Identification of the basolateral targeting determinant of a peripheral membrane protein, MacMARCKS, in polarized cells

Monn Monn Myat*†, Sandy Chang*, Enrique Rodriguez-Boulan‡ and Alan Aderem†

Background: Although the molecular determinants that specify the targeting of transmembrane proteins to the apical or basolateral membrane domains within polarized epithelial cells have been well characterized, very little is known about the targeting of peripheral membrane proteins within these cells. MacMARCKS is a member of the MARCKS family of protein kinase C (PKC) substrates. This myristoylated protein regulates actin structure at cell membranes and is essential for the morphogenic movement of neuroepithelial cells during the formation of the neural tube.

Results: MacMARCKS was specifically targeted to sites of cell–cell contact in the basolateral domain of polarized Madin–Darby canine kidney (MDCK) epithelial cells and was displaced from this location upon activation of PKC. We defined the basolateral targeting determinant of MacMARCKS to be the effector domain, a basic region spanning 24 amino acids and containing the PKC phosphorylation sites as well as binding sites for calmodulin and actin. This domain, in conjunction with a myristoyl moiety, was sufficient to target a non-membrane-associated protein – green fluorescent protein – specifically to the basolateral surface of polarized MDCK cells.

Conclusions: This is the first description of a specific amino acid sequence that specifies targeting of a peripheral membrane protein to the basolateral membrane in polarized epithelial cells.

Addresses: *The Rockefeller University, 1230 York Avenue, New York, New York 10021, USA. †University of Washington, Department of Immunology, Box 357650, Seattle, Washington 98195-7650, USA. ‡Margaret M. Dyson Vision Research Institute, LC-300 Cornell University Medical College, 1300 York Avenue, New York, New York 10021, USA.

Correspondence: Alan Aderem
E-mail: aaderem@u.washington.edu

Background

Cell polarity reflects an asymmetric distribution of plasma membrane constituents; often the membrane is divided into two or more domains which have different protein and lipid compositions [1–3]. The maintenance of cell polarity is crucial in specifying a variety of biological processes including embryonic development, differentiation, activation of the immune response, and vectorial transport of ions and solutes across cells [1–3]. Epithelial cells form a layer at the interface between the organism and the environment, or between an organ and a fluid space, and their polarized morphology facilitates their function both as a physical barrier and as a transport system. In general, polarized epithelial cells have distinct apical and basolateral domains. Although the molecular determinants that specify apical or basolateral targeting of transmembrane proteins within polarized epithelial cells have been well characterized, little is known about the targeting of peripheral membrane proteins to apical and basolateral domains within these cells.

MacMARCKS (also known as MARCKS related protein, MRP) is a member of the MARCKS family of protein kinase C (PKC) substrates and has been implicated in regulating adhesion, phagocytosis, membrane trafficking and the morphogenic movements accompanying embryonic development [4–6]. The protein has a basic effector domain which contains the serine residues that are phosphorylated by PKC and which binds calcium–calmodulin, F-actin and acidic phospholipids such as phosphatidylinositol 4,5-bisphosphate (PIP$_2$) [4,7,8]. MacMARCKS is myristoylated at its amino terminus and it binds to membranes using the cooperative binding energies contributed by the insertion of the myristic acid moiety into the lipid bilayer and by the interaction of the basic effector domain with phospholipids [4,9,10]. Deletion of the MacMARCKS gene in mice prevents cranial neurulation, and this is thought to be due to a defect in actin-based shape changes of the neuroepithelial cells that form the dorsolateral hinges of the developing neural tube [11,12]. The phenotype of the MacMARCKS null mouse prompted us to examine the biology of MacMARCKS in epithelial cells. Here, we report that MacMARCKS is specifically targeted to the basolateral membrane of Madin–Darby canine kidney (MDCK) epithelial cells and that it is displaced from this location after activation of PKC. We also demonstrate that the basolateral targeting determinant of MacMARCKS is contained within its effector domain.

