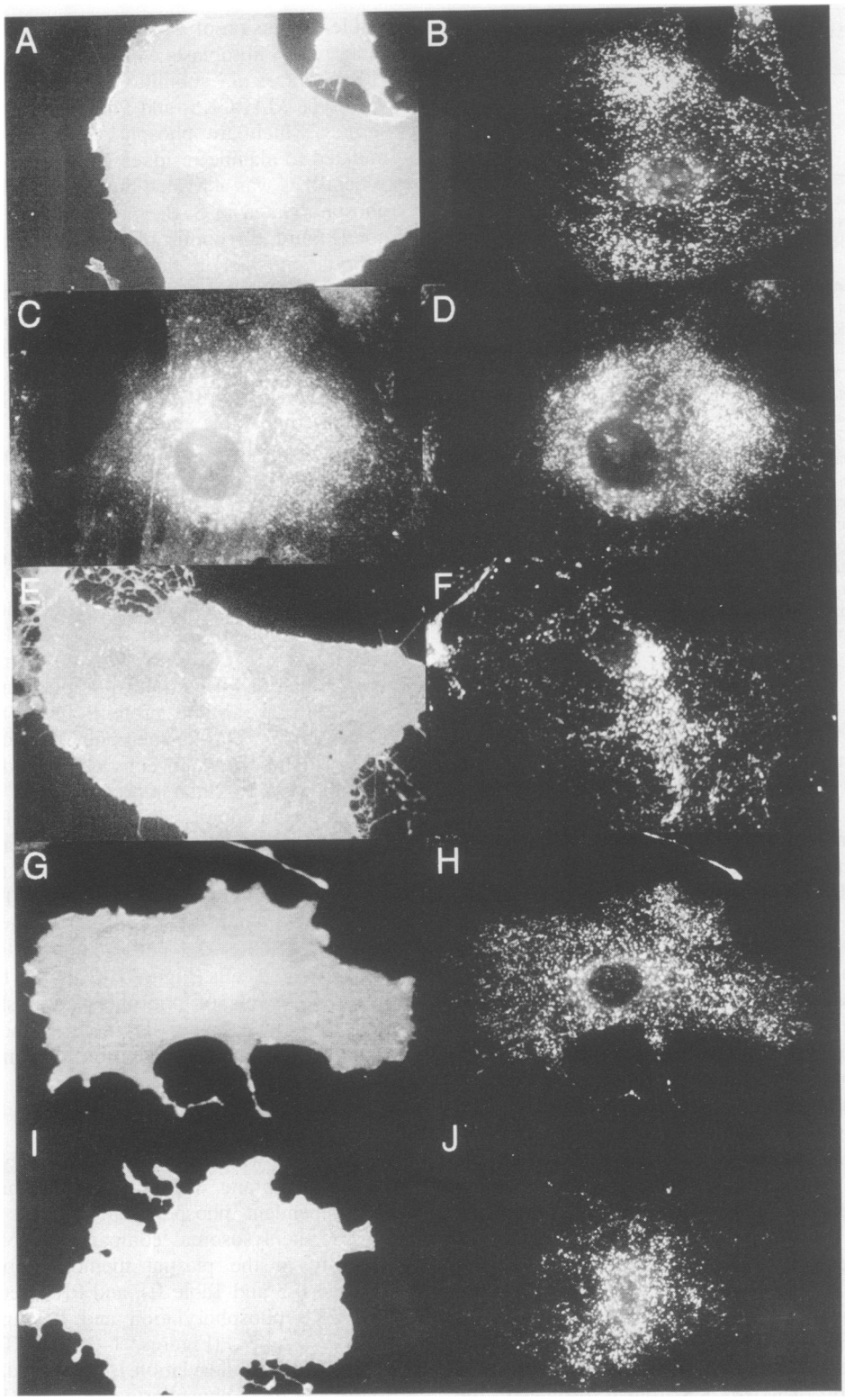

Fig. 4. Inhibition of PKC blocks the association of MARCKS with lysosomes. All cells were fixed, permeabilized and double-stained for MARCKS (A, C, E, G and I) and Lamp-1 (B, D, F, H and J) as described in the legend to Figure 2. (**A** and **B**) Control cells. (**C** and **D**) Cells treated with 200 nM PMA for 15 min. (**E** and **F**) MEFs treated with 200 nM PMA + 0.05 μg/ml staurosporine for 15 min. (**G** and **H**) cells treated with 2 μM PMA overnight to down-regulate PKC. (**I** and **J**) MEFs treated with 200 nM PMA for 15 min prior to addition of 0.05 μg/ml staurosporine for 30 min. The bar in C is 1 μm.

membrane upon drug removal (Figures 6 and 7). The observation that the phos$^-$ protein does not accumulate on lysosomes in NH_4Cl-treated cells (Figure 10) indicates that PKC-mediated phosphorylation is required for this movement of MARCKS. The relatively slow time course of MARCKS accumulation on lysosomes in the presence of these agents suggests that basal levels of PKC activity are sufficient to promote low level flux of MARCKS to

