

Rab13 regulates PKA signaling during tight junction assembly

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The GTPase Rab13 regulates the assembly of functional epithelial tight junctions (TJs) through a yet unknown mechanism. Here, we show that expression of the GTP-bound form of Rab13 inhibits PKA-dependent phosphorylation and TJ recruitment of the vasodilator-stimulated phosphoprotein, an actin remodelling protein. We demonstrate that Rab13GTP directly binds to PKA and

inhibits its activity. Interestingly, activation of PKA abrogates the inhibitory effect of Rab13 on the recruitment of vasodilator-stimulated phosphoprotein, ZO-1, and claudin1 to cell–cell junctions. Rab13 is, therefore, the first GTPase that controls PKA activity and provides an unexpected link between PKA signaling and the dynamics of TJ assembly.

Introduction

During epithelial morphogenesis, the development and organization of cell asymmetry require the assembly of functional tight junctions (TJs). TJs serve as a barrier to the paracellular diffusion and prevent the movement of lipids and membrane proteins between apical and basolateral membranes (Tsukita et al., 2001; Matter and Balda, 2003). They form a network of transmembrane, scaffolding, and signaling molecules that control cell–cell adhesion, cell polarity, proliferation, and differentiation (Zahraoui et al., 2000). The TJ transmembrane proteins, claudins, are required for the establishment of TJ gate function (Tsukita and Furuse, 1999). Scaffolding proteins such as ZO-1, a PDZ-containing protein, connect transmembrane proteins to the underlying actin cytoskeleton and recruit cytosolic proteins, such as kinases, GTPases, and transcription factors to TJs (Itoh et al., 1999; Wittchen et al., 1999; Balda and Matter, 2000). The actin cytoskeleton contributes substantially to maintain TJ integrity (Madara et al., 1992). In CaCo-2 cells, disruption of actin filaments with cytochalasin D decreases, and stabilization of F-actin increases barrier function (Madara et al., 1986; Balda et al., 1993). Recently, vasodilator-stimulated phosphoprotein (VASP), a key actin remodelling protein, has been implicated in epithelial TJ biogenesis (Lawrence et al., 2002). VASP was shown to be

a substrate for both PKA and PKG (Butt et al., 1994), and phosphorylation of VASP abolishes its interaction with actin and inhibits actin polymerization, assigning VASP a key role in controlling actin dynamics (Harbeck et al., 2000). However, the regulatory pathways that link actin cytoskeleton organization to the assembly of TJ still need to be elucidated. Interestingly, activation of the small GTPases, Rho/Rac, induces the reorganization of the perijunctional actin ring and leads to increased paracellular permeability (Takaishi et al., 1997; Benais-Pont et al., 2003).

Another family of small GTPases, Rab proteins, is also involved in regulating TJ dynamics. Rab GTPases seem to regulate specific membrane transport events including vesicle formation, motility via kinesins or myosins, tethering, and fusion (Pfeffer, 2001; Zerial and McBride, 2001). We have shown previously that Rab13 is recruited from a cytoplasmic pool to cell–cell contacts at an early stage during TJ assembly (Zahraoui et al., 1994; Sheth et al., 2000). Expression of the active (Rab13Q67L), but not the inactive (Rab13T22N) mutant of Rab13 in epithelial MDCK cells delays the recruitment of TJ proteins, claudin1 and ZO-1, and alters TJ integrity. Interestingly, it does not impair the recruitment of E-cadherin to the lateral membrane (Marzesco et al., 2002). Nevertheless, how Rab13 interferes with TJ structure and function is still unclear. In the present work, we show that the active mutant Rab13Q67L inhibits PKA-mediated phosphorylation of VASP and its recruitment to cell–cell junctions.

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Abbreviations used in this paper: aPKC ζ , atypical PKC ζ ; P-VASP, phosphorylated VASP; TJ, tight junction; VASP, vasodilator-stimulated phosphoprotein.