Results and discussion

MacMARCKS is targeted to the basolateral membrane

MacMARCKS is expressed endogenously in the MDCK epithelial cell line; a polyclonal MacMARCKS-specific anti-serum immunoprecipitated a protein of the appropriate molecular mass from ^{3}H-myristic-acid-labeled MDCK cell lysates (Figure 1, lane 2). As expected, the phosphorylation of MacMARCKS was increased upon activation of PKC (Figure 1, lanes 3,4), and peptide mapping confirmed that the known PKC phosphorylation sites, serines 93 and 104, were phosphorylated ([4,7]; data not shown). In MDCK cells, serine 101 is not phosphorylated by PKC, and this was confirmed by the demonstration that mutation of serines 93 and 104 resulted in a nonphosphorylatable protein (data not shown). In the absence of PKC activation, approximately 90% of total cellular MacMARCKS protein partitioned with the membrane fraction (Figure 1, lanes 5,6). Upon activation of PKC, only an additional 5–10% of MacMARCKS was released from the membrane to the cytosol (Figure 1, lanes 7,8). This minimal release of MacMARCKS from the membrane has been noted previously in synaptosomes and PC12 cells [13] and contrasts with the release of the related MARCKS protein that is quantitatively released from the membrane when it is phosphorylated by PKC [9,14]. The difference in membrane-binding properties between MARCKS and MacMARCKS might be ascribed to the additional phosphorylation site in the effector domain of MARCKS and to the increased distance between the amino-terminal myristoyl moiety and the effector domain of the MARCKS protein [9].

Figure 1
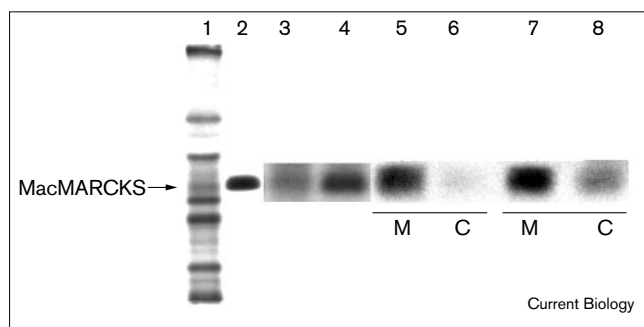
MacMARCKS is expressed endogenously in MDCK cells as analyzed by ^{3}H-myristic-acid-labeling followed by SDS−PAGE and autoradiography. Canine MacMARCKS was immunoprecipitated (lane 2) from ^{3}H-myristic-acid-labeled MDCK cell lysates (lane 1) using an anti-murine MacMARCKS polyclonal antiserum (see Materials and methods). The PKC-mediated phosphorylation of MacMARCKS increased in cells treated with phorbol myristate acetate, PMA (lane 4) compared with untreated cells (lane 3). In untreated MDCK cells (lanes 5,6), approximately 90% of total ^{3}H-myristic acid-labeled MacMARCKS was found in the membrane fraction (M), and 10% was found in the cytosol (C). In cells treated with PMA (lanes 7,8), approximately 80% of MacMARCKS was found associated with the membrane fraction and 20% with the cytosol fraction.

In polarized MDCK cells, endogenous MacMARCKS (Figure 2a) localized to sites of cell–cell contact in the basolateral membrane, where co-staining of MacMARCKS and the adhesion protein E-cadherin was observed (Figure 2b). Additional MacMARCKS staining was also seen on intracellular vesicles in the basolateral domain (Figure 2a). The localization of MacMARCKS to sites of cell–cell contact depended upon ligated E-cadherin (Figure 2c–f). Homotypic interaction between cadherin molecules is calcium-dependent; in low calcium media