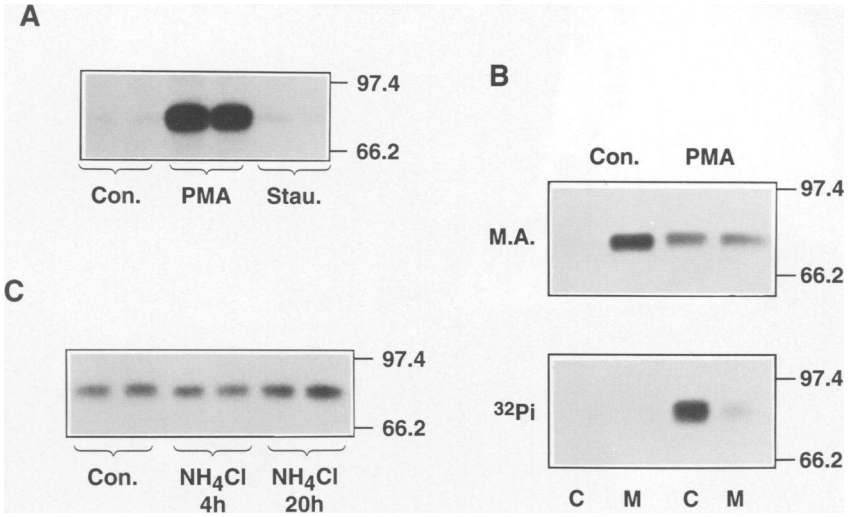

Fig. 5. Biochemical localization of MARCKS in MEFs. (**A**) Staurosporine blocks MARCKS phosphorylation by PKC. Duplicate cultures of cells were labeled for 3 h at 37°C with [^{32}P]orthophosphate, treated as described below, and MARCKS was immunoprecipitated from whole cell lysates using the RIPA method. Samples were resolved by 8% SDS−PAGE and the dried gel was exposed to X-ray film for 4 h at −70°C. Con., untreated cells; PMA, cells treated with 200 nM PMA for 15 min; Stau., cells treated with 200 nM PMA + 0.05 μg/ml staurosporine for 15 min. (**B**) PKC activation results in MARCKS phosphorylation and its release from membranes into the cytosol. Cells labeled with [^{3}H]myristic acid (upper panel) or [^{32}P]orthophosphate (lower panel) were left untreated (Con.) or exposed to 200 nM PMA for 15 min (PMA) prior to fractionation. MEFs were broken using nitrogen cavitation, separated into membrane (M) and cytosolic (C) fractions as described in the Materials and methods, and MARCKS was immunoprecipitated from each fraction as described above. M.A., myristic acid. (**C**) Ammonium chloride does not increase MARCKS phosphorylation. Duplicate cultures of control MEFs (Con.), or cells treated with 15 mM NH_4Cl for 4 or 20 h were labeled with [^{32}P]orthophosphate, and MARCKS was immunoprecipitated from whole cell lysates using the RIPA method. Samples were resolved by 8% SDS−PAGE and the gel was exposed to X-ray film for 16 h at −70°C. Note that the gel in (C) was exposed to film for four times as long as the gel in (A) in order to show the low basal level of MARCKS phosphorylation in MEFs.

Table II. Distribution of MARCKS in subcellular fractions

MEFFs		Percentage of total			
		N	P15	P400	S400
Control	protein	14.2 ± 0.6	25.1 ± 2.7	23.4 ± 3.9	37.3 ± 1.9
	5′-NTase	10.3 ± 3.9	25.0 ± 2.5	62.7 ± 6.9	1.8 ± 1.9
	APase	11.4 ± 1.0	52.5 ± 5.0	13.7 ± 0.6	22.5 ± 5.2
	MARCKS	8.8 ± 3.9	25.4 ± 2.7	46.3 ± 3.1	19.6 ± 3.2
PMA	protein	11.7 ± 1.1	29.8 ± 3.0	22.4 ± 2.4	36.2 ± 2.9
	5′-NTase	7.0 ± 2.7	29.2 ± 2.4	62.0 ± 7.3	1.9 ± 2.3
	APase	11.3 ± 1.4	60.7 ± 3.5	11.4 ± 1.5	16.7 ± 2.8
	MARCKS	9.0 ± 1.5	51.1 ± 1.2	15.2 ± 4.7	24.7 ± 2.4

Control MEFs or cells treated with 200 nM PMA for 60 min were fractionated as described in Materials and methods. Data are presented as the percentage of total activity, and each data set is the average ± standard deviation of four independent preparations. Abbreviations: 5′-nucleotidase (5′-NTase), acid phosphatase (APase), nuclei (N), mitochondria, lysosomes and peroxisomes (P15), plasma membrane, endoplasmic reticulum and microsomes (P400), cytosol (S400).

lysosomes. This is consistent with our electron microscopy data which show that ~12% of MARCKS associates with lysosomes in control cells (Figure 3 and Table I). The observation that soluble/phosphorylated MARCKS does not accumulate in the presence of nocodazole or NH_4Cl (Figure 5C and data not shown) is consistent with the idea that translocation is not synchronous, as is observed with PMA. Taken together, the data suggest that MARCKS cycles between the plasma membrane and lysosomes; MARCKS phosphorylation shifts the equilibrium towards lysosomes, lysosomotropic agents trap the protein in this compartment, and down-regulation of PKC or inhibition of the kinase promotes MARCKS reassociation with the plasma membrane. In addition, the finding that MARCKS transport from lysosomes to the plasma membrane requires intact microtubules (whereas transport in the opposite direction does not) further suggests that recycling of MARCKS to the plasma membrane may occur on vesicles transported along microtubules.