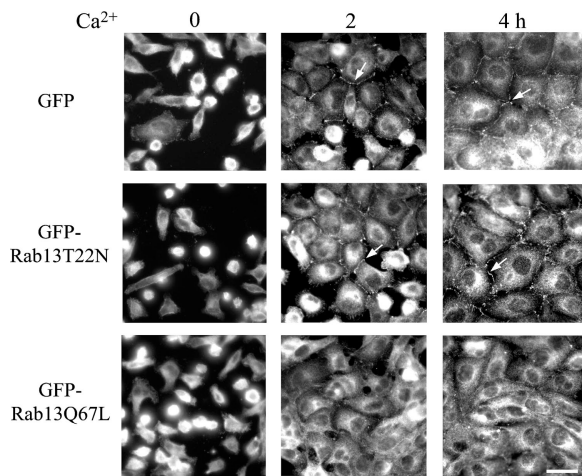


Figure 1. Rab13Q67L inhibits the recruitment of VASP to cell–cell junctions during TJ formation. MDCK cells expressing GFP, GFP-Rab13T22N, or GFP-Rab13Q67L were incubated for 16 h without Ca^{2+} , then Ca^{2+} was added back for the time indicated and the cells stained with a monoclonal anti-VASP antibody. Within 2–4 h after the Ca^{2+} switch, VASP was detected at cell–cell junctions in cells expressing GFP and GFP-Rab13T22N (arrows), but not in those expressing GFP-Rab13Q67L. Bar, 10 μm .

Moreover, Rab13GTP binds to PKA and inhibits its activity. Our data provide new insights into the molecular mechanisms by which Rab13 regulates TJ assembly in epithelial cells.

Results and discussion

Rab13Q67L prevents the recruitment of VASP to TJs

The effect of GFP-Rab13Q67L on TJ protein recruitment (Marzesco et al., 2002) suggests that Rab13 regulates some aspects of actin cytoskeleton–TJ interactions. Because VASP is involved in the establishment of TJ barrier (Lawrence et al., 2002), we tested whether Rab13 mutants could affect VASP localization during TJ assembly. We used the Ca^{2+} switch protocol, a model system to study TJ assembly. Cells were plated in medium without Ca^{2+} for 16 h to dissociate cell–cell junctions, followed by the addition of Ca^{2+} to trigger synchronous junction assembly. The localization of VASP was analyzed by immunofluorescence at 0, 2, and 4 h after induction of cell–cell contacts. In control cells, as well as in cells expressing GFP-Rab13T22N and Q67L mutants, removal of Ca^{2+} resulted in the dissociation of cell–cell junctions and redistribution of VASP into the cytoplasm (Fig. 1). Restoration of physiological Ca^{2+} concentrations induced an increased plasma membrane labeling of VASP at sites of cell–cell contacts in cells expressing GFP or GFP-Rab13T22N. In contrast, in GFP-Rab13Q67L cells, within 4 h after the addition of Ca^{2+} , VASP was not detected at cell–cell contacts and was still cytoplasmic (Fig. 1). This indicated that Rab13Q67L inhibits the recruitment of VASP to sites of cell–cell contacts during junction assembly.

Rab13 controls PKA-dependent phosphorylation of VASP and its subsequent recruitment to cell–cell junctions

Because phosphorylated VASP (P-VASP) accumulates at cell–cell junctions under conditions that enhance barrier re-

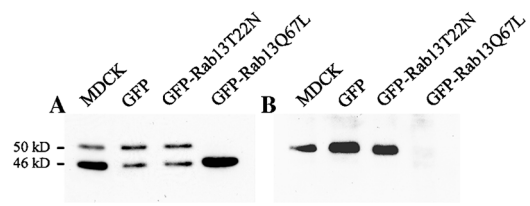


Figure 2. The activated form of Rab13 inhibits phosphorylation of VASP. (A) Lysates from MDCK or cells expressing GFP, GFP-Rab13T22N, or GFP-Rab13Q67L were blotted with the monoclonal anti-VASP antibody recognizing both the phosphorylated (50 kD) and non phosphorylated (46 kD) form of VASP. In GFP-Rab13Q67L cells, the 50-kD band was not detected. (B) The same lysates as in A were blotted with a specific antibody recognizing VASP phosphorylated at Ser157 (P-VASP). Note that P-VASP was absent in cells expressing GFP-Rab13Q67L. Similar results were obtained from three independent GFP-Rab13T22N and GFP-Rab13Q67L mutants.

