© 2021. Published by The Company of Biologists Ltd | Journal of Cell Science (2021) 134, jcs259467. doi:10.1242/jcs.259467

CORRECTION

Correction: Actin remodeling requires ERM function to facilitate AQP2 apical targeting

Grazia Tamma, Enno Klussmann, Johannes Oehlke, Eberhard Krause, Walter Rosenthal, Maria Svelto and Giovanna Valenti

There were errors published in *J. Cell Sci.* (2005) **118**, 3623–3630 (doi:10.1242/jcs.02495).

The authors wish to correct errors in Figs 3A and 4A. In Fig. 4A, the cells in the middle row did not correspond to cells treated with the control peptide as originally labelled, and new panels with the correct cells are now presented. In addition, for clarity, new labels have been added to the top row of Fig. 3A and Fig. 4A to clarify these cells were not treated with peptide. The corrected and original panels are shown below. Both the online full text and PDF versions of the paper have been corrected.

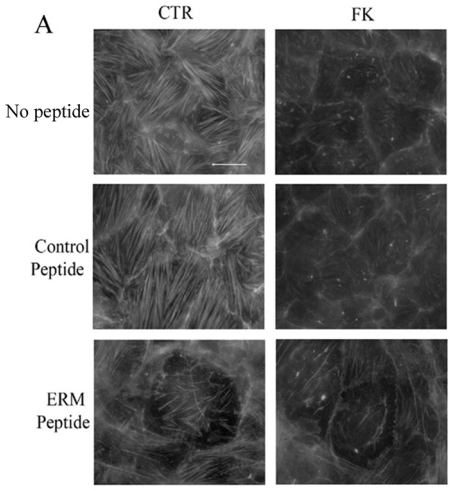

Fig. 3A (corrected panel). (A) Cells were either left untreated (CTR) or stimulated with forskolin (FK) with or without preincubation for 3 hours with the ERM peptide or with the control peptide. F-actin was stained with TRITC-conjugated phalloidin and visualized by epifluorescence microscopy. Bar, 5 µm.

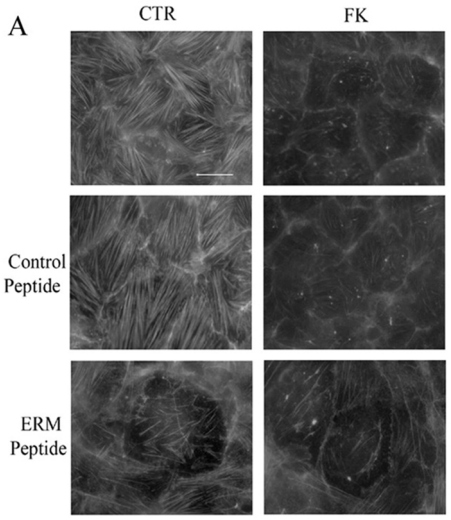

Fig. 3A (original panel). (A) Cells were either left untreated (CTR) or stimulated with forskolin (FK) with or without preincubation for 3 hours with the ERM peptide or with the control peptide. F-actin was stained with TRITC-conjugated phalloidin and visualized by epifluorescence microscopy. Bar, 5 µm.

Journal of Cell Science (2021) 134, jcs259467. doi:10.1242/jcs.259467

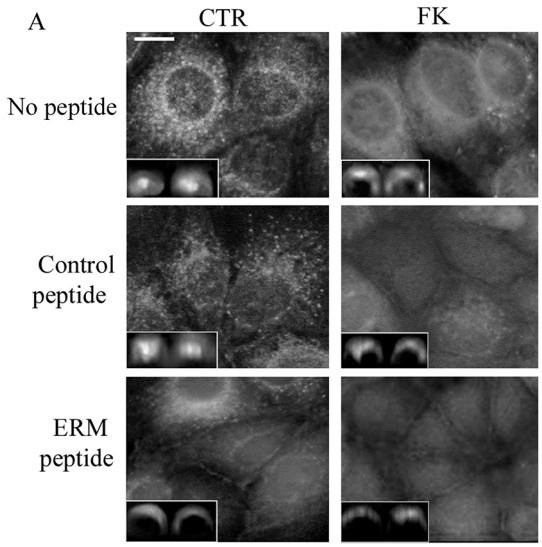

Fig. 4A. (corrected panel). (A) Cells were either left untreated or stimulated with forskolin in the presence or absence of either ERM peptide or the control peptide. Fluorescence was visualized by epifluorescence microscopy and the xz reconstructions were obtained by deconvolution using Autodeblur software (shown in the insets). Forskolin stimulation caused AQP2 translocation from an intracellular pool to the apical membrane. A similar effect was observed on preincubation with the ERM peptide, while control peptide had no effect on AQP2 cellular localization.

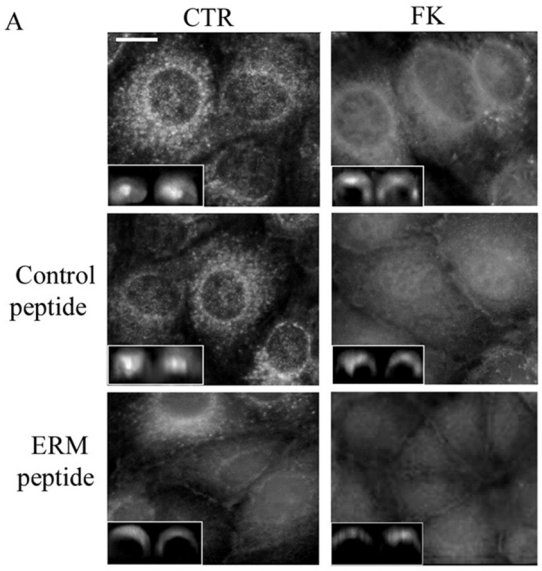

Fig. 4A. (original panel). (A) Cells were either left untreated or stimulated with forskolin in the presence or absence of either ERM peptide or the control peptide. Fluorescence was visualized by epifluorescence microscopy and the xz reconstructions were obtained by deconvolution using Autodeblur software (shown in the insets). Forskolin stimulation caused AQP2 translocation from an intracellular pool to the apical membrane. A similar effect was observed on preincubation with the ERM peptide, while control peptide had no effect on AQP2 cellular localization.

The authors apologise to readers for these errors, which do not impact the results or the conclusions of the article.