MARCKS may be transported from the plasma membrane to lysosomes via a soluble intermediate or by associating with endocytic membranes. We favor a soluble intermediate model for the following reasons. First, MARCKS accumulates much more rapidly on lysosomes in PMA treated cells (5–15 min) than do endocytic markers such as diLDL, lucifer yellow and ruby-dextran (Swanson *et al.*, 1985; Green *et al.*, 1987) (data not shown). Second, subcellular fractionation demonstrates that upon activation of PKC, MARCKS is phosphorylated and released from membranes into the cytosol (Figure 5B). This is consistent with our previous studies in neutrophils and macrophages which demonstrated that MARCKS is released from

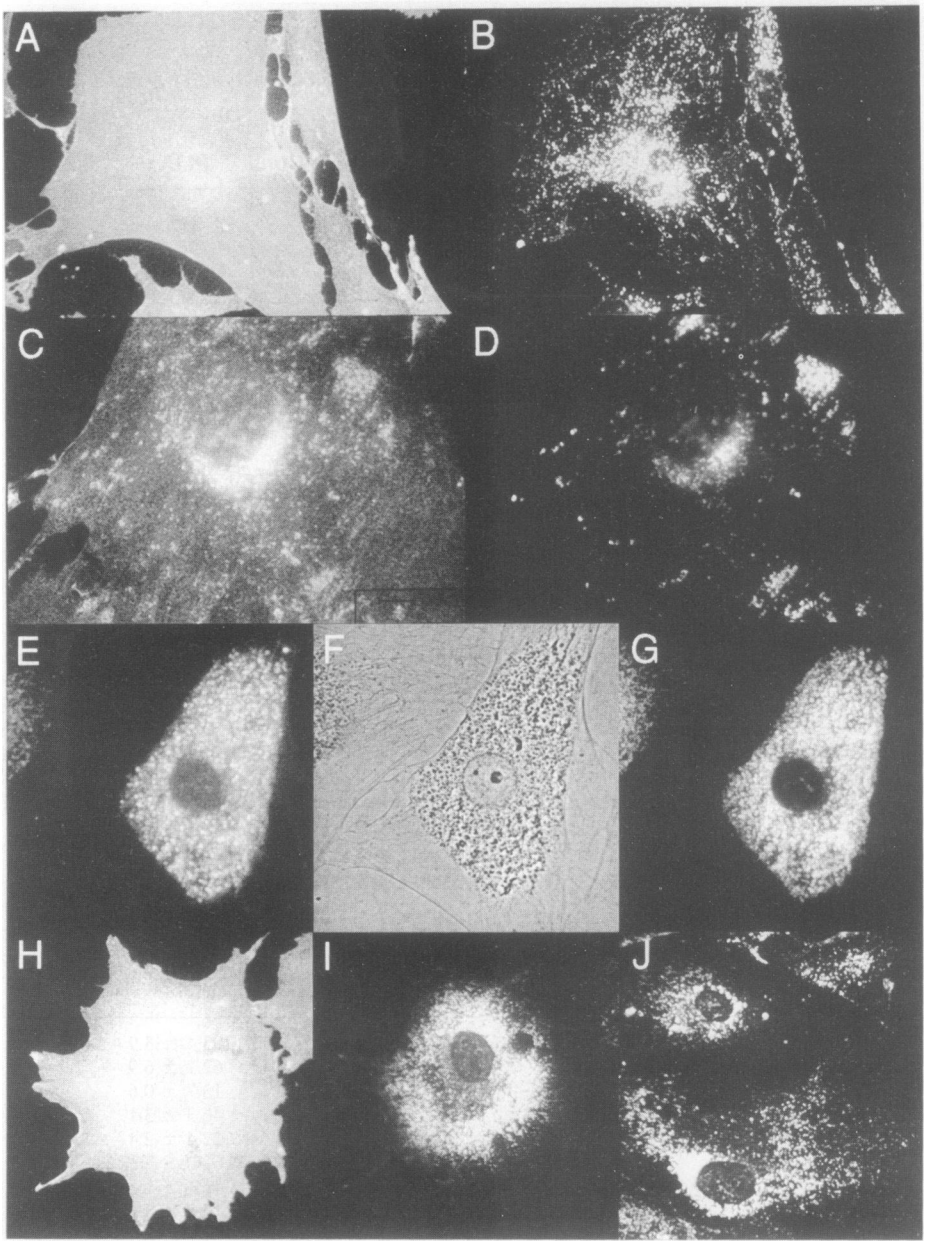

Fig. 6. Ammonium chloride treatment traps MARCKS on vacuolated lysosomes. MEFs were left untreated (**A** and **B**) or exposed to 15 mM NH_4Cl (**E**–**J**) prior to processing for indirect immunofluorescence microscopy. All cells were fixed, permeabilized and then double-stained for MARCKS (A, C, E, H) and Lamp-1 (B, D, G, I and J) using secondary antibodies conjugated to rhodamine and fluorescein, respectively. (**C** and **D**) Cells treated with NH_4Cl for 5 h. (**E**–**G**) Cells treated with NH_4Cl for 24 h. Phase micrograph (F) of the cells shown in (E) and (G) demonstrates that large vacuolated lysosomes are clustered around the nucleus and absent in the cell periphery. (**H**–**J**) Cells treated with NH_4Cl overnight were returned to normal medium for 3 h (H and I) or 12 h (J) prior to processing for immunofluorescence. Note that MARCKS returned to the plasma membrane (H) before lysosomes regained their normal size and distribution (compare I and J). The bar in C is 1 μm.

membranes upon phosphorylation and reassociates with membranes when it is dephosphorylated (Thelen *et al.*, 1991). However, we cannot exclude the possibility that phosphorylated MARCKS may bind loosely to lysosomal membranes such that the protein is released into the cytosol during our fractionation experiments. Third, loss of soluble/phosphorylated MARCKS could also explain the reduced number of anti-MARCKS gold particles detected in cryosections of PMA treated MEFs (Figure 3 and Table I). Fourth, NH_4Cl promotes the accumulation of MARCKS on lysosomes, despite its demonstrated property of inhibiting endosome/lysosome fusion (Mellman *et al.*, 1986). Fifth, MARCKS accumulates on lysosomes in nocodazole-treated cells, even though microtubules are required for transport from early to late endosomes (Gruenberg *et al.*, 1989). Thus, if MARCKS is being delivered to lysosomes via the usual endocytic pathway, NH_4Cl and nocodazole should inhibit its association with lysosomes, not stimulate it.