covery, it was proposed that P-VASP is a component of TJs (Lawrence et al., 2002). VASP phosphorylation on residue Ser157 results in a conformational change concomitant with a shift in SDS-PAGE mobility from 46 to 50 kD (Butt et al., 1994). Thus, we tested whether Rab13Q67L could interfere with VASP phosphorylation. Using a monoclonal anti-VASP antibody that recognizes nonphosphorylated as well as P-VASP, we found that in MDCK cells and cells expressing GFP or GFP-Rab13T22N, both forms of VASP were detected. In contrast, in cells expressing GFP-Rab13Q67L, only the 46-kD band corresponding to the nonphosphorylated form of VASP was detected (Fig. 2 A). This indicates that GFP-Rab13Q67L inhibited phosphorylation of VASP at Ser157. To confirm these results, the same cell lysates used in Fig. 2 A were blotted with an anti-VASP antibody that specifically recognizes VASP phosphorylated at Ser157 (P-VASP). This antibody detected the 50-kD band corresponding to P-VASP in cells expressing GFP-Rab13T22N and in control cells, whereas no P-VASP specific band was observed in GFP-Rab13Q67L cell lysate (Fig. 2 B). Thus, the expression of Rab13Q67L specifically inhibits the phosphorylation of VASP at Ser157 in MDCK cells.

VASP phosphorylation at Ser157 was observed upon stimulation of PKA or PKG (Burkhardt et al., 2000). To determine which kinase is responsible for the Rab13-dependent phosphorylation of VASP, we examined the effect of specific kinase inhibitors and activators on VASP phosphorylation. As shown in Fig. 3 A, incubation with both PKG and PKC inhibitors did not significantly reduce the phosphorylation of VASP in control cells or cells expressing GFP-Rab13T22N. In contrast, incubation with the PKA inhibitor H89 decreased the amount of P-VASP in a dose-dependent manner, indicating that the effect on VASP-phosphorylation is specific. Moreover, treatment of cells expressing Rab13Q67L with 8-Br-cGMP, an activator of PKG, did not induce phosphorylation of VASP in those cells. Conversely, activation of PKA by either 8-Br-cAMP or Forskolin restored VASP phosphorylation in GFP-Rab13Q67L cells as monitored by the presence of the 50-kD band of VASP (Fig. 3 B). These data indicate that Rab13-dependent phosphorylation of VASP in MDCK cells is mediated by PKA, but not PKC or PKG.

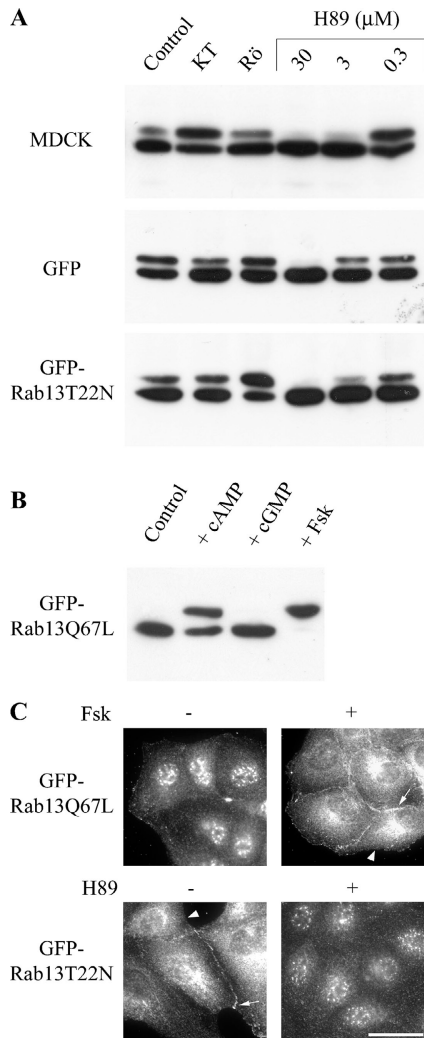


Figure 3. Rab13 regulates PKA-mediated VASP phosphorylation, thereby controlling VASP recruitment to cell–cell junctions. (A) PKA inhibition decreases VASP phosphorylation. MDCK or cells expressing GFP or GFP-Rab13T22N were treated with inhibitors of PKG (KT5823), PKC (Rö-32-0432), or PKA (H89) and the lysates blotted for VASP. (B) Activation of PKA by Forskolin or cAMP, but not that of PKG by cGMP restores VASP phosphorylation in Rab13Q67L cells. Cells expressing GFP-Rab13Q67L were incubated with Forskolin (Fsk), 8-Br-cAMP or 8-Br-cGMP and VASP was detected by immunoblot. Nontreated cells were blotted as a control. (C) Cells expressing GFP-Rab13Q67L or GFP-Rab13T22N were treated with Forskolin (Fsk) or the PKA inhibitor (H89), respectively, and analyzed by immunofluorescence with the anti-P-VASP antibody. P-VASP was not detected at cell–cell junctions in Rab13Q67L cells, but relocated to TJ upon PKA activation. Inhibition of PKA removed P-VASP from cell–cell contacts in cells expressing Rab13T22N. P-VASP was found in cell–cell contacts where junctional complexes developed with neighboring cells (arrows), and absent from the edges of cells devoid of junctional complexes (arrowheads). Bar, 10 μ m.