Figure 2
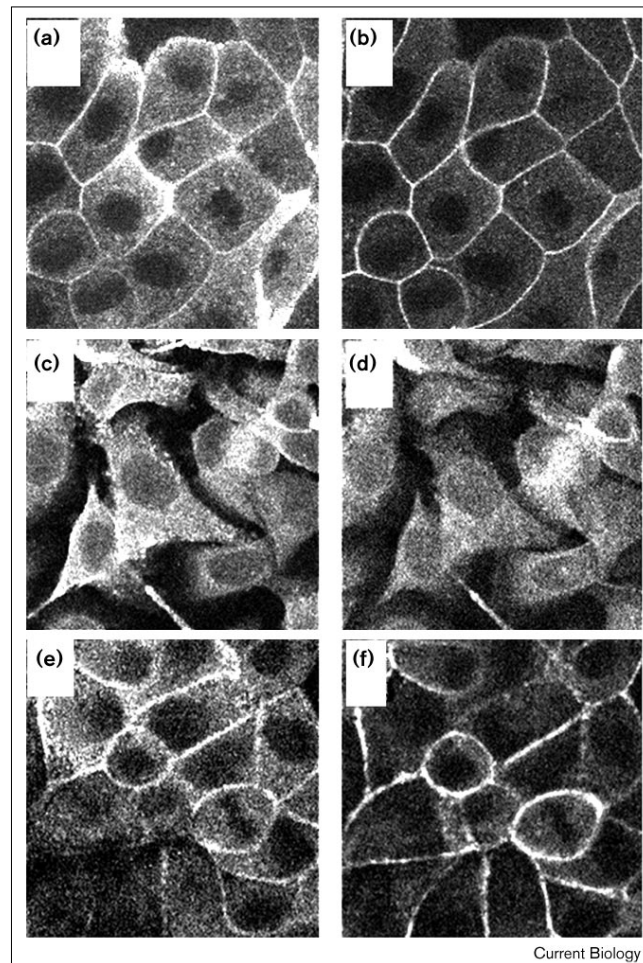
MacMARCKS is targeted to the basolateral membrane in polarized MDCK cells as revealed by immunofluorescence staining of **(a,c,e)** MacMARCKS and **(b,d,f)** E-cadherin, a component of the adherens junctions in the basolateral domain. (a,b) In polarized MDCK cells grown on filter supports for 5 days, endogenous MacMARCKS was found in the basolateral domain at sites of cell–cell contact, where it colocalized with E-cadherin. MacMARCKS also localized to a population of basolateral vesicles. (c,d) When MDCK cells were incubated for 18 h in low calcium medium, MacMARCKS and E-cadherin were found on intracellular vesicles. (e,f) Upon switching the cells from low calcium medium to normal calcium medium, MacMARCKS and E-cadherin once again colocalized at sites of cell–cell contact.

cell–cell contacts are not formed [15]. In cells lacking cell–cell contact due to incubation in low calcium media, MacMARCKS and E-cadherin were distributed throughout the cell in a vesicular pattern (Figure 2c,d). Upon addition of calcium to the medium, cell–cell contacts formed and MacMARCKS and E-cadherin were redistributed to sites of cell–cell contact (Figure 2e,f).

Identification of the basolateral targeting determinant of MacMARCKS

In order to identify the basolateral targeting domain of MacMARCKS, a series of hemagglutinin (HA) epitope-tagged mutant molecules were constructed (Figure 3), and stably expressed in MDCK cells. HA-tagged wild-type MacMARCKS (WT-MM) colocalized with E-cadherin at sites of cell–cell contact in the basolateral domain in an identical pattern to the endogenous protein, indicating that the HA epitope did not interfere with targeting (Figure 4a,b). Pixel intensity measurements revealed that more than 90% of MacMARCKS was localized to the basolateral domain (Figure 4h). The targeting of Mac-MARCKS to the basolateral membrane was confirmed biochemically in purified basolateral and apical membranes [16]. Densitometric measurements showed that approximately 70% of MacMARCKS partitioned with basolateral membranes, 25% associated with apical membranes, and 5% was found in the cytosol (Figure 4g and data not shown). The 25% of MacMARCKS found in the apical membranes was probably due to contamination of this preparation with basolateral membranes, because it paralleled the distribution of E-cadherin (approximately 20%; Figure 4g and data not shown). Extraction of MDCK cells with 0.5% Triton X-100 showed that 40% of Mac-MARCKS (Figure 4c) remained associated with the basolateral cytoskeleton identified by E-cadherin (Figure 4d and data not shown).

The MH2 domain and the effector domain of Mac-MARCKS are highly conserved among different species [7]. The MH2 domain does not participate in targeting MacMARCKS to the basolateral membrane, because an HA-tagged mutant version of MacMARCKS that lacks the MH2 domain (MM-ΔMH2) was targeted to the basolateral membrane (Figure 4e,f). This result was confirmed by analyzing purified apical and basolateral membrane preparations and by a pixel intensity profile of optical sections of MDCK cells (Figure 4g,i).

Activation of PKC by phorbol esters prevented the specific targeting of MacMARCKS to the basolateral membrane, thereby allowing it to be distributed to both the apical and the basolateral membranes (Figure 5). PKC activation did not alter the expression level of Mac-MARCKS, nor was the redistribution of MacMARCKS affected by protein synthesis inhibitors, indicating that the MacMARCKS found in the apical membranes was not