Actin remodeling requires ERM function to facilitate AQP2 apical targeting

Grazia Tamma[1], Enno Klussmann[2], Johannes Oehlke[2], Eberhard Krause[2], Walter Rosenthal[2,3], Maria Svelto[1] and Giovanna Valenti[1,*]

[1]Dipartimento di Fisiologia Generale ed Ambientale, University of Bari, Via Amendola 165/A, 70126 Bari, Italy
[2]Forschungsinstitut für Molekulare Pharmakologie, 13125 Berlin, Germany
[3]Freie Universität Berlin, Institut für Pharmakologie, 14195 Berlin, Germany
*Author for correspondence (e-mail: g.valenti@biologia.uniba.it)

Accepted 13 May 2005
Journal of Cell Science 118, 3623-3630 Published by The Company of Biologists 2005
doi:10.1242/jcs.02495

Summary

This study provides the first evidence that actin reorganization during AQP2 vesicular trafficking to the plasma membrane requires the functional involvement of ERM (ezrin/radixin/moesin) proteins cross-linking actin filaments with plasma membrane proteins. We report that forskolin stimulation was associated with a redistribution of moesin from intracellular sites to the cell cortex and with a concomitant enrichment of moesin in the particulate fraction in renal cells. Introduction of a peptide reproducing a short sequence of moesin within the binding site for F-actin induced all the key effects of forskolin stimulation, including a decrease in F-actin, translocation of endogenous moesin, and AQP2 translocation. A straightforward explanation for these effects is the ability of the peptide to uncouple moesin from its putative effector. This modifies the balance between the active and inactive forms of moesin. Extraction with Triton X-100, which preserves cytoskeletal associated proteins, showed that forskolin stimulation or peptide introduction reduced the amount of phophorylated moesin, a molecular modification known to stabilize moesin in an active state. Our data point to a dual role of moesin in AQP2 trafficking: it might modulate actin depolymerization and it participates in the reorganization of F-actin-containing cytoskeletal structures close to the fusion sites of the AQP2-bearing vesicles.

Key words: Aquaporin, ERM, Actin cytoskeleton

Introduction

ERM (ezrin/radixin/moesin) proteins cross-link actin filaments with the plasma membrane. They are involved in the formation of microvilli, cell-cell adhesion, maintenance of cell shape, cell motility and membrane trafficking. Recent analyses reveal that they are not only involved in cytoskeleton organization but also in signaling pathways (Ivetic and Ridley, 2004; Speck et al., 2003).

ERM proteins share a high degree of homology among themselves. However, their tissue distribution and primary structure suggest that these are not simply redundant proteins (Tsukita and Yonemura, 1997). ERM proteins promote the organization of the cortical actin-based cytoskeleton through their C-terminal domain and of the cell membrane through the N-terminal FERM domain (Bretscher et al., 2002; Jankovics et al., 2002; Mangeat et al., 1999; Tsukita and Yonemura, 1999). The FERM domain of ERM proteins interacts directly with the intracellular domain of several membrane proteins, as well as indirectly through apical scaffolding proteins (Reczek and Bretscher, 1998). In the C-terminal region of the ERM family proteins, an actin binding site has been identified. In particular, a site interacting with F-actin and highly conserved among ERM members has been detected in the 34 C-terminal amino acids using a truncated ezrin fused with glutathione-*S*-transferase (Turunen et al., 1994). The association between the ERM proteins and the underlying cytoskeleton is crucial for determining cell shape, apical junction (AJ) assembly during development, motility and several plasma membrane processes, including endocytosis and exocytosis (Mangeat et al., 1999; Tsukita and Yonemura, 1999; Van Furden et al., 2004). Indeed, endocytosis and exocytosis are processes that are strictly dependent on actin dynamics, and ERM proteins participate not only in controlling actin dynamics but also in signal transduction pathways (Bretscher et al., 2002; Ivetic and Ridley, 2004). Interestingly, high level expression of the carboxyl-terminal domain (aa 319-583) of radixin disrupts normal cytoskeletal structure and function and causes a strong desegregation of ventral actin filaments in NIH-3T3 cells (Henry et al., 1995). Small GTP binding proteins of the Rho family are key players in regulating actin assembly and ERM proteins play an important role in the activation of members of the Rho family by recruiting their regulators. The FERM domain of ERM binds signaling molecules of the Rho pathway, including Rho guanine dissociation inhibitors (Rho-GDI) and the Rho GDP/GTP exchange protein Dbl (Bretscher, 1999). The observation that ERM proteins function both upstream and downstream of Rho GTPases implies that there could be a feedback loop for Rho-pathway autoregulation (Hirao et al., 1996; Mammoto et al., 2000; Takahashi et al., 1997). The ability of ERM to reversibly recruit the regulators of the Rho family is a consequence of

the molecular conformation. In fact, the intramolecular interaction between the N- and C-terminal domains of ERM proteins masks several binding sites including signaling molecules of the Rho pathway, leading to a dormant protein in closed monomers and the intermolecular interaction in head-to-tail hetero- and homo-oligomers (Gary and Bretscher, 1993; Gautreau et al., 2000; Ivetic and Ridley, 2004). We have recently shown that cAMP-dependent translocation of the water channel aquaporin-2 (AQP2) into the apical membrane in renal principal cells is associated with RhoA inactivation, resulting in actin depolymerization (Klussmann et al., 2001; Tamma et al., 2001; Tamma et al., 2003). This event is a prerequisite for proper targeting of AQP2-bearing vesicles into the apical membrane of renal collecting duct cells, leading to an increase in osmotic water permeability. These observations suggest that actin could negatively regulate AQP2 targeting, forming a physical barrier that must be removed for vesicular fusion to occur. However, while actin cytoskeleton and actin-regulatory proteins have an increasingly recognized role in the assembly of vesicular transport and in their translocation, the mechanisms underlying this regulation are still largely unknown. ERM proteins proposed to link transmembrane proteins to the actin cytoskeleton in the apical domain regulate cell signalling events that affect actin organization.

This study represents the first report investigating the functional involvement of moesin, one of the ERM proteins expressed in renal principal cells in the regulation of AQP2 trafficking.

Materials and Methods

Cell culture

CD8 cells were established by stably transfecting the RC.SV3 rabbit cortical collecting duct cells with cDNA encoding rat AQP2. CD8 cells were grown at 37°C as described in a hormonally defined medium (Valenti et al., 1996). CD8 cells respond to cAMP-elevating compounds like vasopressin or forskolin with an increase in the osmotic water permeability coefficient (Pf) and the subsequent AQP2 redistribution from an intracellular pool to the apical surface (Tamma et al., 2001; Valenti et al., 1996; Valenti et al., 2000; Valenti et al., 1998). Confluent monolayers were used at days 3-5 after plating.

Peptide synthesis and introduction into the cells

A peptide (ERM peptide) corresponding to a short sequence in the carboxyl terminal region of the ERM family (RDKYKTLRQIRQGN), including the threonine 558 residue, was synthesized (Fig. 2). This peptide is connected by a disulphuric bond to the cysteine of a chain comprising 20 hydrophobic amino acids which may constitute a permeable helix through the plasma membrane. A dansyl residue was added to the hydrophobic chain to visualize the presence of the peptide in the cells. In addition, a control peptide with a reversed sequence with respect to the peptide described above was synthesized (NGQRIQRLTKYKDR). To get the peptide into the cells, it was employed at a final concentration of 2 μM in the cell culture medium and incubated for 3 hours. Fluorescence analysis then confirmed that it was internalized into the cells, probably by endocytosis.

