Quantitative Proteomics Analysis of Human Endothelial Cell Membrane Rafts

EVIDENCE OF MARCKS AND MRP REGULATION IN THE SPHINGOSINE 1-PHOSPHATE-INDUCED BARRIER ENHANCEMENT

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Endothelial cell barrier dysfunction results in the increased vascular permeability observed in inflammation, tumor metastasis, angiogenesis, and atherosclerosis. Sphingosine 1-phosphate (S1P), a biologically active phosphorylated lipid growth factor released from activated platelets, enhances the endothelial cell barrier integrity in vitro and in vivo. To begin to identify the molecular mechanisms mediating S1P-induced endothelial barrier enhancement, quantitative proteomics analysis (iTRAQ™) was performed on membrane rafts isolated from human pulmonary artery endothelial cells in the absence or presence of S1P stimulation. Our results demonstrated that S1P mediates rapid and specific recruitment (1 μM, 5 min) of myristoylated alanine-rich protein kinase C substrate (MARCKS) and MARCKS-related protein (MRP) to membrane rafts. Western blot experiments confirmed these findings with both MARCKS and MRP. Finally, small interfering RNA-mediated silencing of MARCKS or MRP or both attenuates S1P-mediated endothelial cell barrier enhancement. These data suggest the regulation of S1P-mediated endothelial cell barrier enhancement via the cell specific localization of MARCKS and MRP and validate the utility of proteomics approaches in the identification of novel molecular targets. Molecular & Cellular Proteomics 6:689–696, 2007.

The pulmonary endothelium serves as a semipermeable cellular barrier between blood and the interstitium and airspaces of the lung. Endothelial cell (EC) barrier dysfunction results in increased vascular permeability, which is a cardinal feature of inflammation and an essential component of tumor metastasis, angiogenesis, and atherosclerosis. Proteins and lipids released after platelet activation have long been appreciated as enhancing the integrity of the microcirculation (1, 2). Sphingosine 1-phosphate (S1P), a biologically active phosphorylated lipid growth factor released from platelet, has multiple EC effects, including promoting barrier integrity in vivo and in vitro across both human and bovine pulmonary artery and lung microvascular ECs (3, 4). Our previous studies demonstrated S1P-mediated barrier enhancement to be dependent on S1P binding to its major surface receptor, S1P₁; activation of the small GTPase, Rac1; and rearrangement of the cortical actin cytoskeleton (3, 5, 6). Very recently, we further detailed the essential involvement of PI 3-kinase, Tiam1, and α-actinin in specialized membrane domains (i.e. membrane rafts) in S1P-treated EC barrier regulation (7). However, the underlying signaling mechanisms by which S1P increases vascular integrity via signaling to the endothelial cytoskeleton remain poorly understood.

As defined by the recent Keystone Symposium on lipid rafts and cell function (March 23–28, 2006, in Steamboat Springs, CO) (8), “Membrane rafts are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions.” Many biophysical, biochemical, and microscopy studies suggest that membrane rafts truly exist and are implicated in diverse cellular processes including signal transduction (9–12). Currently, two commonly used methods to isolate membrane rafts, resistant to either high pH or nonionic detergents, involve separation of membrane rafts from other proteins by density gradient centrifugation, with detergent resistance the more widely utilized method.

To begin to identify the underlying signaling mechanisms by which S1P increases vascular integrity, we chose to identify protein changes in membrane rafts isolated from human pulmonary artery ECs in the presence or absence of S1P treatment (1 μM, 5 min) using quantitative proteomics analysis. The time period and concentration for S1P treatment were based on previous studies (3, 4). Because of the extremely hydrophobic nature of membrane rafts, we chose a solution-based...
proteomics approach, isobaric tagging for relative and absolute quantitation (iTRAQ™) method. The protein changes in membrane rafts were confirmed by Western blot analysis. Linking these proteins to EC barrier enhancement was then explored using siRNA transfection and measurement of transendothelial electrical resistance (TER).

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human pulmonary artery ECs were obtained from Cambrex Bioscience (East Rutherford, NJ) and cultured as described previously (8) in EGM-2 BulletKits (Cambrex Bioscience) supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO₂, 95% air.