The precise mechanism by which phosphorylation would promote the translocation of MARCKS from the plasma membrane to lysosomes via a soluble intermediate is not clear. At least two possibilities exist. First, it is possible that MARCKS contains specific lysosomal targeting sequences that are unmasked by phosphorylation. Alternatively, phosphorylation may indirectly promote the

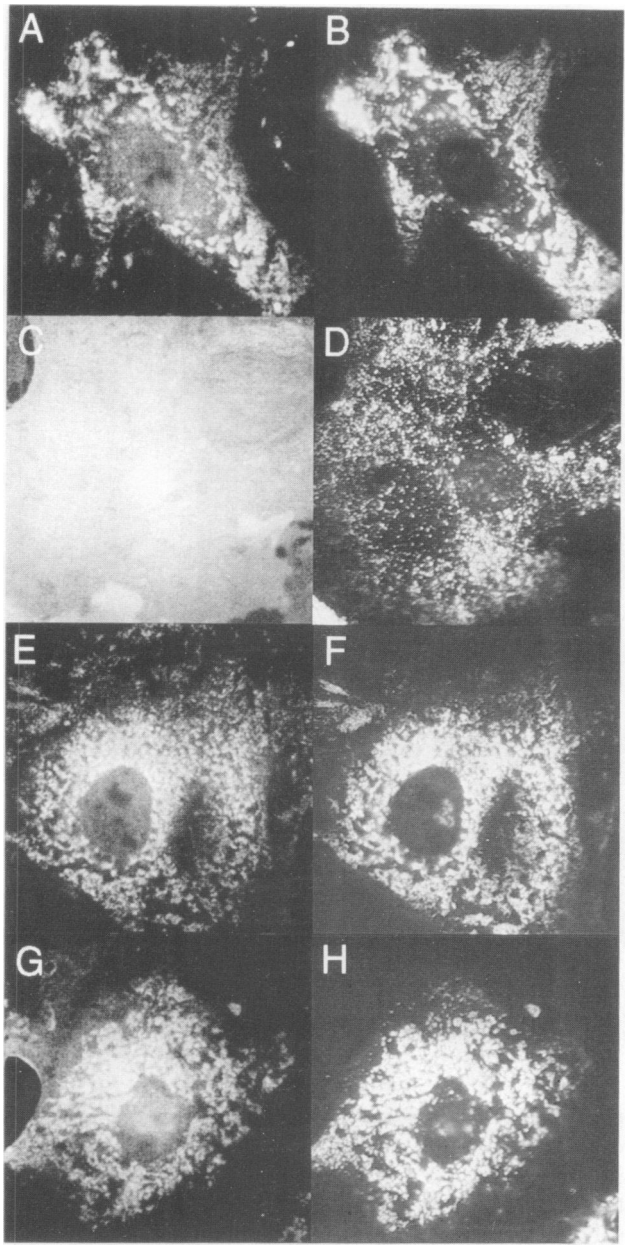

Fig. 7. Microtubules are required to return MARCKS to the plasma membrane. MEFs were exposed to 2 μg/ml nocodazole for 8 h (**A** and **B**), or nocodazole followed by a 4 h wash in drug-free medium (**C** and **D**). Alternatively, MEFs were treated with 10 mM NH_4Cl for 8 h, and then 2 μg/ml nocodazole + 10 mM NH_4Cl for 8 h (**E** and **F**). In (**G**) and (**H**) cells were treated as in (E) and (F), and then the NH_4Cl was washed out for 4 h in the continued presence of nocodazole. In all cases, cells were double-stained for MARCKS (A, C, E and G) and Lamp-1 (B, D, F and H) described for Figure 1.

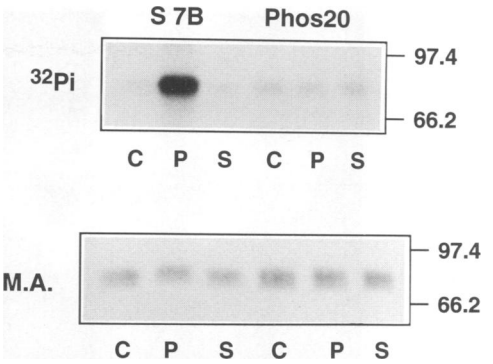

Fig. 8. Phos$^-$ MARCKS is no longer a PKC substrate. Cultures of S7B and Phos20 were labeled with [^{3}H]myristic acid or [^{32}P]orthophosphate at 37°C. MARCKS was immunoprecipitated from whole cell lysates using the RIPA method, samples were resolved by 8% SDS−PAGE, and gels were exposed to X-ray film at −70°C. Control cells (C), cells treated with 200 nM PMA for 15 min (P), cells treated with 200 nM PMA + 0.05 μg/ml staurosporine for 15 min (S). M.A., myristic acid.

binding of MARCKS to lysosomes by preferentially preventing its association with the plasma membrane. This second model would still require that MARCKS specifically targets to the plasma membrane and lysosomes, since the protein does not associate randomly with all intracellular membranes. MARCKS has two membrane-binding determinants: its myristoylated N-terminus confers hydrophobicity on the molecule; and the basic effector domain binds electrostatically to acidic phospholipids (George and Blackshear, 1992; Taniguchi and Manenti, 1993; Kim *et al.*, 1994). Phosphorylation prevents the electrostatic interaction by introducing negative charges into the effector domain, thereby markedly decreasing the affinity of MARCKS for the membrane. However, neither of these membrane-binding determinants confer specificity and it is likely that other domains of the protein specify targeting. This hypothesis is supported by the observation that a mutant form MARCKS, in which the amino acids between the myristoylation site and the effector domain have been deleted, is targeted incorrectly although its still associates tightly with membranes (J.Seykora *et al.*, submitted).