Immunofluorescence

CD8 cells were grown on glass coverslips and fixed with 4% paraformaldehyde in phosphate buffer saline (PBS) for 20 minutes. Cells were washed 3 times for 5 minutes in PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 minutes. After blocking in 0.1% gelatin in PBS for 20 minutes, cells were incubated at room temperature for 1 hour, either with affinity-purified anti-AQP2 antibodies or with monoclonal anti-moesin or with anti-phosphorylated moesin (Thr 558) antibodies. Cells were washed three times for 5 minutes with 0.1% gelatin in PBS and incubated for 60 minutes with fluorescein-conjugated goat anti-rabbit/mouse IgG (10 μg/ml in PBS), followed by washing twice for 1 minute in PBS containing 2.7% NaCl (PBS high salt) and twice in regular PBS. Alternatively, after blocking, the actin cytoskeleton was visualized by incubation with phalloidin-TRITC (100 μg/ml, 45 minutes). The coverslips were mounted in 50% glycerol in 0.2 M Tris-HCl, pH 8.0, containing 2.5% *n*-propyl gallate to retard quenching of the fluorescence. Moesin or the actin cytoskeleton were detected by epifluorescence microscopy (Leica DMRXA microscope). Alternatively, AQP2 and moesin were detected with an epifluorescence microscope (TE 2000S, Nikon Instruments, Florence, Italy) equipped with a CCD camera (Princeton Instruments MicroMax 512BFT, NJ) using a Delta RAM Highspeed Multiwavelength Illuminator for excitation (Photo Technology International PTI, NJ). The xz planes were obtained by deconvolution using Autodeblur software (Universal Imaging Corporation, PA). The fluorescence intensity was analyzed by using Metamorph software and the statistical analysis was performed by using a one-way Anova and Tukey's multiple comparison test.

Actin polymerization assay

Actin polymerization was analyzed as previously described (Hall et al., 1988; Knetsch et al., 2001; Peracino et al., 1998). Briefly, CD8 cells were left untreated or stimulated with forskolin (10^{-4} M). Alternatively, cells were either preincubated with the ERM peptide (2 μM) for 3 hours or pretreated with the control peptide. The treatments were stopped by adding 450 μl of 3.7% paraformaldehyde, 0.1% Triton X-100, 0.25 μM TRITC-phalloidin in 20 mM potassium phosphate, 10 mM PIPES, 5 mM EGTA and 2 mM $MgCl_2$, pH 6.8. After staining for 1 hour the cells were washed three times with PBS and 800 μl of methanol were added overnight. The fluorescence (540/565 nm) was read in a RF-5301PC fluorimeter. The values obtained were analyzed by Student's *t*-test.

Analysis of association with actin cytoskeleton

The interaction of moesin with actin cytoskeleton was measured by its solubility in Triton X-100. Briefly, CD8 cells were seeded 2 days before the experiments and grown to confluence. The Triton soluble fraction was extracted by a 1 minute incubation with 500 μl of a Triton X-100 buffer (80 mM PIPES, 5 mM EGTA, 1 mM $MgCl_2$, 0.5% Triton X-100, 50 mM NaF, pH 6.4, and calyculin 1 μM), which preserves the cytoskeleton association of proteins. Proteins were resolved in a 13% polyacrylamide gel and then transferred onto immobilon-P (Millipore) by standard procedures.

Cell fractionation

CD8 cells were homogenized with a glass/Teflon homogenizer in ice-cold buffer containing 130 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.5, and 1 mM PMSF, 2 mg/ml leupeptin and 2 mg/ml pepstatin A. Nuclei were removed by centrifugation at 800 ***g*** for 10 minutes. Membrane and cytosol fractions were obtained by centrifugation for 1 hour at 4°C at 150,000 ***g*** in Beckman Instruments ultracentrifuge. The pellets (particulate fractions) were resuspended in homogenization buffer and the supernatant (soluble fractions) was concentrated using centricon tubes (10,000 Da cut-off).

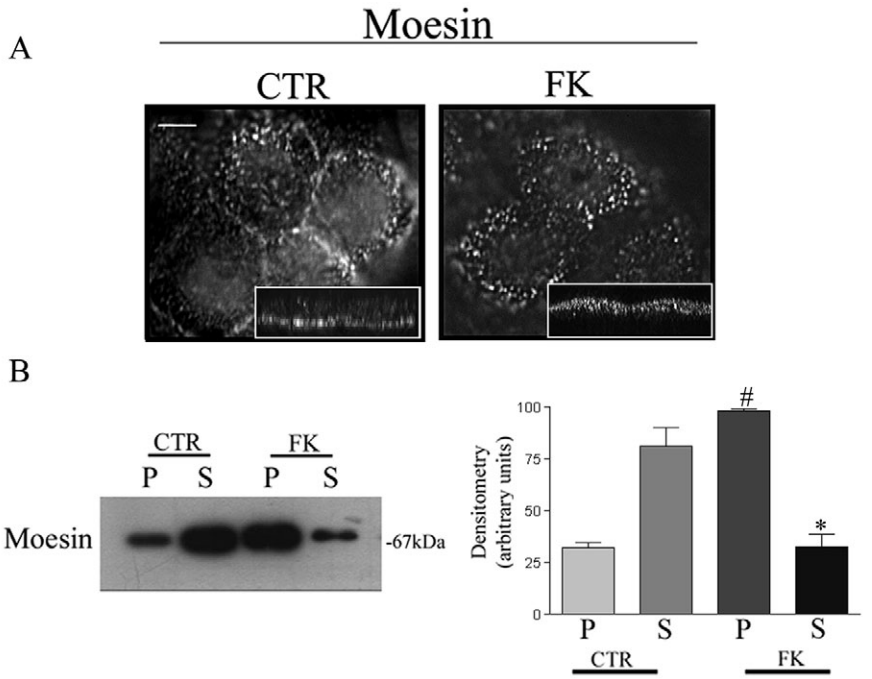

Fig. 1. Immunolocalization of moesin in CD8 cells. (A) Cells were either left untreated (CTR) or stimulated with forskolin (FK). After fixation, cells were immunostained with anti-moesin antibody (1:100 dilution). Fluorescence was visualized by epifluorescence microscopy and the xz reconstructions were obtained by deconvolution using Autodeblur software (insets). In untreated cells, moesin was predominantly localized at cell adhesion sites. In FK-stimulated cells, moesin was mainly expressed at cell-surface structures resembling apical microvilli. Bar, 5 μm. (B) Moesin distribution in subcellular fractions. Equal amounts of protein (30 μg/lane) from particulate (P, 150,000 ***g*** pellet) and soluble (S, 150,000 ***g*** supernatant) fractions from control and forskolin-stimulated cells were separated by gel electrophoresis and immunoblotted with anti-moesin antibody. Equal loading was confirmed by Coomassie blue staining. On the right, the densitometric analysis of the revealed bands is shown (**P*<0.01, #*P*<0.001, with respect to control; *n*=3).