Figure 3
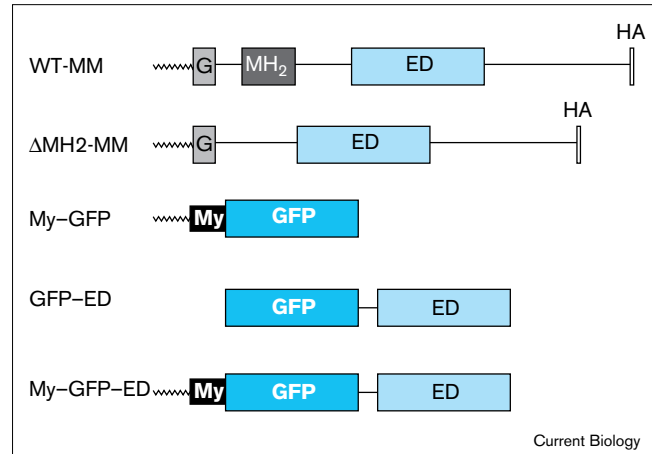
Schematic diagram of wild-type and mutant MacMARCKS proteins, and green fluorescent protein (GFP) fusion proteins. Wild-type MacMARCKS (WT-MM) was epitope-tagged at the carboxyl terminus with the 9 amino-acid hemagglutinin (HA) epitope tag. ΔMH2-MM represents a deletion mutant of WT-MM, lacking the MH2 domain. My–GFP contains 12 amino acids of the myristoylation sequence of recoverin (My) fused to the amino terminus of GFP. GFP–ED contains the effector domain (ED) of MacMARCKS fused to the carboxyl terminus of GFP. My–GFP–ED represents GFP flanked at the amino terminus with the recoverin myristoylation sequence and at the carboxyl terminus with the effector domain of MacMARCKS.

derived from new protein synthesis (data not shown). To assess the role of MacMARCKS phosphorylation in preventing its specific association with the basolateral cytoskeleton, we generated a nonphosphorylatable form of MacMARCKS. This was achieved by mutating serines 93 and 104, which are phosphorylated by PKC in MDCK cells, to alanine residues (M.M.M., E.R-B. and A.A., unpublished observations). This nonphosphorylatable MacMARCKS mutant was also not targeted exclusively to the basolateral membrane, and instead localized to the entire plasma membrane (M.M.M., E.R-B. and A.A., unpublished observations). In data to be reported elsewhere, we demonstrate that MDCK cells expressing the nonphosphorylatable MacMARCKS mutant are unable to establish normal polarity, rendering it impossible to compare the localization of the mutant MacMARCKS to its wild-type counterpart. This therefore suggests that phosphorylation of MacMARCKS abrogates its specific targeting to the basolateral membrane. It is also possible that PKC phosphorylates other cellular components that prevent the specific localization of MacMARCKS to the basolateral membrane.