**Isolation of Membrane Rafts from Human Pulmonary Artery ECs**—ECs were grown to confluence at passage 7 in T150 flasks (1.5 × 10⁷ cells each flask), washed with PBS, and serum-starved in EB2 media (Cambrex Bioscience) for 1 h. Confluent EC monolayers were either untreated (control) or treated with 1 μM S1P for 5 min, after which the cells were washed three times with cold PBS (4 °C) and stored at ~80 °C. Membrane rafts were isolated using the low-density detergent-resistant fractionation as described (13). Briefly, 750 μl of cold (−4 °C) TKM (50 mM Tris-HCl, pH 8, 25 mM KC1, 5 mM MgCl₂, 1 mM EDTA) in 1% Triton X-100 solution containing protease inhibitors was added to each flask while still cold. The cells were then scraped and homogenized using a Dounce homogenizer. The concentration of Triton X-100 in the suspension was adjusted to 1% by the addition of 10% Triton X-100, and the suspension was placed on ice for ~30 min with pipetting every 10 min. Next, 0.5 ml of the lysate was mixed with equal volume of 80% sucrose and overlaid with 6 ml of 38% sucrose and 4 ml of 5% sucrose in an ultracentrifuge tube. The gradients were centrifuged (40,200 rpm, SW60 rotor) for 12 h at 4 °C. Fractions of 1 ml were taken from the top to bottom, and Western blots were performed to verify the localization of membrane rafts marker caveolin-1. Membrane rafts fractions (fraction 4, 5, and 6 from the top) were pooled and precipitated in trichloroacetic acid (TCA), and the pellet was dissolved in 1% SDS, 100 mM triethylammonium bicarbonate (TEAB), pH 8.5. A BCA assay was performed to measure the protein concentration.

**Protein Digest, iTRAQ Labeling, and Strong Cation Exchange Fractionation**—Control and S1P-treated membrane rafts in 1% SDS, 100 mM TEAB were diluted 5-fold with 100 mM TEAB. The samples (50 μg each) were reduced, alkylated, and digested with trypsin with a protein to enzyme ratio of 20:1 at 37 °C overnight. Each digest was concentrated to 15 μl in a speed vac followed by the addition of 15 μl of 1 % TEAB. The iTRAQ reagent was dissolved in 70 μl of ethanol and added to the digest, and the mixture was incubated at room temperature for 1 h. Control and S1P-treated membrane rafts labeled with different iTRAQ reagents were mixed and dried down to a volume of 50 μl. The combined peptide mixture was fractionated by strong cation exchange (SCX) chromatography on an Ultimate HPLC system (LC Packings) using a polysulfoethyl A column (2.1 × 100 mm, 5 μm, 300 Å, PolyLC, Columbia, MD). The sample was then dissolved in 1 ml of SCX loading buffer (25% v/v acetonitrile, 10 mM KH₂PO₄, pH 2.8), and pH was adjusted to 2.8 by adding 1 N hydrochloric acid. The entire sample was loaded onto the column and washed isocratically for 30 min at 200 μl/min. Peptides were eluted with a linear gradient of 0–500 mM KC1 (25% v/v acetonitrile, 10 mM KH₂PO₄, pH 2.8) over 30 min at a flow rate of 200 μl/min. The absorbance at 214 nm was monitored, and 15 fractions were collected along the gradient.

**LC-MS Analysis**—Each SCX fraction was dried down, dissolved in 0.1% formic acid, and analyzed on Ostar Pulsar™ (Applied Biosystems-MDS Sciex) interfaced with an Agilent 1100 HPLC system. Peptides were separated on a reverse-phase column packed with 10 cm of C18 beads (360 × 75 μm, 5 μm, 120 Å, YMC ODS-AQ, Waters Associates, Milford, MA) with an emitter tip (New Objective, Woburn, MA) attached. The HPLC gradient was 5–40% B for 60 min (A, 0.1% formic acid; B, 90% acetonitrile in 0.1% formic acid) at a flow rate of 300 nL/min. Survey scans were acquired from m/z 400–1200 with up to three precursors selected for MS/MS using a dynamic exclusion of 45 s. A rolling collision energy was used to promote fragmentation, and the collision energy range was ~20% higher than that used for unlabeled peptides because of iTRAQ tags.