To investigate whether VASP phosphorylation is required for its recruitment to cell–cell junctions, MDCK cells expressing GFP-Rab13Q67L or GFP-Rab13T22N were incubated with Forskolin and H89, respectively, and P-VASP localization was examined by immunofluorescence using the antibody recognizing P-VASP. In the absence of Forskolin, no P-VASP could be detected at cell–cell junctions in cells

expressing GFP-Rab13Q67L (Fig. 3 C). However, activation of PKA by Forskolin restored the recruitment of P-VASP to cell–cell junctions in those cells. In contrast, in cells expressing GFP-Rab13T22N, P-VASP was detected at cell–cell junctions. Treatment of those cells with the specific PKA inhibitor H89 removed P-VASP from cell–cell junctions (Fig. 3 C). Overall, our results demonstrate that activation of Rab13 inhibits PKA-mediated phosphorylation of VASP and prevents its association with intercellular junctions.

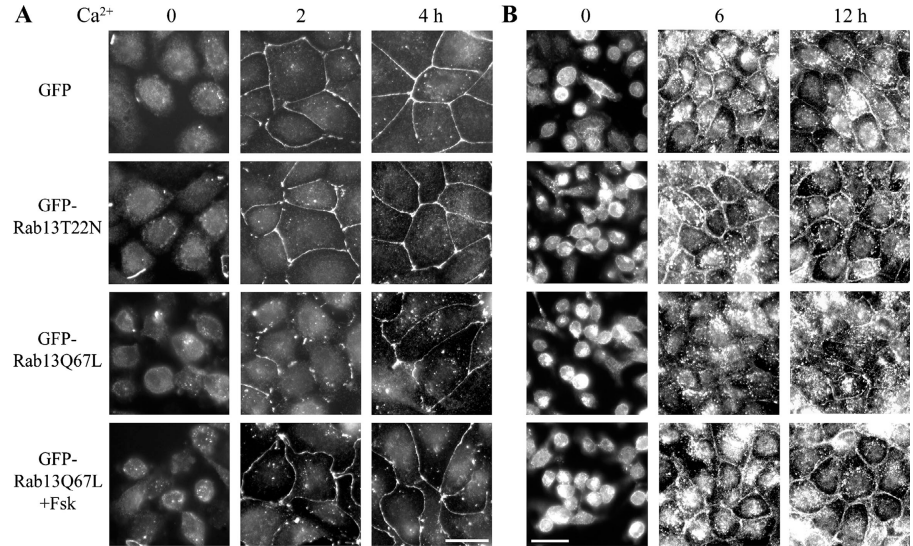
Activation of PKA abolishes the inhibitory effect of Rab13Q67L on the recruitment of TJ proteins, ZO-1 and claudin1

To determine whether the effect of Rab13 on claudin1 and ZO-1 recruitment could be mediated by PKA, we examined the consequences of PKA activation on the recruitment of ZO-1 and claudin1 in cells expressing GFP-Rab13Q67L using the Ca^{2+} switch assay. In control cells as well as in cells expressing GFP-Rab13T22N or GFP-Rab13Q67L, removal of Ca^{2+} resulted in the dissociation of cell–cell junctions and redistribution of ZO-1 and claudin1 into the cytoplasm (Fig. 4). By 2 h after addition of Ca^{2+} , ZO-1 immunoreactivity was already detected at cell–cell junctions in cells expressing GFP and GFP-Rab13T22N. In contrast, expression of GFP-Rab13Q67L delayed slightly the recruitment of ZO-1; ZO-1 labeling was discontinuous along the lateral membrane. By 4 h after readdition of calcium, ZO-1 staining was detected at the plasma membrane, but some ZO-1 immunoreactivity was still observed in the cytoplasm in cells expressing GFP-Rab13Q67L (Fig. 4 A). Similarly, plasma membrane recruitment of claudin1 was delayed in cells expressing GFP-Rab13Q67L. Within 6–12 h after readdition of calcium, most of the claudin1 staining was still cytoplasmic in GFP-Rab13Q67L cells, whereas claudin1 was detected at the plasma membrane in cells expressing GFP or GFP-Rab13T22N (Fig. 4 B). Significantly, activation of PKA with Forskolin restored the kinetics of both ZO-1 and claudin1 recruitment to the lateral membrane in GFP-Rab13Q67L cells (Fig. 4). This indicates that PKA activation abolished the inhibitory effect of Rab13GTP on the recruitment of TJ proteins, leading to their accumulation at cell–cell junctions. Our data establish that the Rab13–PKA signaling complex plays a key role in TJ biogenesis. They further confirm that PKA-dependent phosphorylation is implicated in the dynamics of epithelial TJ.