Targeting of MacMARCKS–GFP fusion proteins
The observations described above implicate the effector domain in basolateral targeting of MacMARCKS, as phosphorylation of this region disrupts the restriction of the protein to the basolateral domain. An effector domain
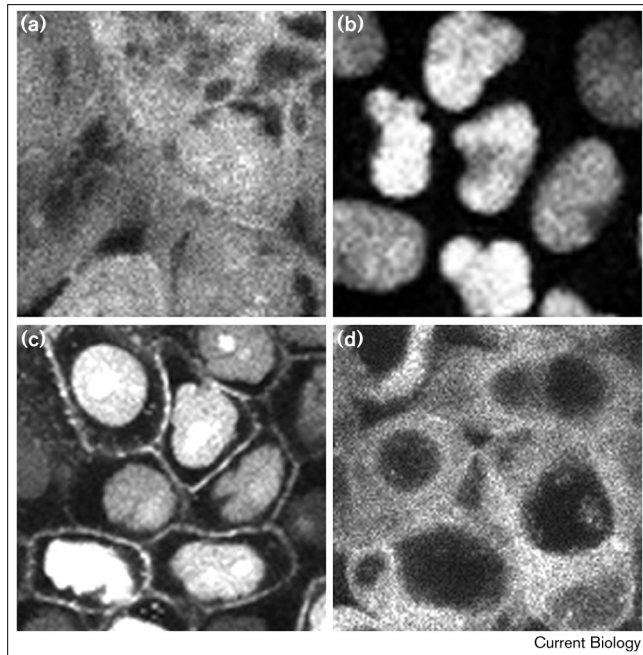
Figure 4
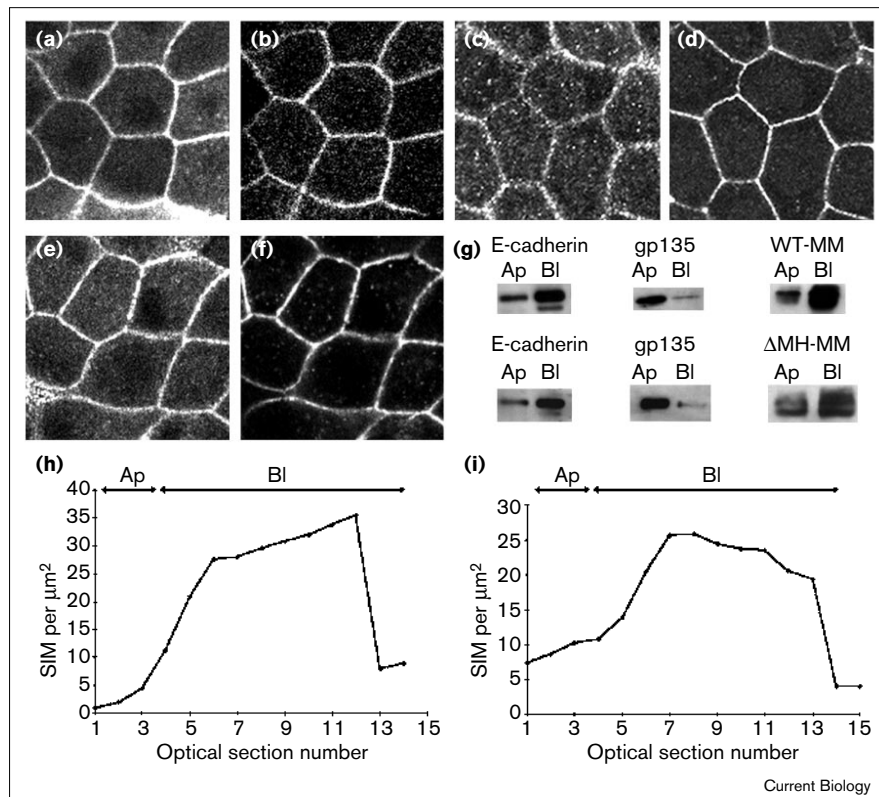
Localization of HA-tagged wild-type and ΔMH2 MacMARCKS. Immunofluorescence staining of **(a,c,e)** MacMARCKS proteins and **(b,d,f)** E-cadherin in transfected MDCK cells. (a,b) WT-MM colocalized with E-cadherin at sites of cell–cell contact in the basolateral domain. (c,d) In cells extracted with 0.5% Triton X-100, a pool of WT-MM remained Triton-insoluble on the basolateral membrane marked by E-cadherin. (e,f) ΔMH2-MM also colocalized with E-cadherin at sites of cell–cell contact in the basolateral domain. **(g)** Immunoblot of the apical marker gp135, the basolateral marker E-cadherin, and WT-MM and ΔMH2-MM in purified apical (Ap) and basolateral (Bl) membranes. **(h,i)** Pixel intensity profiles of MDCK cells expressing (h) WT-MM or (i) ΔMH2-MM. SIM represents the sum of intensity measurements.

deletion mutant of MacMARCKS could not be expressed in MDCK cells (data not shown). We therefore attempted to target green fluorescent protein (GFP) to the basolateral domain by fusing it to the effector domain of Mac-MARCKS. GFP alone was not targeted to a distinct domain in polarized MDCK cells, and instead was found in a diffused pattern throughout the cytoplasm (Figure 6a).

Fusion of the MacMARCKS effector domain to the car-boxyl terminus of GFP (GFP–ED; see Figure 3) targeted the fusion protein to the nucleus (Figure 6b). This occurs because the basic effector domain resembles a nuclear localization signal, which is usually masked by the carboxyl terminus of MacMARCKS (A.A., unpublished observations). Because myristoylation is absolutely required for