Results

Forskolin stimulation results in moesin redistribution from the soluble to the particulate fraction

Because ERM protein can reversibly interact with actin, and Rho-dependent actin rearrangements occur during forskolin stimulation in renal CD8 cells, the localization of the ERM protein moesin was analyzed in CD8 cells at rest and in forskolin-stimulated cells.

Immunofluorescence localization of moesin in CD8 cells revealed a predominant expression at cell adhesion sites (Fig. 1A, CTR). These results are in agreement with previous observations showing that ERM proteins are localized at specific regions where actin filaments interact with plasma membranes, organizing actin enriched structures like microvilli, cleavage furrows and ruffling membranes. In forskolin-stimulated cells, the antibody stained mainly cell-surface structures that may represent apical microvilli, in agreement with similar localization in other cell lines (Hayashi et al., 1999; Henry et al., 1995; Yonemura et al., 1998) (Fig. 1A, FK). Western blotting analysis was performed following cell fractionation. In control cells, the majority of immunodetectable moesin was associated with the soluble fraction (S), compared with the particulate fraction (P). Forskolin stimulation resulted in a significant increase in moesin in the particulate fraction and was associated with a parallel decrease in the soluble fraction (Fig. 1B and densitometric analysis shown on the right). These findings suggest a relocalization of moesin from the soluble to the particulate fraction upon forskolin stimulation.

A peptide corresponding to the actin-binding domain of moesin induces actin remodeling

ERM proteins consist of three domains: a globular NH_2-terminal membrane-binding domain followed by an extended α-helical domain and a positively charged COOH-terminal domain. The C-terminal region of ERM proteins has a very high affinity for F-actin through its major actin-binding sites, which consist of 34 amino-acids and which are highly conserved among these proteins. Nevertheless, the lack of six C-terminal amino acids completely prevents any actin binding (Turunen et al., 1994). To investigate the role of moesin in regulating the actin cytoskeleton in CD8 cells, a peptide corresponding to a highly conserved sequence in the ERM family at the C-terminal domain (ERM peptide) was synthesized and introduced into CD8 cells as described by Oehlke (Oehlke et al., 1998; Scheller et al., 1999) (Fig. 2A). A peptide having a reversed sequence with respect to the ERM peptide was also synthesized, and was used as a control (Fig. 2A, control peptide).

The actual peptide internalization into the cells was confirmed by its fluorescence localization (Fig. 2B). Preincubation of the cells with the ERM peptide was associated with a strong, and time-dependent, decrease in moesin content in the soluble fraction. By contrast, preincubation with the control peptide for 3 hours had no effect on moesin abundance in the soluble fraction. In addition, forskolin treatment resulted in a decrease in moesin abundance in the soluble fraction (Fig. 2C). As we have previously shown that forskolin stimulation results in a partial depolymerization of actin cytoskeleton (Klussmann et al., 2001; Tamma et al., 2001; Tamma et al., 2003), we next tested whether the effect of the ERM peptide is associated with actin remodeling. Incubation of CD8 cells with the ERM peptide for 3 hours resulted in a dramatic reduction in stress fibers in the absence of forskolin stimulation (Fig. 3A, +ERM peptide), an effect similar to that obtained in forskolin-stimulated cells (Fig. 3A, FK, –peptide). Pretreatment with the control peptide had no effect on actin organization (Fig. 3A, +control peptide). Semi-quantitative analysis of the amount of F-actin evaluated with the actin polymerization assay confirmed that F-actin content significantly decreased

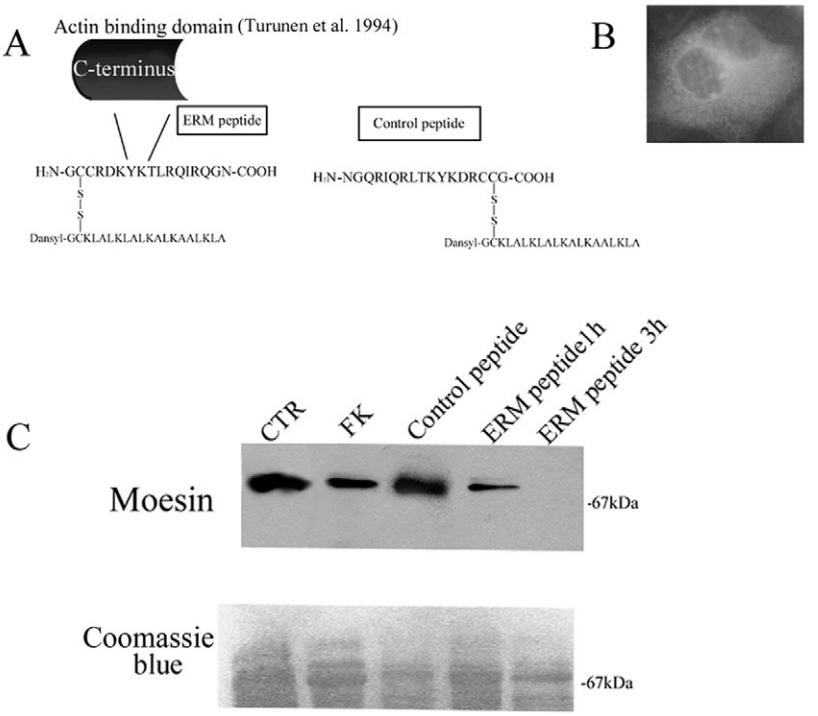

Fig. 2. (A) Primary structure of the ERM peptide which reproduces a short sequence located in the C-terminal region of the ERM proteins within the binding site for F-actin (Turunen et al., 1994). An irrelevant peptide having a reversed sequence was used as a control, the control peptide.
(B) Fluorescence localization of the ERM peptide in CD8 cells (2 μM, for 3 hours). (C) Moesin distribution in the soluble fraction. Cells were left untreated either stimulated with forskolin or pretreated for 1 or 3 hours with the ERM peptide or preincubated with the control peptide. After homogenization, the amount of protein in the 150,000 ***g*** supernatant was determined. Equal amounts of protein (30 μg/lane) were separated by gel electrophoresis and immunoblotted with anti-moesin antibody. Equal loading was confirmed by Coomassie blue staining. Notably, preincubation with the ERM peptide was associated with a strong and time-dependent decrease in moesin abundance in the soluble fraction – an effect similar to, although more potent than, that observed with forskolin treatment.

on preincubation with the ERM peptide (58.04±8.49 peptide; 85.13±2.33 control, $P<0.01$), an effect similar to that obtained with forskolin stimulation (forskolin 62.58±2.90, $P<0.001$) (Fig. 3B). Introduction of a control peptide with a reversed sequence had no effect on F-actin content (Fig. 3B, control peptide).