**Data Analysis**—The MS/MS spectra were extracted and searched against Uniprot-sprot database (version 4, total number of entries 230093, entries for Homo sapiens 14515) using ProteinPilot™ software (version 1.0, revision 33087, Applied Biosystems) with the Paragon™ method utilizing the following search parameters: Homo sapiens as species, trypsin as enzyme (one missed cleavage allowed), cysteine static modification with methylemethanethiolosulfate and iTRAQ (peptide labeled at N terminus and lysine) as sample type. Mass tolerance was set to 0.15 atomic mass units for precursor and 0.1 atomic mass units for fragment ions. The raw peptide identification results from the Paragon™ Algorithm (Applied Biosystems) searches were further processed by the Pro Group™ Algorithm (Applied Biosystems) within the ProteinPilot software before final display. The Pro Group Algorithm uses the peptide identification results to determine the minimal set of proteins that can be reported for a given protein confidence threshold. For each protein, Pro Group Algorithm reports two types of scores for each protein: unused ProtScore and total ProtScore. The total ProtScore is a measurement of all the peptide evidence for a protein and is analogous to protein scores reported by other protein identification softwares. The unused ProtScore, however, is a measurement of all the peptides evidence for a protein that is not better explained by a higher ranking protein. In other words, the unused ProtScore is calculated by using the unique peptides (peptides that are not used by the higher ranking protein), and it is a true indicator of protein evidence. This is how a single-protein member of a multiprotein family has been singled out.

The protein confidence threshold cutoff for this study is ProtScore 2.0 (unused) with at least one peptide with 99% confidence. The mean, standard deviation, and p values to estimate statistical significance of the protein changes were calculated by Pro Group. Proteins identified with changes (ratio >1.2 or <0.8) that were consistent between two independent biological experiments were manually validated and quantified. Peak areas for each of the signature ions (114, 115, 116, and 117) were obtained and corrected according to the manufacturer’s instructions to account for isotopic overlap. Only those signature ions with intensities less than 1500 counts were used for quantitation. In our experiment, we tend to get a 1:1 ratio if the intensities of the signature ions are higher than 1500 counts because the detector gets saturated.

**Western Blot Analysis**—To verify iTRAQ data, samples (1 μg) were separated by SDS-PAGE (4–12%) and transferred to PVDF membranes. After blocking 1 h at room temperature with blocking buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20 (TBS/Tween 20), and 5% nonfat milk powder), membranes were incubated overnight at 4 °C in primary antibody in blocking buffer. The membranes were washed with TBS/Tween 20, incubated for 1 h at room temperature in secondary antibody (alkaline phosphatase-conjugated AffiniPure Donkey Anti-Rabbit IgG, Jackson ImmunoResearch Laboratories Inc., West Grove, PA) with 1:10000 dilution in blocking buffer, and finally washed with TBS/Tween 20. Blots were developed with Immuno-Star™ AP substrate Pack (BioRad) and scanned on an Epson Scan (Agilent Technologies, Foster City, CA) scanner within the linear range of detection. Primary antibodies were anti-MARKCS (1:2000 dilution), anti-phospho-MARKCS (pSer115/116/117) (1:1000 dilution), anti-MRP (1:500 dilution) rabbit polyclonal antibodies (Calbiochem) and anti-
caveolin-1 (N-20) Sc-894 (1:1000 dilution) (Santa Cruz Biotechnology). For verification of membrane raft fractions after ultracentrifugation using anti-caveolin-1 antibody, 10 μl of each fraction (1 ml) was loaded.