Figure 5
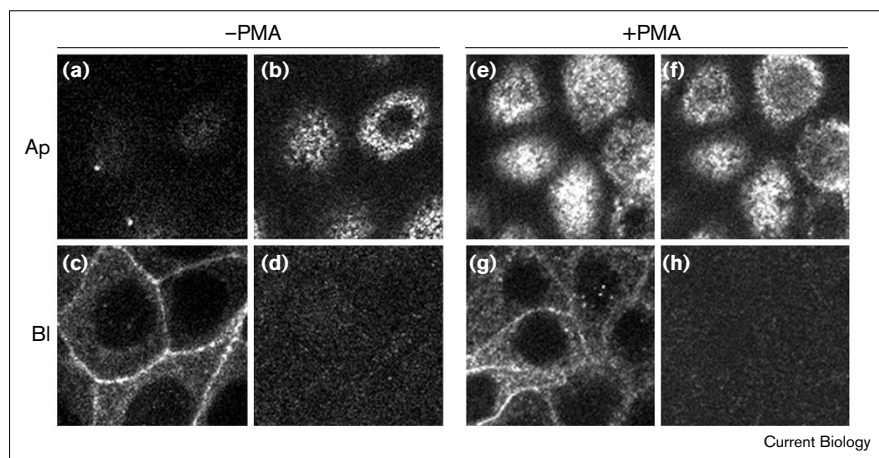
Activation of PKC prevents the specific targeting of MacMARCKS as revealed by immunofluorescence staining of **(a,c,e,g)** WT-MM and **(b,d,f,h)** the apical marker gp135 in (a,b,e,f) apical sections and (c,d,g,h) basolateral sections of MDCK cells. (a–d) In control untreated MDCK cells (–PMA), WT-MM was absent from the apical domain, marked by gp135, and was present in the basolateral domain where gp135 was absent. (e–h) In PMA-treated cells (+PMA), WT-MM was found in the apical domain marked by gp135, and in the basolateral domain where gp135 was absent. The apical optical sections identified by the gp135 marker were separated by 6 μm from the basolateral sections identified by E-cadherin (data not shown). Ap, apical; Bl, basolateral.

Figure 6

The effector domain of MacMARCKS targets myristoylated GFP to the basolateral domain. MDCK cells were transfected with the GFP constructs shown in Figure 3. **(a)** GFP alone was found in a diffuse pattern in the cytoplasm, whereas **(b)** GFP–ED was targeted to the nucleus. **(c)** My–GFP–ED was targeted to sites of cell–cell contact in the basolateral domain, and was also found in the nucleus. **(d)** My–GFP localized exclusively to intracellular membranes.

the membrane binding of MacMARCKS, we added a myristoyl moiety to the GFP–ED fusion protein (My–GFP–ED; see Figure 3) to determine whether membrane binding is a requisite for basolateral targeting. The myristoylation consensus sequence of the unrelated protein, recoverin [17], was used to avoid introducing possible targeting signals of MacMARCKS that may reside at its amino terminus. Indeed, the MacMARCKS effector domain, in conjunction with a generic myristoylation sequence, targeted GFP to sites of cell–cell contact in the basolateral domain, as well as to the nucleus (Figure 6c), but not to the apical domain (data not shown). Activation of PKC resulted in the phosphorylation of My–GFP–ED, and prevented its association with the basolateral membrane (data not shown). However, in these cells the bulk of the fusion protein was still found in the nucleus (data not shown). Most of the nuclear fraction of the My–GFP–ED fusion protein was not myristoylated (data not shown). Myristoylated GFP lacking the MacMARCKS effector domain (My–GFP; see Figure 3) associated mainly with intracellular membranes (Figure 6d and data not shown).

Conclusions

The effector domain of MacMARCKS targets the protein to the basolateral membrane of polarized cells. PKC-mediated phosphorylation of this domain prevents this interaction and allows the protein to distribute randomly on cellular membranes. PKC activation in polarized MDCK cells is accompanied by weakened cell–cell interactions, as evidenced by decreased electrical resistance across the monolayer [18,19]. In data to be reported elsewhere, we show that expression in MDCK cells of a MacMARCKS mutant in which the PKC phosphorylation sites within the effector domain have been changed from serine to alanine profoundly modifies cell–cell contact. Thus, targeting of MacMARCKS to sites of cell–cell contact at the basolateral membrane of polarized epithelial cells places it in the correct context to mediate the effects of PKC on cell–cell adhesion.

Materials and methods

Radiolabeling and immunoprecipitation of MacMARCKS
MDCK cells, grown to confluence on plastic dishes, were incubated overnight with 40 μCi/ml ^{3}H-myristic acid [7]. For ^{32}P-orthophosphate labeling, MDCK cells were incubated in phosphate-free RPMI (Life Technologies) for 1.5 h, and labeled for 1.5 h with 0.1 mCi ^{32}P-orthophosphate per confluent 35 mm dish of cells. Where indicated, labeled cells were treated with 200 nM PMA for 30 min prior to lysis [7]. Immunoprecipitations of MacMARCKS from radiolabeled MDCK cell lysates were performed using a polyclonal antibody raised against a purified GST–MacMARCKS fusion protein [7], as previously described [13]. Two-dimensional thermolytic phosphopeptide mapping of Mac-MARCKS immunoprecipitated from MDCK cells was carried out as described previously [7]. Protein synthesis was inhibited by incubating MDCK cells with 0.05 mg/ml cycloheximide for 1 h and immunoblotting the cell lysates for MacMARCKS as described previously [13].