Introduction of the peptide causes AQP2 translocation to the plasma membrane

Actin depolymerization is an important prerequisite for cAMP-dependent translocation of the water channel protein AQP2 to the plasma membranes in renal principal cells (Klussmann et al., 2001; Tamma et al., 2001; Valenti et al., 2000). Therefore,

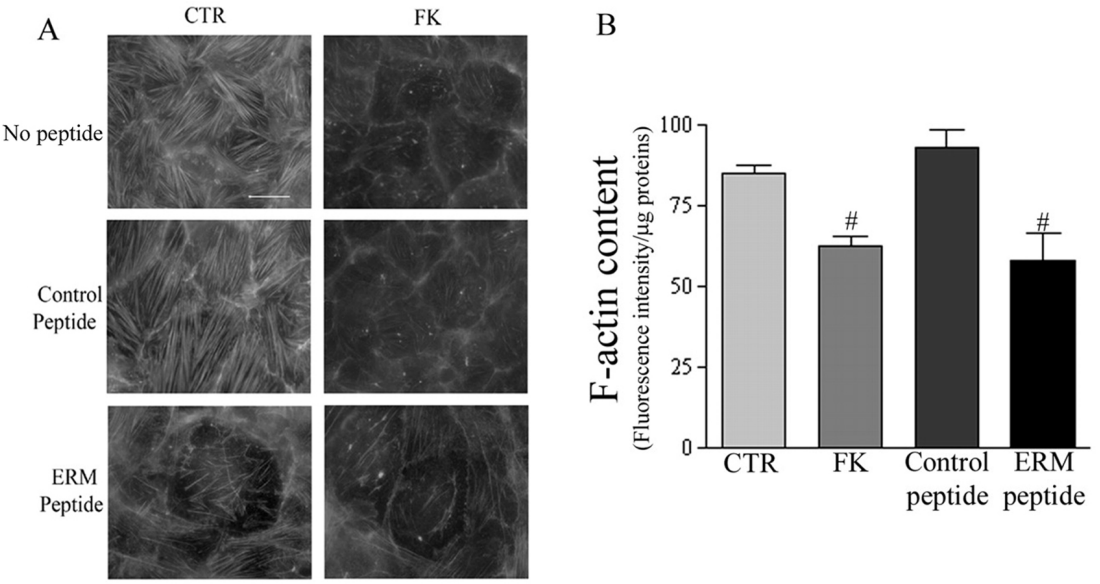

Fig. 3. Effect of the ERM peptide on actin organization. (A) Cells were either left untreated (CTR) or stimulated with forskolin (FK) with or without preincubation for 3 hours with the ERM peptide or with the control peptide. F-actin was stained with TRITC-conjugated phalloidin and visualized by epifluorescence microscopy. Bar, 5 μm. (B) F-actin quantization by actin polymerization assay. Confluent cells were either left untreated (CTR) or stimulated with forskolin (FK) or preincubated for 3 hours with the ERM peptide. As internal control, cells were incubated with a control peptide with a reversed sequence with respect to the ERM peptide. After staining with TRITC-phalloidin, cells were extracted with cold methanol and the fluorescence absorbance of extracts was read (540/565 nm) in a RF-5301PC fluorimeter. The values obtained were compared by a one-way Anova and Tukey's multiple comparison test (#$P<0.001$).

we next tested whether a plasma membrane relocalization of the water channel AQP2 occurs upon peptide preincubation. Immunofluorescence experiments were performed in the presence of the ERM and the control peptides. Preincubation with the ERM peptide induced a partial relocalization of AQP2 to the plasma membrane, which was completed upon forskolin treatment (Fig. 4, ERM peptide and xz reconstruction in the inset). As expected, forskolin stimulation caused AQP2 translocation from an intracellular pool to the apical membrane coincident with a decrease in intracellular staining due to the relocalization of AQP2 to the apical plasma membrane (Fig. 4, FK and xz reconstruction in the inset). Pretreatment with the control peptide did not affect the cellular localization of AQP2 (Fig. 4, control peptide and xz reconstruction in the inset).

Peptide-induced actin depolymerization is paralleled with a dissociation of moesin from the actin cytoskeleton and a consistent reduction in P-Thr-moesin abundance

AQP2 translocation, in the presence of the ERM peptide, might be due to the uncoupling effect of endogenous moesin from its effector/s, leading to actin depolymerization. Because phosphorylation of conserved threonine in ERM proteins influences the differential capacity to associate with actin cytoskeleton, we next evaluated the association dynamics between actin filaments and moesin in response to the introduction of the ERM peptide and during forskolin stimulation. To this end, we used an extraction procedure with Triton X-100 buffer, which preserves the cytoskeleton and the cytoskeleton-associated proteins. When Triton X-100 buffer-extracted material was compared by immunoblotting, we found that, relative to the control, the amount of actin-dissociated moesin was significantly increased both upon forskolin stimulation (densitometry of the signals: 2.667±0.48 fold, n=9, P<0.05 compared with control) and also after the ERM peptide treatment (3.84±0.64 fold, n=4, P<0.001). Interestingly, a similar effect was observed in response to the selective inhibitor of Rho kinase, Y27632 (3.083±0.31 fold, n=3, P<0.05 compared with control) (Fig. 5A). Compared with the control, preincubation with the control peptide had no effect on the amount of actin dissociated moesin (1.11±0.11 fold, n=3, P>0.05). The distribution of endogenous P-Thr-moesin was then examined in the Triton-soluble fractions. Compared with the control condition no significative difference in the band intensity was observed when the cells were either stimulated with forskolin or preincubated with the ERM peptide, whereas the phosphorylation level decreased significantly on Y27632 treatment (Fig. 5B). When the amount of P-Thr-moesin was normalized to the total amount of moesin of each Triton-soluble fraction, it seemed clear that overall, dissociation of moesin from actin cytoskeleton was associated with a consistent reduction in P-Thr-moesin abundance (Fig. 5C). In addition, these data confirm that actin depolymerization is paralleled with a dissociation of moesin from the actin cytoskeleton. Immunofluorescence localization of P-Thr-moesin revealed that at rest, it was localized not only at the cell-cell adhesion sites but also along intracellular filaments depicting actin stress fibers (Fig. 5D, CTR, arrowheads). This particular staining, as well as that at the cell-cell adhesion sites, disappeared after forskolin stimulation (Fig. 5D, FK).