Rab13GTP binds to the PKA catalytic subunit and inhibits its activity in vitro

Given that Rab13 interferes with PKA signaling in the regulation of TJ assembly, we wondered whether and how Rab13 interacts with PKA. Thus, we performed a GST pull-down assay from MDCK cell lysates using GST-Rab13 fusion proteins. GST alone or GST-Rab13 were immobilized on glutathione beads, preloaded either with GDP or GTP γ S (a poorly hydrolysable GTP analogue), and incubated with MDCK cell lysates. The proteins retained on the beads were separated on SDS-PAGE and blotted with an antibody against the α catalytic subunit of PKA. As shown in Fig. 5 A, the α catalytic subunit of PKA, but not atypical PKC ζ (aPKC ζ), was preferentially recovered with GST-Rab13

Figure 4. PKA activation abolishes the inhibitory effect of Rab13Q67L on the recruitment of ZO-1 and claudin1. Cells expressing GFP, GFP-Rab13T22N, or GFP-Rab13Q67L were grown without Ca^{2+} for 16 h and subsequently incubated in normal medium for the time indicated in the absence or presence of Forskolin (Fsk). The localization of (A) ZO-1 and (B) claudin1 was analyzed by immunofluorescence. In cells expressing Rab13Q67L, the recruitment of ZO-1 and claudin1 to cell–cell junctions was delayed. Forskolin treatment restored ZO-1 and claudin1 recruitment in those cells. Bars, 10 μm .



loaded with $\text{GTP}\gamma\text{S}$, whereas GST alone did not interact with PKA or $\text{aPKC}\zeta$. To verify whether the Rab13–PKA interaction is direct, GST–Rab13 loaded with GDP, $\text{GTP}\gamma\text{S}$, or GST alone were incubated with a purified preparation of PKA catalytic subunit. Blotting with the anti-PKA antibody revealed that PKA preferentially bound to GST–Rab13– $\text{GTP}\gamma\text{S}$, but neither to GST alone, nor to GST–Rab6 $\text{GTP}\gamma\text{S}$ (Fig. 5 B), indicating that Rab13 interacts directly and specifically with the PKA catalytic subunit.

Because the GTP bound form of Rab13 inhibits PKA-dependent VASP phosphorylation *in vivo*, we next determined whether Rab13GTP interferes with PKA activity *in vitro*. VASP was immunoprecipitated from cells expressing GFP–Rab13Q67L and used as a physiologically relevant substrate for PKA in a kinase assay. PKI, a potent peptide inhibitor of PKA, was used as a positive control in these experiments. Incubation of VASP with the catalytic subunit of PKA induced VASP phosphorylation *in vitro*, resulting in a shift in SDS-PAGE mobility from 46 to 50 kD (Fig. 5 C). Similarly to PKI, GST–Rab13– $\text{GTP}\gamma\text{S}$ caused a strong inhibition of PKA activity, whereas GST alone had no effect. The results *in vitro* are concordant with those *in vivo* and demonstrate that Rab13 is able to inhibit PKA activity.

In this paper, we identified PKA as a new effector for the small GTPase Rab13. Our data demonstrate that the GTP bound form of Rab13 directly binds and negatively controls PKA activity both *in vitro* and *in vivo*. This is the first demonstration of a biochemical and functional link between a small Rab GTPase and the PKA catalytic subunit.