Fractionation of MDCK cells
MDCK cells were disrupted by hypotonic shock by placing them in a buffer containing 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.09 TIU/ml aprotinin, 0.5 mg/ml leupeptin, and 1 mM PMSF. Bulk membrane and cytosol fractions were prepared by centrifuging the lysed cells at $100,000 \times g$ for 30 min to separate the cytosol fraction (supernatant) from the membrane fraction (pellet). The membrane pellet was resuspended in the homogenization buffer, and equivalent amounts of protein were analyzed by SDS–PAGE and immunoblotting. The amount of MacMARCKS in the cytosol and membrane fraction was quantified by densitometry.

Immunofluorescence
MDCK cells grown in DMEM with 1% L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin (all from JRH Biosciences) and 10% heat-inactivated FBS (HyClone), were plated at a density of 1×10^5 cells per 12 mm Transwell filter insert (Costar) and allowed to grow for 5 days before processing for microscopy. Cells were fixed in 2% paraformalde-hyde and permeabilized in 0.075% saponin. After blocking in PBS supplemented with 0.1 mM CaCl$_2$, 1 mM MgCl$_2$, and 0.05% milk, cells were incubated in primary and secondary antibodies. Processed filters were then mounted onto microscope slides in Vectashield (Vector Laboratories). Endogenous MacMARCKS was detected with an affinity-purified polyclonal antibody raised against a purified GST–MacMARCKS fusion protein [7]. HA-tagged MacMARCKS proteins were detected with a polyclonal rabbit anti-HA antibody (Babco). E-cadherin was detected with a pan-cadherin antibody (Sigma) and gp135 with a monoclonal anti-gp135 antibody (a kind gift of G. Ojakien). F-actin was detected either with FITC–phalloidin or TRITC–phalloidin (Molecular Probes). Rabbit polyclonal antibodies were visualized with either a Texas-red-conjugated goat anti-rabbit secondary antibody (Molecular Probes) or a FITC-conjugated goat anti-rabbit (Fab'$_2$) immunoglobulin (Ig) G antibody (Tago) and mouse monoclonal antibodies were visualized with a FITC-conjugated goat

anti-mouse IgG plus IgM antibodies (Jackson ImmunoResearch Laboratories) or Texas-red-conjugated goat anti-mouse antibodies (Molecular Probes). Where indicated, cells were treated with 200 nM PMA (LC Services), for 30 min prior to processing for immunofluorescence. Cells containing GFP fusion proteins were fixed and mounted onto microscope slides as described above.

Confocal microscopy and measurement of pixel intensity profiles
Confocal images were acquired using a Zeiss Axiovert microscope and the MRC-1024 Lasersharp software (Bio-Rad Laboratories). For measurements of pixel intensity profiles, a series of 0.2 μm confocal optical sections were acquired and the pixel intensity profiles of selected areas were obtained using the MRC-1024 Lasersharp Software. The sum of intensity measurements/μm^2 was quantitated and graphs were generated using Microsoft Excel software.

Calcium switch assay
MDCK cells were plated at 1×10^5 cells per 12 mm Transwell filter and allowed to adhere for 2 h in DMEM (10% FBS) containing a normal concentration (1.8 mM) of calcium. Cells were then washed gently in low calcium medium (LCM; MEM modified for suspension cultures which lacks CaCl$_2$ and contains less than 5 μM Ca^{2+}, Gibco BRL). Cells were incubated for 18 h in LCM containing dialyzed 5% FBS (Gibco BRL). The calcium switch was begun by replacing LCM with DMEM (1.8 mM Ca^{2+}) containing 10% FBS for the indicated times before visualization by microscopy.