**Construction and Transfection of siRNA against MARCKS and MRP**—The siRNA sequence(s) targeting human against MARCKS and MRP were generated using mRNA sequences from GenBank™ (gi:11125771 and gi:32401423, respectively). For each mRNA (or scramble), two targets were identified. Specifically, MARCKS target sequence 1 (5'-AACTACACTTGGCTCTTTT-3'), MARCKS target sequence 2 (5'-AAAAATTTTATCCCGAGTG-3'), MRP target sequence 1 (5'-AAAAAGAATTGGAGACTTATCC-3'), MRP target sequence 2 (5'-AAGAGAAAGAAGAAATTCTC-3'), scramble sequence 1 (5'-AGAAGAGAATCGAAACCAGAAA-3') and scramble sequence 2 (5'-AGAACCCTTTACTTGCGCAAG-3') were utilized. Sense and antisense oligonucleotides were provided by Integrated DNA Technologies, Inc. (Coralville, IA). For construction of the siRNA, a transcription-based kit from Ambion was used (Silencer™ siRNA construction kit). Human lung EC were then transfected with siRNA using siPORT™, the transfection reagent (Ambion, TX) according to the protocol provided by Ambion. Cells (~40% confluent) were serum-starved for 1 h followed by incubation with 3 μM (1.5 μM of each siRNA) of target siRNA (or scramble siRNA or no siRNA) for 6 h in serum-free media. The serum-containing medium was then added (1% serum, final concentration) for 42 h before biochemical experiments and/or functional assays were conducted.

**Measurement of TER**—EC were grown to confluence in polycarbonate wells containing evaporated gold microelectrodes, and TER measurements were performed using an electrical cell substrate impedance sensing system (Applied Biophysics, Troy, NY) as described previously (7). TER values from each microelectrode were pooled at discrete time points and plotted versus time as the mean ± S.E.

**RESULTS**

**Quantitative Proteomics Analysis of Membrane Rafts after S1P Stimulation**—In this study, iTRAQ-based stable isotope labeling of membrane rafts was carried out to obtain insight into the underlying mechanism of S1P-mediated EC barrier enhancement. The experimental strategy is shown in Fig. 1. Human pulmonary artery ECs were cultured in T150 flasks to confluence, either left untreated (control, 5 min) or treated with S1P (1 μM, 5 min). Sucrose gradient centrifugation was performed to isolate membrane rafts, and the membrane rafts fractions were verified using Western blot of EC membrane rafts marker caveolin-1 (Fig. 2). Membrane rafts fractions (fraction 4, 5, and 6 from the top) were then pooled and precipitated. Aliquots (50 μg) from each sample were then reduced and alkylated, and the resulting peptides were labeled with iTRAQ reagents (114 and 115). The two samples were mixed and analyzed by LC/MS/MS. The MS/MS fragmentation of the iTRAQ-labeled peptides results in signature peaks (114.1 and 115.1) for quantitation, and the fragmentation along the peptide backbone results in b- and y-type fragments, which may be used to identify the peptide sequence. The second independent experiment was performed in identical fashion with the exception that samples were labeled with iTRAQ reagents 116 and 117. A total of 245 non-redundant proteins were identified from EC membrane rafts (supplemental Table S1). The MS/MS spectra for the single peptide that was used to match a protein were displayed in supplemental Fig. S1. Two proteins, MARCKS and MRP, were specifically recruited to the membrane rafts upon S1P treatment in both independent biological experiments. The recruitment of these two proteins was specific as no other protein was found to be consistently altered outside the range of 0.8 to 1.2. Table I depicts the MARCKS, MRP, and caveolin-1 peptide sequences, peptide confidence, peptide ratios, protein ratios, standard deviations, and p values from the ProteinPilot software from the two independent experiments. Representative MS/MS spectra for peptides identified from MARCKS, MRP, and caveolin-1 are shown in Fig. 3, with the peaks for the signature ions shown in the insets.

**Confirmation of S1P-induced MARCKS and MRP Recruitment to Membrane Rafts in Human EC**—To validate our iTRAQ results, Western blot experiments were performed. Fig. 4 shows that under control conditions, a minimal level of MARCKS and MRP is present in the isolated membrane rafts (Fig. 4, A and C) and that with S1P treatment of human pulmonary EC (1 μM, 5 min) there is an extensive recruitment of MARCKS and MRP to the membrane rafts.

As MARCKS is known to be phosphorylated (14, 15), the role of phosphorylation of this protein was investigated using an anti-phospho-MARCKS antibody recognizing specifically MARCKS phosphorylated at Ser159, Ser163, and Ser179. Clearly, a trace amount of phospho-MARCKS protein was present in the control membrane rafts, and there was a sig-
significant reduction in phospho-MARCKS after S1P challenge (Fig. 4B). The level of caveolin-1 was not altered by S1P stimulation (Fig. 4D), findings consistent with the iTRAQ data and previous results (7).