Nevertheless, we cannot exclude the possibility that a portion of MARCKS is internalized from the plasma membranes onto endosomes. The electron micrographs demonstrate that after PKC activation, there is an increase in the amount of MARCKS associated with Lamp-1-negative vesicles (Figure 3, right panels, and Table I). However, these small vesicles may be involved in transporting MARCKS from lysosomes to the plasma membrane. This hypothesis is particularly attractive since microtubule destabilizing agents inhibit the movement of MARCKS from lysosomes to the plasma membrane. We do not detect MARCKS on Lamp-1-negative vesicles using indirect immunofluorescence microscopy, perhaps because of the limited resolution of the light microscope. Bidirectional trafficking between lysosomes and the plasma membrane occurs at a high level in macrophages (Steinman *et al.*, 1983), but it may also occur at lower levels in other cell types. The integral membrane glycoprotein LEP100 is known to cycle between lysosomes and the plasma membrane in chicken embryo fibroblasts (Lippincott-Schwartz and Fambrough, 1987). In contrast to MARCKS, however, LEP100 is depleted from lysosomes in the presence of lysosomotropic agents and accumulates in the plasma membrane and endosomes (Lippincott-Schwartz and Fambrough, 1987).

It is unlikely that fibroblasts contain two independent pools of MARCKS, one on the plasma membrane, the other on lysosomes, and that phosphorylation promotes the release of the plasma membrane fraction resulting in an apparent increase in MARCKS on lysosomes. First, MARCKS is found in trace amounts on lysosomes of

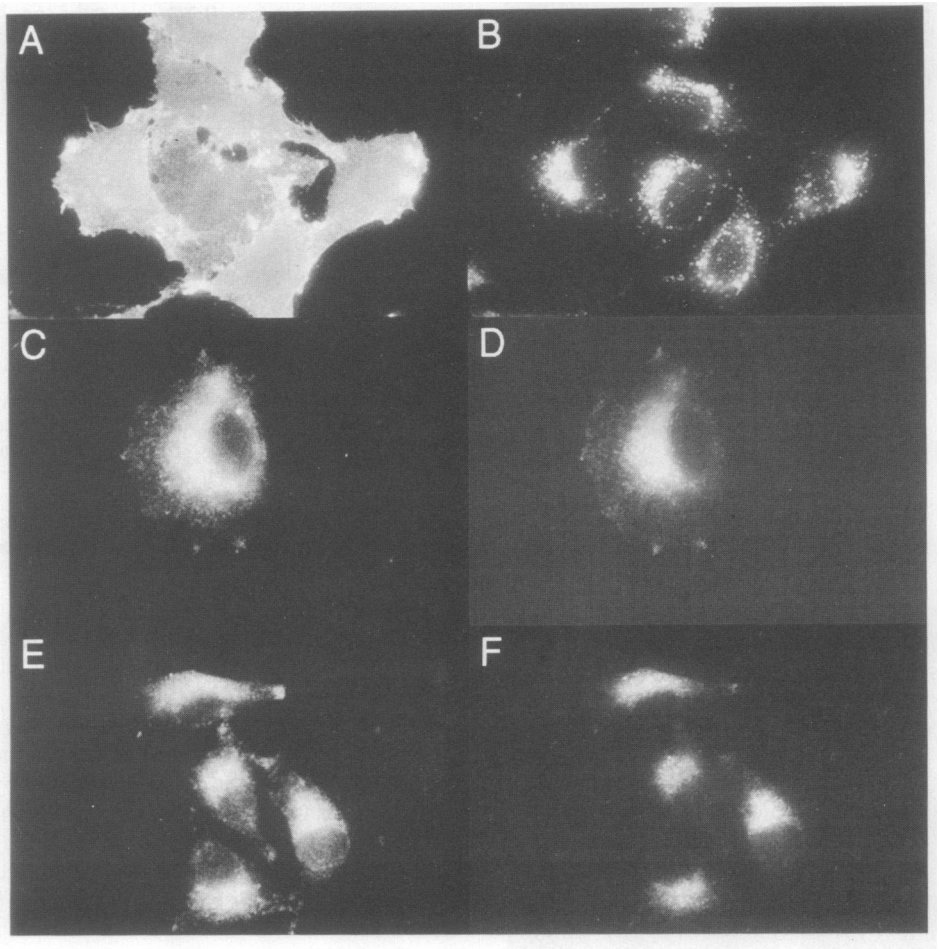

Fig. 9. Localization of wild-type MARCKS in S7B using indirect immunofluorescence microscopy. Cells were treated as described below, fixed and permeabilized, and double-stained for MARCKS (**A**, **C** and **E**) and Lamp-1 (**B**, **D** and **F**), as described in the legend to Figure 1. Cells were left untreated (A and B), treated with 200 nM PMA for 15 min (C and D), or treated with 30 mM NH_4Cl for 3 h (E and F) prior to processing for indirect immunofluorescence microscopy.

control MEFs by confocal microscopy, and increases dramatically upon treatment of cells with PMA (Figure 2). Second, the number of anti-MARCKS gold particles associated with lysosomes increases 4-fold upon activation of PKC (Figure 3 and Table I). Third, the distribution of MARCKS shifts from a light, plasma membrane-enriched membrane fraction to a dense, lysosome-enriched membrane fraction in PMA-treated cells (Table II). Fourth, MARCKS is detected exclusively in the plasma membrane in cells expressing the phos$^-$ protein (Figure 10) and in wild-type cells treated with staurosporine (Figure 4). Fifth, MARCKS accumulates on lysosomes in nocodazole- or NH_4Cl-treated cells, even though these agents do not increase the levels of soluble/phosphorylated MARCKS (Figure 5 and data not shown). These data suggest that the plasma membrane and lysosomal pools of MARCKS are in equilibrium, rather than being independent.