Protein phosphorylation/dephosphorylation events are required for the regulation of TJ dynamics (Sakakibara et al., 1997; Matter and Balda, 2003). In these processes, VASP may play a key role due to its differential actin binding capacities depending on its state of phosphorylation. Phosphorylation of VASP by PKA reduces VASP–actin interactions and promotes the association of P-VASP with cell–cell junctions. Interestingly, VASP is required for the establishment of cell–cell contacts (Vasioukhin et al., 2000). Although Rab13 does not affect the recruitment of E-cadherin (Marzesco et al., 2002) and β -catenin to cell–cell junctions (Fig. S1, available at <http://www.jcb.org>),

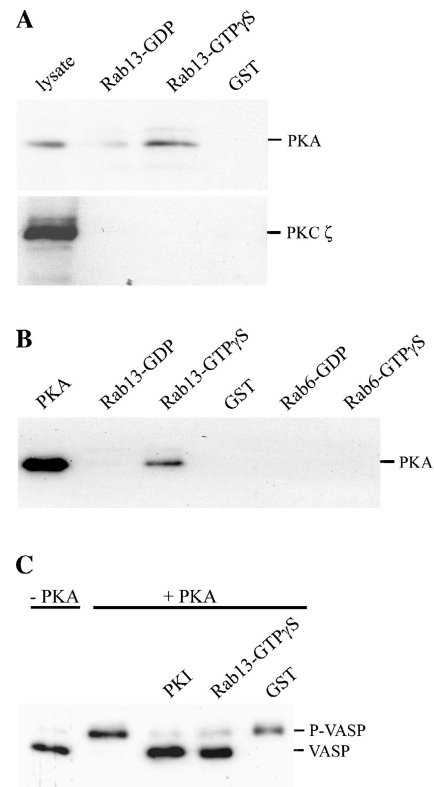


Figure 5. Rab13GTP interacts *in vitro* directly with PKA and inhibits its activity. (A) MDCK cell lysates were incubated with GST or GST–Rab13 fusion proteins preloaded with GDP or $\text{GTP}\gamma\text{S}$. The retained proteins were analyzed by SDS-PAGE and immunoblotted with antibodies against the α catalytic subunit of PKA or $\text{aPKC}\zeta$. Equal loading was confirmed by ponceau staining. (B) GST–Rab13 or GST–Rab6 preloaded with GDP, $\text{GTP}\gamma\text{S}$, or GST was incubated with the purified catalytic subunit of PKA. Proteins bound to the beads were separated by SDS-PAGE and blotted with the anti-PKA antibody. 48% of the PKA was recovered with GST–Rab13– $\text{GTP}\gamma\text{S}$. (C) VASP immunoprecipitate from cells expressing GFP–Rab13Q67L was incubated in an *in vitro* kinase assay with the purified catalytic subunit of PKA in the presence of PKI (a peptide inhibitor of PKA), GST–Rab13– $\text{GTP}\gamma\text{S}$, or GST. Samples were subjected to SDS-PAGE and immunoblotted with the anti-VASP antibody recognizing both phosphorylated and non-P-VASP.

www.jcb.org/cgi/content/full/jcb.200312118/DC1), we cannot rule out the possibility that Rab13 alters TJ assembly through regulating the recruitment of other adherens junction components. We suggest that Rab13GTP could locally influence actin rearrangement by controlling PKA-dependent VASP phosphorylation and hence inhibit TJ integrity. Our previous data suggested that Rab13, in its GTP-bound form, interacts with an effector that inhibits the recruitment of claudin1 and, to a lesser extent, that of ZO-1 to TJ. Conversely, the inactive form Rab13T22N, unable to bind the effector, favors the establishment of TJ gate and fence functions (Marzesco et al., 2002). The inhibitory effect of the Rab13Q67L mutant on TJ assembly and function could thus be explained by the inhibition of PKA activity. The Rab13–PKA signaling complex may as well regulate vesicle docking near cell–cell junctions. Indeed, PKA activity has been shown to be required for membrane trafficking (Pimplikar and Simons, 1994; Rodionov et al., 2003). Therefore, our observations allow us to postulate the existence of a new signaling cascade at the TJ implicating Rab13–PKA in the dynamics of TJ assembly. They also highlight novel insights into the role of Rab proteins in membrane–cytoskeleton interactions.

Materials and methods

Antibodies

Anti-VASP mAb was purchased from BD Transduction Laboratories; monoclonal anti-VASP antibody specific for VASP phosphorylated at Ser157 was purchased from NanoTools; polyclonal anti-ZO-1 and anti-claudin-1 antibodies were purchased from Zymed Laboratories; anti-PKA α cat and anti-PKC ζ pAbs were purchased from Santa Cruz Biotechnology, Inc. Secondary antibodies coupled to Cy2 or Cy5 were purchased from Jackson ImmunoResearch Laboratories.