The Effects of MARCKS and MRP on S1P-mediated Human EC Barrier Function—The effects of membrane rafts-associated MARCKS and MRP on S1P-mediated EC barrier regulation was then investigated using an siRNA approach. As shown in Fig. 5, siRNA transfection effectively silenced MARCKS and MRP protein expression in human EC. Whereas silencing of either MARCKS or MRP in control EC did not produce any alteration in basal barrier integrity as measured by TER, silencing either MARCKS (Fig. 6A) or MRP (Fig. 6B) significantly attenuated the rapid increase in S1P-induced EC barrier enhancement. Furthermore, dually silencing of both MARCKS and MRP (Fig. 6C) attenuated both the rapid TER increase as well as the prolonged enhancement of S1P-mediated EC barrier function. These results suggest an important, non-redundant role of MARCKS and MRP in regulating S1P-induced EC barrier protection.

DISCUSSION

Since our report of S1P-induced endothelial cell activation (16), there has been increasing evidence for a role of S1P in the maintenance and restoration of the dynamic semipermeable endothelial cellular barrier in the vasculature. S1P-mediated enhancement of endothelial junctional integrity via prominent cytoskeletal rearrangement involving the translocation of key actin-binding proteins, such as cortactin and myosin light chain kinase to the cell periphery and the formation of a strong cortical actin ring (3, 5), has been defined. An emerging concept from these studies is that a marked increase in polymerized cortical actin represents a common and essential feature of barrier-protective agents such as S1P (4). Consistent with this concept, the Rho family of small GTPases, critical regulators of the non-muscle cytoskeleton, is intimately involved in S1P-mediated cytoskeletal rearrangement and distribution/assembly of intercellular adherens complexes and focal adhesions (17).

Among the proteins that have been proposed to interact with actin and to cross-link actin at the plasma membrane (18) are MARCKS (30–32 kDa) and its homologue MRP (20 kDa), which are essential proteins and products of single genes with no known isoforms (19–21). Both proteins contain three highly conserved domains including a myristoylated N terminus and an effector domain (22). The unusual biochemical properties and multiple interactions of MARCKS and MRP have led to a

| Protein name | Peptide sequence | Peptide confidence | Peptide ratio (S1P:control) | Protein ratio (S1P:control) | p value |
|--------------|-----------------|--------------------|-----------------------------|-----------------------------|---------|
| Quantification: sample 1 | AAEEPSKVEEK | 99 | 1.42 |
| | EAPAEGEAEPGSPTAEG | 99 | 1.31 |
| | EAASAASSSTSSPK | 99 | 1.5 | 1.41 ± 0.07 | 0.0001 |
| MARCKS | GEAARPEGAEVASSPSK | 99 | 1.38 |
| | VNGDASPAAAEAGSAK | 99 | 1.41 |
| | VNGDASPAAAEAGSAK | 99 | 1.8 |
| | Geaaaapeagaspvek | 99 | 1.03 |
| MRP | GDVTADEEAGASPAK | 99 | 1.77 |
| | EidlnrDpk | 99 | 0.71 |
| Caveolin-1 | EQGNIYKPNNK | 99 | 0.86 |
| | IDFEDVIAEPEGTHSFGIWK | 99 | 0.82 |
| | YVDSEGHLYTVPIR | 99 | 0.93 |
| Quantification: sample 2 | EAAPAEGEAEPGSPTAEG | 99 | 1.33 |
| | EAASAASSSTSSPK | 99 | 1.25 |
| MARCKS | GEAARPEGAEVASSPSK | 99 | 1.25 |
| | GEAARPEGAEVASSPSK | 99 | 1.8 |
| | NGDASPAAAEAGSAK | 99 | 1.38 |
| | Geaaaapeagaspvek | 99 | 1.10 |
| MRP | GDVTADEEAGASPAK | 99 | 1.8 |
| | IDFEDVIAEPEGTHSFGIWK | 99 | 0.82 |
| | YVDSEGHLYTVPIR | 99 | 1.18 |
| Caveolin-1 | AMADELSEK | 99 | 1.22 |
| | EidlnrDpk | 99 | 1.27 |
| | EQGNIYKPNNK | 99 | 1.03 |
| | HLNDDDV | 99 | 1.04 |