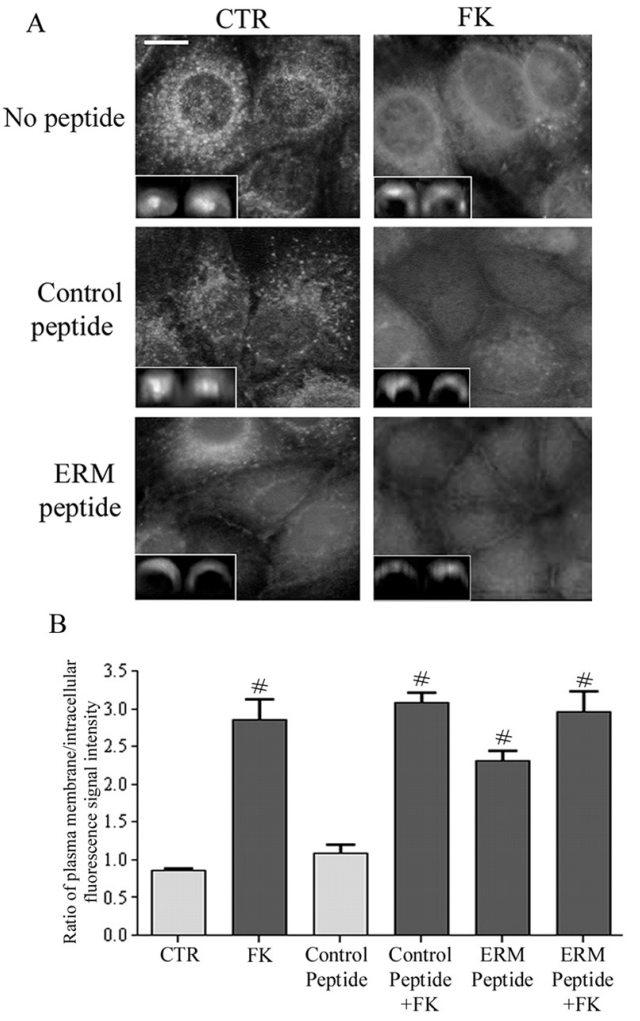

Fig. 4. Immunolocalization of AQP2 in cells pretreated with the ERM peptide. (A) Cells were either left untreated or stimulated with forskolin in the presence or absence of either ERM peptide or the control peptide. Fluorescence was visualized by epifluorescence microscopy and the xz reconstructions were obtained by deconvolution using Autodeblur software (shown in the insets). Forskolin stimulation caused AQP2 translocation from an intracellular pool to the apical membrane. A similar effect was observed on preincubation with the ERM peptide, while control peptide had no effect on AQP2 cellular localization. (B) Ratios of cell membrane/intracellular membrane fluorescence signals. Signal immunofluorescence intensities were detected from deconvoluted cells (Autodeblur software). The intracellular and cell membrane immunofluorescence signal intensities were calculated by using Metamorph software and normalized to the background signal intensities (n=15 for the control, n=13 for forskolin, n=13 for peptide control, n=13 for peptide control in the presence of forskolin, n=15 for ERM peptide, n=15 for ERM peptide in the presence of forskolin). Ratios greater than 1 indicate a cell membrane localization of AQP2. (#P<0.001 with respect to control). Values are expressed as means ± s.e.

Discussion

In this study we propose that moesin, a member of the ERM family known to cross-link actin filaments with plasma membranes, and to be implicated in signalling pathways, is a

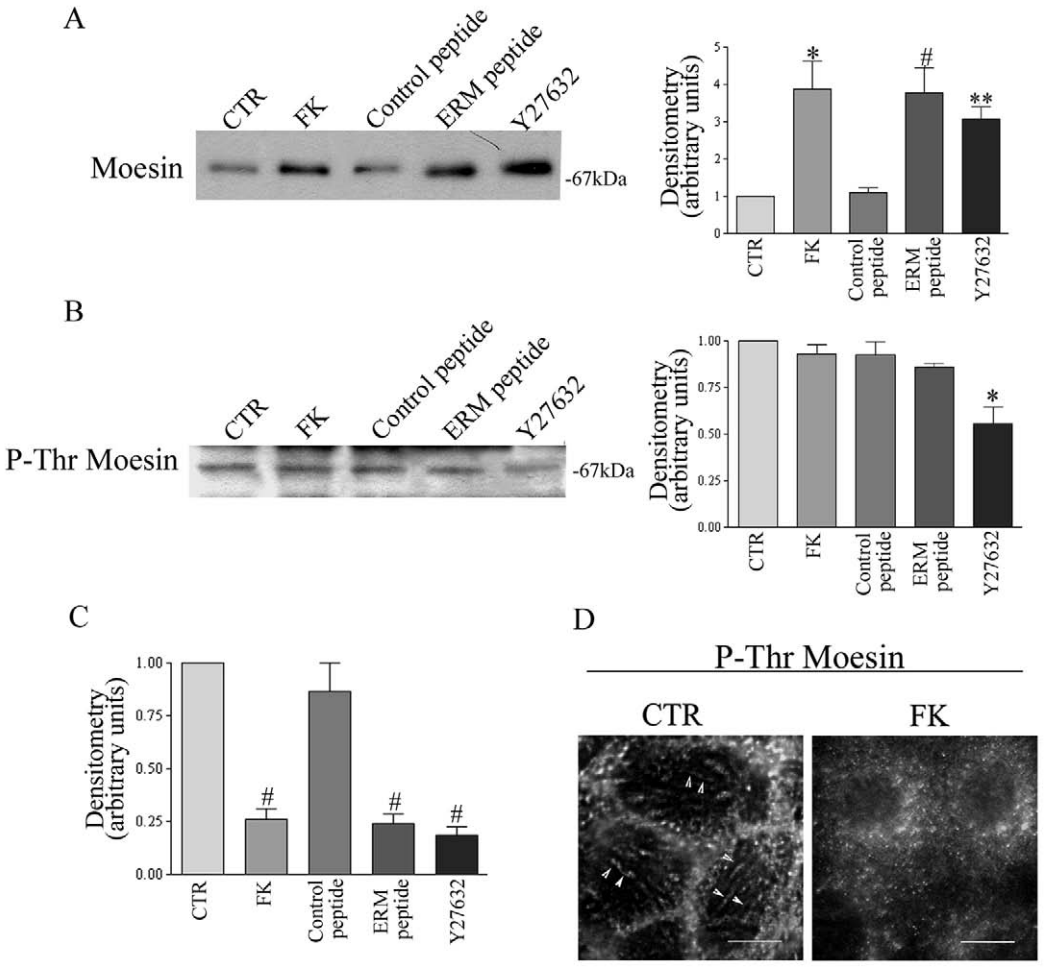

Fig. 5. Moesin association with actin cytoskeleton. The association of moesin with actin cytoskeleton was determined by its solubility in Triton X-100, which preserves the cytoskeleton and the cytoskeleton-associated proteins. Confluent cells were either left untreated (CTR) or stimulated with forskolin (FK) or preincubated for 3 hours with the control peptide or with the ERM peptide or with Y27632, an inhibitor of Rho kinase. Cells were incubated in Triton X-100 buffer and extracted proteins were immunoblotted with moesin antibody (A) or with P-Thr-moesin antibodies (B); on the right of each panel, the densitometric analysis of the bands obtained was shown. Histograms show the ratios between the densitometric signals of P-Thr-moesin and total moesin in each Triton-soluble fraction (C). (*P<0.01, **P<0.05, #P<0.001). Actin depolymerization is accompanied by a dissociation of moesin from actin cytoskeleton with a consistent reduction in P-Thr-moesin abundance. (D) Immunolocalization of P-Thr-moesin in CD8 cells. Cells were either left untreated or stimulated with forskolin. Fluorescence was visualized by epifluorescence microscopy. In untreated cells, P-Thr-moesin was localized at the cell-cell adhesion sites and along intracellular filaments depicting actin stress fibers (arrowheads). This particular staining disappeared in forskolin-stimulated cells. Bars, 5 μm.