MacMARCKS/MRP, another member of the MARCKS family of myristoylated PKC substrates (Umekage and Kato, 1991; Blackshear *et al.*, 1992; Li and Aderem, 1992), also cycles between lysosomes and the plasma membrane. MacMARCKS is associated with the plasma membrane of quiescent macrophages, redistributes to lysosomes when the cells encounter aggregated bacterial lipopolysaccharides, and returns to the plasma membrane following activation of PKC (J.Li *et al.*, manuscript in preparation). Therefore, PKC-dependent phosphorylation drives MARCKS from the plasma membrane to lysosomes in fibroblasts, and drives MacMARCKS from lysosomes to the plasma membrane in macrophages. There are subtle differences in the membrane-binding domains of MARCKS and MacMARCKS, and they target differently in macrophages (L.Allen *et al.*, manuscript in preparation). This is reminiscent of the *rab* low molecular weight GTP-binding proteins, in which similar effector domains associate with different intracellular membranes (Bucci *et al.*, 1992; Van der Sluijs *et al.*, 1992; Simons and Zerial, 1993).

Dephosphorylated MARCKS crosslinks actin filaments, whereas phosphorylated MARCKS, or MARCKS bound to calcium/calmodulin, can bind actin filaments but cannot crosslink them (Hartwig *et al.*, 1992). MARCKS is phosphorylated during cell movement, phagocytosis and neurosecretion (Wu *et al.*, 1982; Rozengurt *et al.*, 1983; Blackshear *et al.*, 1986; Aderem *et al.*, 1988; Thelen *et al.*, 1990), where it has been postulated to regulate actin structure at the membrane as well as actin–membrane interactions (Aderem, 1992). MARCKS is found on phagosomes in macrophages (L.Allen *et al.*, manuscript in preparation), and is highly enriched in presynaptic junctions (Wang *et al.*, 1989), both systems in which the actin cytoskeleton is closely associated with membrane traffic

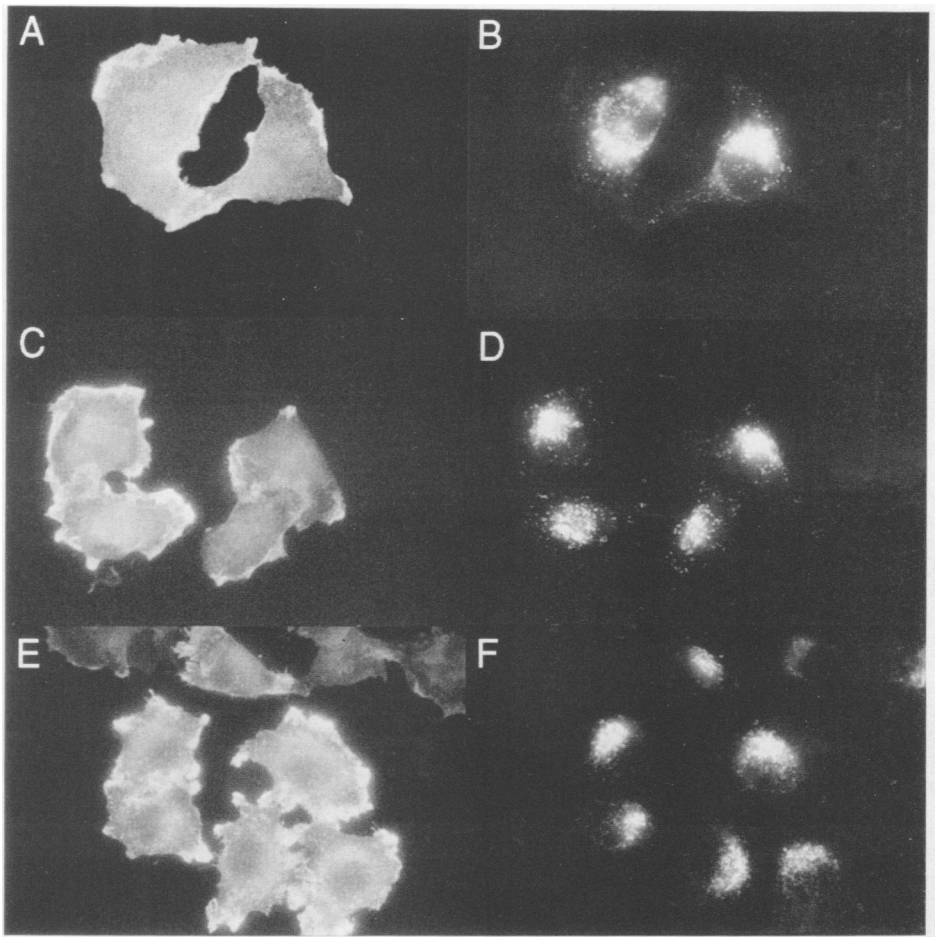

Fig. 10. Phos$^-$ MARCKS remains in the plasma membrane of cells treated with PMA or NH_4Cl. Cells were treated as described below, fixed and permeabilized, and double-stained for MARCKS (**A**, **C** and **E**) and Lamp-1 (**B**, **D** and **F**), as described in the legend to Figure 1. Cells were left untreated (A and B), treated with 200 nM PMA for 15 min (C and D), or treated with 30 mM NH_4Cl for 3 h (E and F) prior to processing for indirect immunofluorescence microscopy.

and membrane fusion. Indeed, MARCKS is phosphorylated and released from membranes during synaptic transmission (Wang *et al.*, 1989). Similarly, the synapsins are actin crosslinking proteins which are phosphorylated during synaptic transmission and which have a role in synaptic vesicle transport (Greengard *et al.*, 1993). It is intriguing that MARCKS, an actin-binding protein, cycles between the plasma membrane and lysosomes, given that trafficking between these compartments usually occurs on microtubules. Numerous recent studies suggest that there is extensive crosstalk between actin filaments and microtubules (reviewed by Goldstein and Vale, 1992). Thus, MARCKS provides additional complexity to a growing body of evidence demonstrating the interplay between the actin cytoskeleton and the microtubules. The fact that the interaction between MARCKS and actin, MARCKS and the plasma membrane, and MARCKS and lysosomes is regulated by PKC-dependent phosphorylation and by calcium/calmodulin suggests that this protein may have a role in linking these major signal transduction pathways to traffic between lysosomes and the plasma membrane.