Generation of MacMARCKS mutants and their expression in MDCK cells
A wild-type MacMARCKS cDNA construct (WT-MM) containing a HA epitope YPYDVPDYA sequence (in single-letter amino acid code) at the 3′ end was generated by PCR using the 5′ oligo A1 5′-GCGGGATC-CATGGGCAGCCAGAGCTCT-3′ and the 3′ oligo A2 5′-GCGGG-ATCCTCACAAGCTAGCGTAATCTGGAACATCGTATGGGTACTCAT TCTGCTCAGCACT-3′. Mouse MacMARCKS cDNA [7] served as the template. For high levels of expression in MDCK cells, all HA-tagged constructs were subcloned into the pcDNA3 vector (Invitrogen) under the control of the cytomegalovirus promoter. The serine to alanine mutations within the MacMARCKS effector domain (nonphosphorylatable MacMARCKS) were generated using two DNA fragments spanning this domain that were generated by PCR using WT-MM as the template. The first fragment encoded amino acids 1–100, and was generated with the 5′ A1 oligo and 3′ B1 oligo (5′-AAGCTTGAAAGGCTTCTTGAAACGA-GAATTTCTTC-3′), and the second DNA fragment encoded amino acids 99–200 as well as the 11 amino acid HA epitope, and was generated with the 5′ B2 oligo 5′-AAGCTTAGTGGCCTGGCCTTCAAGAGAAAT-3′ and the 3′ A2 oligo. All PCR reaction products were subcloned into pBlueScript. For construction of the ΔMH2-MM mutant, an *Mlu*I site was engineered into PCR primers to generate a PCR fragment containing sequences carboxy-terminal of the MH2 domain. This fragment was then ligated to the existing *Mlu*I site at the amino terminus of the MH2 domain.

To generate the My–GFP construct, the MMXC1 oligo (5′-AATTCATGGGAACAGCAAGAGTGGGGCCCTGCA-3′), and the MMXC2 oligo (5′-GGGCCCCACTCTTGCTGTTCCCCATG-3′) that corresponded to the amino-terminal myristoylation sequence of recoverin [17] were annealed and linked to the 5′ end of the GFP gene in the pEGFP vector (Clontech). To generate the GFP–ED construct, the effector domain of MacMARCKS was first obtained by PCR using the MMXC3 (5′-ATTGTACAAGGGGGAGGTCGCCCCCAAGGAG-3′) and MMXC4 (5′-ATTGTACATTATCACCCCCCACCCGCCGGC-3′) oligos, and wild-type HA-tagged MacMARCKS as the template. The effector domain was then ligated onto the 3′ end of the GFP gene in the pEGFP vector. The My–GFP–ED construct was generated by ligating the effector domain of MacMARCKS to the 3′ end of the My–GFP construct. For high levels of expression in MDCK cells, all HA-tagged constructs were subcloned into the pcDNA3 vector (Invitrogen) under

the control of the cytomegalovirus promoter. Stable MDCK cell lines were generated using the calcium-phosphate transfection method [20].

Triton X-100 extraction
For immunofluorescence, polarized MDCK cells grown on Transwell filters were extracted in CSK (cytoskeleton) buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.01% NaN$_3$, 2 mM EDTA with protease inhibitors, aprotinin, leupeptin and PMSF) with 0.5% Triton X-100 for 10 min at 4°C, and washed twice with PBS-CM (PBS supplemented with 0.1 mM CaCl$_2$ and 1 mM MgCl$_2$) before being processed for immunofluorescence. For immunoblots, polarized cells grown for 5 days on 12 mm Transwell filters were extracted in CSK buffer as described above and cell lysates were centrifuged at 48,000 rpm for 10 min. The supernatants (Triton-soluble) were removed, and the Triton-insoluble pellets were resuspended in CSK buffer. Equivalent amounts of total protein for Triton-soluble and Triton-insoluble samples were analyzed by immunoblotting, as described above.

Purification of apical and basolateral membranes
Semi-purified apical and basolateral membranes were obtained as described previously [16]. Cells grown on 24 mm Transwell filters (Corning) were coated with cationized colloidal silica (a kind gift of B. Jacobson) in Mes/saline (Sigma) and polyacrylic acid (Polysciences), and overlaid with a glass slide that had been previously coated with poly-L-lysine (Sigma). The filter and the glass slide were pressed together using a rolling pin, the filter was peeled apart rapidly, and the transferred apical membranes on the glass slide were scraped into homogenization buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, and 1 mM EGTA). The apical membranes were precipitated in 10% TCA and washed three times with −20°C acetone. Equivalent amounts of total protein for apical and basolateral samples were analyzed by immunoblotting as described previously [13]. E-cadherin, gp135, HA-tagged proteins, wild-type and ΔMH2-MM MacMARCKS were detected with the antibodies described above.

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