new player regulating cAMP-induced AQP2 trafficking in renal principal cells. We report that forskolin-induced elevation of intracellular cAMP is associated with a redistribution of the endogenous ERM protein moesin, from the soluble (inactive) to the membrane (active) compartment. The functional role of moesin in cAMP-induced AQP2 translocation was analyzed by uncoupling moesin from its putative downstream effector. To this end, a peptide of 14 amino acids within the binding site for F-actin, reproducing the C-terminal domain of moesin, was synthesized and introduced into the cells. The designed peptide contains threonine 558, a residue that is thought to stabilize moesin in an active (open) state when phosphorylated.

Peptide introduction in CD8 cells induced a decrease in F-actin content, an effect similar to that obtained with forskolin stimulation. Notably, this caused AQP2 relocation to the plasma membrane which was associated with a time-dependent decrease of moesin abundance in the soluble fraction.

Therefore, incubation with the ERM peptide mimicked some of the key effects associated with hormonal or forskolin stimulation, including a decrease in F-actin content, moesin translocation from a soluble compartment to a particulate fraction, and AQP2 translocation to the cell surface. This suggests that moesin itself is involved in the signal transduction cascade leading to AQP2 targeting by having a possible role in the organization of cortical actin cytoskeleton.

Actin remodeling plays a crucial role in vesicular AQP2 trafficking. Indeed, we have previously shown that RhoA inhibition with specific toxins also causes depolymerization of the actin network, resulting in AQP2 translocation in the absence of hormonal stimulation (Klussmann et al., 2001; Tamma et al., 2001). ERM proteins are activated by signals

mediated through Rho (Matsui et al., 1998; Matsui et al., 1999; Ramesh, 2004; Shaw et al., 1998). ERM proteins positively regulate Rho activity by binding to RhoGDI and releasing the inactive Rho from GDI, thereby allowing activation of Rho (Ramesh, 2004). The interaction between the regulatory factor RhoGDI occurs at the N-terminal domain of ERM proteins only when they are active (Hirao et al., 1996; Takahashi et al., 1997). As mentioned previously, phosphorylation of ERM stabilizes the proteins in an open (active) conformation which does not affect the ability to interact with F-actin but rather prevents intramolecular interaction with the FERM domain, by stabilizing the activated ERM proteins (Matsui et al., 1998; Yonemura et al., 2002). We might expect that the introduction of the exogenous peptide, which contains threonine 558, phosphorylated by either Rho kinase or by protein kinase C, prevents the phosphorylation of the endogenous and functional proteins. The impairment of endogenous phosphorylation might influence the balance between the active and inactive forms of the ERM family proteins with the consequent release of regulatory factors such as RhoGDI. The RhoGDI released could directly interact with and inactivate Rho proteins leading to partial depolymerization of the actin cytoskeleton which facilitates AQP2 insertion (Klussmann et al., 2001; Tamma et al., 2001).

To conclude, we provide here the first evidence for a putative functional involvement of the ERM family protein moesin in AQP2 trafficking. Our data point to a dual role of moesin in this process: it might modulate actin depolymerization through Rho signalling due to its ability to reversibly bind RhoGDI and it participates in the reorganization of F-actin-containing cytoskeletal structures close to the AQP2-bearing vesicles fusion sites. Both processes seem to be reversibly modulated by moesin phosphorylation.

This work was supported by a grant from the EU-TMR network (proposal n° ERB 4061 PL 97-0406), from the Italian 'Ministero della Ricerca Scientifica e Tecnologica' and from the CEGBA (Centro di Eccellenza di Genomica in campo Biomedico ed Agrario), from the Vigoni program (1999-2001). W.R. and E.K. were supported by the Deutsche Forschungsgemeinschaft (Ro 597/6, Ro597/9-1 and Kl1415/1), the European Union (QLK3-CT-2002-02149 and QLRT-2000-00987), and the Fonds der Chemischen Industrie. We thank our colleague Anthony Green for proofreading and providing linguistic advice.

References

Bretscher, A. (1999). Regulation of cortical structure by the ezrin-radixin-moesin protein family. *Curr. Opin. Cell Biol.* **11**, 109-116.

Bretscher, A., Edwards, K. and Fehon, R. G. (2002). ERM proteins and merlin: integrators at the cell cortex. *Nat. Rev. Mol. Cell Biol.* **3**, 586-599.

Gary, R. and Bretscher, A. (1993). Heterotypic and homotypic associations between ezrin and moesin, two putative membrane-cytoskeletal linking proteins. *Proc. Natl. Acad. Sci. USA* **90**, 10846-10850.

Gautreau, A., Louvard, D. and Arpin, M. (2000). Morphogenic effects of ezrin require a phosphorylation-induced transition from oligomers to monomers at the plasma membrane. *J. Cell Biol.* **150**, 193-203.

Hall, A. L., Schlein, A. and Condeelis, J. (1988). Relationship of pseudopod extension to chemotactic hormone-induced actin polymerization in amoeboid cells. *J. Cell Biochem.* **37**, 285-299.

Hayashi, K., Yonemura, S., Matsui, T. and Tsukita, S. (1999). Immunofluorescence detection of ezrin/radixin/moesin (ERM) proteins with their carboxyl-terminal threonine phosphorylated in cultured cells and tissues. *J. Cell Sci.* **112**, 1149-1158.

Henry, M. D., Gonzalez Agosti, C. and Solomon, F. (1995). Molecular dissection of radixin: distinct and interdependent functions of the amino- and carboxy-terminal domains. *J. Cell Biol.* **129**, 1007-1022.

Hirao, M., Sato, N., Kondo, T., Yonemura, S., Monden, M., Sasaki, T., Takai, Y. and Tsukita, S. (1996). Regulation mechanism of ERM (ezrin/radixin/moesin) protein/plasma membrane association: possible involvement of phosphatidylinositol turnover and Rho-dependent signaling pathway. *J. Cell Biol.* **135**, 37-51.

Ivetic, A. and Ridley, A. J. (2004). Ezrin/radixin/moesin proteins and Rho GTPase signalling in leucocytes. *Immunology* **112**, 165-176.

Jankovics, F., Sinka, R., Lukacsovich, T. and Erdelyi, M. (2002). MOESIN crosslinks actin and cell membrane in Drosophila oocytes and is required for OSKAR anchoring. *Curr. Biol.* **12**, 2060-2065.