Materials and methods

Materials

[^{3}H]Myristic acid (0.4–2.2 TBq/mmol), [^{32}P]orthophosphate (320 Tbq/mmol), [^{125}I]protein A (370 kBq/μg) and En^{3}hance were obtained from New England Nuclear (Boston, MA). [2-^{3}H]Adenosine 5′-monophosphate (851 Gbq/mmol) was from Amersham (Arlington Heights, IL). Leupeptin was obtained from Boehringer Mannheim (Indianapolis, IN). Hydromount was obtained from National Diagnostics (Manville, NJ). PMA was purchased from LC Services, Corp. (Woburn, MA). Unless indicated otherwise, all other chemicals were purchased from Sigma (St Louis, MO).

Cells and culture conditions

MEFs were prepared from 12–15 day ICR mouse embryos (Charles River Laboratories) by digestion with collagenase. MEFs were routinely used at passage two-six. S7B is a clone of Ltk$^-$ cells stably transfected with wild-type murine MARCKS cDNA, and Phos20 is a clone of Ltk$^-$ cells stably transfected with murine MARCKS cDNA in which the four serines in the phosphorylation domain have been mutated to alanine residues (J.Seykora *et al.*, submitted). MEFs were cultured at 37°C in DME supplemented with 1% L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin (all from JRH Biosciences, Lenexa, KS) and 10% heat-inactivated FBS (HyClone, Logan, UT) in an atmosphere of 5% CO_2 in air. S7B and Phos20 were cultured in the same medium supplemented with 400 μg/ml G418 (Gibco, Grand Island, NY). All cells were passaged using trypsin–EDTA.

PMA was used at 200 nM or 2 μM from a 0.5 mM stock solution, and staurosporine was used at 0.05 μg/ml from a 1 mg/ml stock solution, both in DMSO. NH_4Cl was used at 10–30 mM from a 450 mM stock solution in culture medium. Nocodazole was used at 2 μg/ml from a 2 mg/ml stock solution in ethanol. A 3 h incubation with nocodazole was sufficient to depolymerize all microtubules.

Immunofluorescence microscopy

Cells were grown on acid-washed round glass coverslips (Propper Manufacturing Company, Inc., Long Island City, NY) for 24–72 h prior

to analysis. After desired treatments, cells were fixed for 10 min at room temperature in 10% neutral buffered formalin solution (Sigma, St Louis, MO) and permeabilized in −20°C acetone for 5 min. Cells were incubated with primary antibodies for 1 h at room temperature in a humidified chamber. Coverslips were washed sequentially in eight medicine cups of PBS supplemented with 0.2 g/l sodium azide and 5 g/l bovine serum albumin (PAB), incubated with secondary antibodies for an additional hour, and then washed eight more times in PAB. Cells were attached to microscope slides using hydromount. Specificity of staining was assessed by omission of primary antibodies. All antibodies were diluted in PAB.

MARCKS was visualized using an affinity purified rabbit anti-murine MARCKS antibody (Rosen *et al.*, 1989, 1990) and a 1:600 dilution of rhodamine-conjugated goat anti-rabbit (Fab')2 secondary antibody (Tago, Burlingame, CA) or a 1:1000 dilution of a Texas red-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Eugene, OR). Lamp-1 was detected using a 1:100 dilution of rat monoclonal antibody 1D4B (NIH Developmental Studies Hybridoma Bank, Baltimore, MD) and a 1:40 dilution of FITC-conjugated goat anti-rat (Fab')2 secondary antibody (Tago, Burlingame, CA). The cation-independent mannose-6-phosphate receptor (CI-M6PR) was detected using 60 μg/ml of a rabbit anti-bovine antibody (the generous gift of Dr William J.Brown, Cornell University, Ithaca, NY) and a 1:600 dilution of a rhodamine-conjugated goat anti-rabbit (Fab')2 secondary antibody (Tago, Burlingame, CA). Antibody ABL-70, which recognizes a 125 kDa medial Golgi protein, and antibody H69, which recognizes a 76 kDa RER glycoprotein, were obtained from the NIH Developmental Studies Hybridoma Bank (Baltimore, MD).

Stained cells were photographed on a Zeiss Axiophot microscope using 63× Plan-Apochromat or 100× Plan-Neofluar objectives, rhodamine or fluorescein optics, and Kodak professional ASA 200 slide film. Confocal microscopy was performed using the Molecular Dynamics system and Image Space software in conjunction with a Zeiss Axioskop microscope. No MARCKS or Lamp-1 staining was detected in unpermeabilized cells.