**a** This ratio was excluded because the reporter ions are higher than 5000 counts.

**b** This ratio was excluded because the reporter ions are higher than 1500 counts.
FIG. 3. Representative MS/MS spectra for peptides derived from MARCKS, MRP, and caveolin-1. For each MS/MS spectrum, b- and y-type fragment ions enable peptide identification, whereas the peak areas for each of the iTRAQ signature ions (insets) enable quantification of the peptides and proteins. A, MS/MS spectrum of AEEPSKVEEK from MARCKS; B, MS/MS spectrum of GEAAERPGEAAVASPSK from MARCKS; C, MS/MS spectrum of GDVTAEEAAGASPAK from MRP; D, MS/MS spectrum of YVDSEGHLYTVPIR from caveolin-1.

FIG. 4. Western blot analysis of MARCKS, phospho-MARCKS, MRP, and caveolin-1 upon S1P stimulation in human EC membrane rafts. Confluent human ECs were either untreated (Control) or challenged with S1P (1 μM, 5 min), and lipid raft fractions were prepared. An aliquot (1 μg) of each sample was separated on 4–12% SDS-PAGE, and Western blots were carried out for MARCKS (A), phospho-MARCKS (B), MRP (C), and caveolin-1 (D).

Fig. 5. Characterization of siRNA treatment of human EC. Cellular lysates from untransfected (Control, no siRNA), scramble siRNA (siRNA that does not target any known human mRNA), MARCKS siRNA, or MRP siRNA transfection were analyzed using Western blot with anti-MARCKS antibody (A), anti-MRP antibody (B) or anti-actin antibody (C) as described in “Experimental Procedures.”
variety of proposed functions at the molecular level. These include binding to phospholipids including phosphatidylinositol-4,5-biphosphate (23, 24), phosphorylation by protein kinase C (14, 15), and binding and cross-linking of actin filaments (18).

Upon phosphorylation, MARCKS is translocated from membrane to cytosol (25), and the actin cross-linking ability is also lost (26). MARCKS and MRP are implicated in the coordination of membrane-cytoskeletal signaling events, such as cell adhe-
sion, migration, secretion, and phagocytosis in a variety of cell types (22, 27). However, MARCKS and MRP have not been associated with S1P-mediated barrier enhancement in EC.

Using quantitative proteomics analysis, we identified a total of 245 proteins from membrane rafts, including well known lipid raft markers such as annexin A2, caveolin-1, and flotillin-1; numerous proteins involved in cytoskeletal rearrangements; signal transduction molecules; and proteins harboring putative post-translational modifications that favor this localization in the lipid raft environment. Consistent with previous membrane rafts studies (28, 29), we also had non-rafts proteins in our membrane rafts because of the biochemical isolation of membrane rafts. However, we were able to identify the differences from rafts proteins, and the only differences in this study were from rafts proteins. We found that MARCKS and MRP are specifically recruited into membrane rafts upon S1P stimulation and that the phosphorylation of MARCKS is inhibited. Western blot experiments validated these findings. Based on our proteomics studies and biochemistry results, we propose that upon S1P stimulation, MARCKS and MRP are recruited to membrane rafts and in this locale, stabilize the newly generated rigid F-actin cytoskeleton, thereby increasing the EC barrier protection. S1P also inhibits MARCKS from phosphorylation, an event potentially linked to F-actin release and barrier regulation. Our results are not novel in the sense that these cytoskeletal proteins (MARCKS and MRP) have been reported previously as localized in membrane rafts (30). However, our proteomics, cell biologic, and physiologic data strongly suggest a paradigm where MARCKS and MRP binding and cross-linking of F-actin filaments in membrane rafts provide a novel mechanism of S1P-mediated barrier protection. Our siRNA experiments confirmed the regulation role of MARCKS and MRP in S1P-induced EC barrier enhancement and suggested that MARCKS and MRP are non-redundant in S1P-induced EC barrier protection.

In summary, although mechanisms of vascular barrier enhancement by agonists such as S1P are poorly understood, using proteomics analysis in combination of biochemistry tools, we now show that S1P-mediated cortical actin rearrangement and barrier protection are critically dependent on recruitment of MARCKS and MRP into membrane rafts.

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