Klussmann, E., Tamma, G., Lorenz, D., Wiesner, B., Maric, K., Hofmann, F., Aktories, K., Valenti, G. and Rosenthal, W. (2001). An inhibitory role of Rho in the vasopressin-mediated translocation of aquaporin-2 into cell membranes of renal principal cells. *J. Biol. Chem.* **276**, 20451-20457.

Knetsch, M. L., Schafers, N., Horstmann, H. and Manstein, D. J. (2001). The Dictyostelium Bcr/Abr-related protein DRG regulates both Rac- and Rab-dependent pathways. *EMBO J.* **20**, 1620-1629.

Mammoto, A., Takahashi, K., Sasaki, T. and Takai, Y. (2000). Stimulation of Rho GDI release by ERM proteins. *Methods Enzymol.* **325**, 91-101.

Mangeat, P., Roy, C. and Martin, M. (1999). ERM proteins in cell adhesion and membrane dynamics: Authors' correction. *Trends Cell Biol.* **9**, 289.

Matsui, T., Maeda, M., Doi, Y., Yonemura, S., Amano, M., Kaibuchi, K. and Tsukita, S. (1998). Rho-kinase phosphorylates COOH-terminal threonines of ezrin/radixin/moesin (ERM) proteins and regulates their head-to-tail association. *J. Cell Biol.* **140**, 647-657.

Matsui, T., Yonemura, S. and Tsukita, S. (1999). Activation of ERM proteins in vivo by Rho involves phosphatidyl-inositol 4-phosphate 5-kinase and not ROCK kinases. *Curr. Biol.* **9**, 1259-1262.

Oehlke, J., Scheller, A., Wiesner, B., Krause, E., Beyermann, M., Klauschenz, E., Melzig, M. and Bienert, M. (1998). Cellular uptake of an alpha-helical amphipathic model peptide with the potential to deliver polar compounds into the cell interior non-endocytically. *Biochim. Biophys. Acta* **1414**, 127-139.

Peracino, B., Borleis, J., Jin, T., Westphal, M., Schwartz, J. M., Wu, L., Bracco, E., Gerisch, G., Devreotes, P. and Bozzaro, S. (1998). G protein beta subunit-null mutants are impaired in phagocytosis and chemotaxis due to inappropriate regulation of the actin cytoskeleton. *J. Cell Biol.* **141**, 1529-1537.

Ramesh, V. (2004). Merlin and the ERM proteins in Schwann cells, neurons and growth cones. *Nat. Rev. Neurosci.* **5**, 462-470.

Reczek, D. and Bretscher, A. (1998). The carboxyl-terminal region of EBP50 binds to a site in the amino-terminal domain of ezrin that is masked in the dormant molecule. *J. Biol. Chem.* **273**, 18452-18458.

Scheller, A., Oehlke, J., Wiesner, B., Dathe, M., Krause, E., Beyermann, M., Melzig, M. and Bienert, M. (1999). Structural requirements for cellular uptake of alpha-helical amphipathic peptides. *J. Pept. Sci.* **5**, 185-194.

Shaw, R. J., Henry, M., Solomon, F. and Jacks, T. (1998). RhoA-dependent phosphorylation and relocalization of ERM proteins into apical membrane/actin protrusions in fibroblasts. *Mol. Biol. Cell* **9**, 403-419.

Speck, O., Hughes, S. C., Noren, N. K., Kulikauskas, R. M. and Fehon, R. G. (2003). Moesin functions antagonistically to the Rho pathway to maintain epithelial integrity. *Nature* **421**, 83-87.

Takahashi, K., Sasaki, T., Mammoto, A., Takaishi, K., Kameyama, T., Tsukita, S. and Takai, Y. (1997). Direct interaction of the Rho GDP dissociation inhibitor with ezrin/radixin/moesin initiates the activation of the Rho small G protein. *J. Biol. Chem.* **272**, 23371-23375.

Tamma, G., Klussmann, E., Maric, K., Aktories, K., Svelto, M., Rosenthal, W. and Valenti, G. (2001). Rho inhibits cAMP-induced translocation of aquaporin-2 into the apical membrane of renal cells. *Am. J. Physiol. Renal Physiol.* **281**, F1092-F1101.

Tamma, G., Klussmann, E., Procino, G., Svelto, M., Rosenthal, W. and Valenti, G. (2003). cAMP-induced AQP2 translocation is associated with RhoA inhibition through RhoA phosphorylation and interaction with RhoGDI. *J. Cell Sci.* **116**, 1519-1525.

Tsukita, S. and Yonemura, S. (1997). ERM proteins: head-to-tail regulation of actin-plasma membrane interaction. *Trends Biochem. Sci.* **22**, 53-58.

Tsukita, S. and Yonemura, S. (1999). Cortical actin organization: lessons from ERM (ezrin/radixin/moesin) proteins. *J. Biol. Chem.* **274**, 34507-34510.

Turunen, O., Wahlstrom, T. and Vaheri, A. (1994). Ezrin has a COOH-

terminal actin-binding site that is conserved in the ezrin protein family. *J. Cell Biol.* **126**, 1445-1453.

Valenti, G., Frigeri, A., Ronco, P. M., D'Ettorre, C. and Svelto, M. (1996). Expression and functional analysis of water channels in a stably AQP2-transfected human collecting duct cell line. *J. Biol. Chem.* **271**, 24365-24370.

Valenti, G., Procino, G., Carmosino, M., Frigeri, A., Mannucci, R., Nicoletti, I. and Svelto, M. (2000). The phosphatase inhibitor okadaic acid induces AQP2 translocation independently from AQP2 phosphorylation in renal collecting duct cells. *J. Cell Sci.* **113**, 1985-1992.

Valenti, G., Procino, G., Liebenhoff, U., Frigeri, A., Benedetti, P. A., Ahnert-Hilger, G., Nurnberg, B., Svelto, M. and Rosenthal, W. (1998). A heterotrimeric G protein of the Gi family is required for cAMP-triggered trafficking of aquaporin 2 in kidney epithelial cells. *J. Biol. Chem.* **273**, 22627-22634.

Van Furden, D., Johnson, K., Segbert, C. and Bossinger, O. (2004). The C. elegans ezrin-radixin-moesin protein ERM-1 is necessary for apical junction remodelling and tubulogenesis in the intestine. *Dev. Biol.* **272**, 262-276.

Yonemura, S., Hirao, M., Doi, Y., Takahashi, N., Kondo, T. and Tsukita, S. (1998). Ezrin/radixin/moesin (ERM) proteins bind to a positively charged amino acid cluster in the juxta-membrane cytoplasmic domain of CD44, CD43, and ICAM-2. *J. Cell Biol.* **140**, 885-895.

Yonemura, S., Matsui, T. and Tsukita, S. (2002). Rho-dependent and -independent activation mechanisms of ezrin/radixin/moesin proteins: an essential role for polyphosphoinositides in vivo. *J. Cell Sci.* **115**, 2569-2580.