Immunoelectron microscopy

Control MEFs or cells treated with 200 nM PMA for 15 min were washed twice with serum-free DME. Cells were fixed *in situ* with PLP fixative (McLean and Nakane, 1974) containing 4% paraformaldehyde and 0.01% glutaraldehyde for 1 h. The fixed cells were scraped off the dish with a rubber policeman, the pellet was rinsed with PBS and then resuspended in warm 10% gelatin in PBS. The pellet was fixed for another hour with the same fixative and infused with 2.3 M sucrose in PBS. The samples were then frozen in liquid nitrogen until use (Tokuyasu, 1973). Cryo-ultrathin sections were made with a glass knife in a Reichert-Jung FC-4E cryo-ultramicrotome (Microscopical Optical Consulting, Inc., Valley Cottage, NY) and rainbow colored sections were collected on Formvar carbon-coated nickel grids. The sections were treated with PBS containing 0.5% BSA and incubated with the affinity-purified anti-MARCKS antibody or the anti-Lamp-1 monoclonal antibody diluted in the same buffer. After buffer wash, the grids were incubated with goat anti-rabbit IgG 10 nm gold for MARCKS, or goat anti-rat 5 nm gold for Lamp-1. Gold conjugates were obtained from Amersham Life Sciences, Arlington Heights, IL. For dual labeling, the reagents were mixed together before incubation. All procedures were carried out at ambient temperature. Grids were processed and stained using published methods (Griffiths *et al.*, 1983). All micrographs shown are 50 000× magnification. A number of other protocols were also tested. However, none of these protocols preserved both membrane structure and MARCKS antigenicity.

Subcellular fractionation

Cells were grown in 100 mm dishes in 8 ml medium to ~80% confluence and labeled with [^{3}H]myristic acid or [^{32}P]orthophosphate as follows. For each dish of cells [^{3}H] myristic acid was dried under a stream of nitrogen, resuspended in 10 μl of ethanol and then sonicated into 200 μl culture medium. Cells were allowed to incorporate [^{3}H] myristic acid (20–40 μCi/ml) overnight prior to fractionation (see below). To label cells with $^{32}P_i$, MEFs were cultured overnight in serum-free DME. Serum-starved cells were depleted of phosphate by a 2 h incubation in phosphate-free RPMI (Gibco, Grand Island, NY) and subsequently labeled for 2 h in phosphate-free RPMI supplemented with 150 μCi/dish [^{32}P]orthophosphate. In some cases labeled cells were treated with 200 nM PMA for 5–60 min prior to fractionation.

MEFs labeled with $^{32}P_i$ or [^{3}H]myristic acid were fractionated into total membranes and cytosol as follows. Cell monolayers were rinsed twice with cold PBS and then scraped off each dish into 1 ml homogenization buffer (HB, 250 mM sucrose, 20 mM HEPES pH 7.2 and 1 mM EDTA) supplemented with protease inhibitors (0.09 TIU/ml aprotinin, 0.5 mg/ml leupeptin, 1 mM PMSF and 1 mM diisopropyl fluorophosphate). Cells were disrupted at 4°C by nitrogen cavitation (600 p.s.i., 10 min). Nuclei and unbroken cells were pelleted at 400 *g* for 10 min. The post-nuclear supernatants were centrifuged at 400 000 *g* for 20 min at 4°C in a Beckman TLA-100.2 rotor. Supernatants (cytosol) were removed and membrane pellets were resuspended by sonication into 1 ml HB. Membrane and cytosolic fractions were concentrated by precipitation with 10% TCA on ice and the TCA pellets were washed twice by sonication into −20°C acetone. Acetone pellets were resuspended in 30 μl dIH_2O and MARCKS was immunoprecipitated from each fraction with 6 μl of rabbit anti-murine MARCKS antiserum (Rosen *et al.*, 1989) using the RIPA method. Boiled immunoprecipitates were separated by 8% SDS−PAGE. ^{32}P was visualized by autoradiography and ^{3}H was visualized by fluorography of en^{3}hanced gels.

Cell lysates were prepared from 35 mm dishes of cells labeled with [^{32}P]orthophosphate or [^{3}H]myristic acid as described above. Cells were scraped into 100 μl of lysis buffer (10 mM Tris–HCl, pH 7.5, 15 mM EDTA, 50 mM KF, 50 mM NaH_2PO_4, 10 mM sodium pyrophosphate and 1% NP-40) supplemented with the protease inhibitors listed above. Nuclei were pelleted at 400 *g* for 5 min and MARCKS was immunoprecipitated from the postnuclear supernatants using the RIPA method.

For differential centrifugation experiments, four 100 mm or two 150 mm dishes of MEFs were used. Some cells were labeled with [^{3}H]myristic acid and/or treated with 200 nM PMA (as described above) prior to fractionation. Cell were detached by incubating monolayers in PBS + 1 mM EDTA for 15 min at 25°C. This treatment caused the cells to round up, thereby increasing the soluble pool of MARCKS over what is seen when spread cells are scraped off the culture surface (compare Figure 5B and Table II), but was necessary for efficient homogenization and preservation of organelle integrity. Cells were washed twice with HB to remove salts. Cell pellets were resuspended in 2 ml HB supplemented with 0.09 TIU/ml aprotinin, 0.5 mg/ml leupeptin, 1 mM PMSF, 1 mM diisopropyl fluorophosphate, 1 μg/ml antipain and 1 μg/ml pepstatin A, and broken by nitrogen cavitation (300 p.s.i., 10 min, 4°C) followed by five passses through a 27G needle. Nuclei and unbroken cells were pelleted at 400 *g* for 10 min at 4°C. Postnuclear supernatants were centrifuged at 15 000 *g* for 5 min at 4°C in a Beckman JA20 rotor to pellet mitochondria, lysosomes and peroxisomes. Remaining membranes were pelleted by centrifugation at 400 000 *g* for 20 min at 4°C in a Beckman TLA100.2 rotor.

Assays

Protein was determined using the Coomassie Plus Kit (Pierce, Rockford, IL). Acid phosphatase was assayed using a Sigma Acid Phosphatase Kit according to the manufacturer's instructions. 5'-nucleotidase was assayed according to the method of Avruch and Wallach (1971). The amount of 5'-nucleotidase associated with intact cells was comparable with that of cell lysates, indicating that the majority of the enzyme was associated with the plasma membrane. MARCKS was quantified by scanning fluorographs on a LKB UltroScan Enhanced Laser Densitometer.

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