Cell culture

MDCK cells (clone II) were cultured in DME supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 10 mg/ml streptomycin. The cells were incubated at 37°C under 10% CO₂ atmosphere. MDCK cells stably expressing GFP, GFP-Rab13T22N, or GFP-Rab13Q67L were described previously (Marzesco et al., 2002).

Incubation with kinase inhibitors/activators

500,000 cells were plated onto 3.5-cm diam culture plates overnight and incubated as follows: 1 h at 37°C with 30 μ M of PKA inhibitor H-89; 30 min with the PKC and PKG inhibitors R \ddot{o} -32-0432 (10 μ M) or KT5823 (5 μ M; Calbiochem); 30 min with 100 μ M cAMP or cGMP; or 20 min with 10 μ M Forskolin (Sigma-Aldrich). Cells were washed three times in PBS and subjected to either immunofluorescence or immunoblotting procedures. We confirmed that R \ddot{o} -32-0432 is effective in inhibiting PKC activity under these conditions.

Ca²⁺ switch experiments

MDCK cells expressing GFP or GFP-Rab13T22N and GFP-Rab13Q67L mutants were plated at a density of 10,000,000 cells/cm², and incubated for 16 h in S-MEM medium without Ca²⁺. Cells were rinsed with normal DME, incubated in DME for the indicated time and analyzed by immunofluorescence. When the effect of PKA activation/inhibition was studied, Forskolin and H89 were added to DME after the Ca²⁺ depletion.

Immunofluorescence microscopy

Immunofluorescence was performed as described previously (Marzesco et al., 2002). The samples were analyzed with a fluorescence microscope (Carl Zeiss MicroImaging Inc.) and further processed with Adobe Photoshop Software.

Immunoprecipitation and immunoblotting

500,000 MDCK or cells expressing GFP, GFP-Rab13T22N, or GFP-Rab13Q67L were grown on 3.5-cm diam culture plates for 16 h, washed three times with ice-cold PBS, and extracted in 0.5% Triton, 10 mM Tris-

HCl, pH 7.6, 120 mM NaCl, 25 mM KCl, 1.8 mM CaCl₂, 1 mM sodium vanadate, 50 mM NaF, and a mixture of protease inhibitors (Sigma-Aldrich) on a rocker platform for 30 min at 4°C. In the GST pull-down experiments, cells were grown on 10-cm diam culture plates for 3 d, washed in PBS, and extracted in the same buffer containing 1% NP-40 instead of Triton X-100. Solubilized material was recovered by pelleting at 18,000 g for 15 min at 4°C. Supernatants were collected and protein concentration determined using the protein assay kit (Bio-Rad Laboratories). For immunoprecipitation, cells expressing GFP-Rab13Q67L were grown for 3 d on 10-cm diam culture plates, washed with PBS, and extracted in IP buffer (50 mM NaCl, 25 mM Tris, pH 8, 1 mM EDTA, 0.25% Triton, 1 mM sodium vanadate, 50 mM NaF, and protease inhibitors). After centrifugation, supernatants were incubated with 5 μ g of anti-VASP antibody overnight at 4°C. Protein G agarose beads were added for 2 h and the beads washed three times with IP buffer. Equal amount of protein were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose filters. Filters were probed with anti-VASP or anti-P-VASP antibodies before ECL detection according to the manufacturer's protocols (Pierce Chemical Co.).

GST pull-down assay

GST-Rab13 fusion protein was expressed in *E. coli* and purified according to the manufacturer's protocol (Amersham Biosciences). GST-Rab13 bound to glutathione beads was loaded with 1 mM GDP or GTP γ S (a poorly hydrolyzable GTP analogue) for 90 min at RT in incubation buffer (100 mM NaCl, 20 mM Tris, 10 mM EDTA, 5 mM MgCl₂, and 1 mM DTT, pH 7.6). After washing, beads were incubated overnight with MDCK cell extracts at 4°C. To prove the direct interaction of Rab13 with PKA, 100 μ g of purified PKA catalytic subunit from bovine heart (Sigma-Aldrich) was incubated with a 10-fold molar excess of purified GST, GST-Rab13-GDP, or GST-Rab13-GTP γ S proteins in the incubation buffer overnight at 4°C. GST-Rab6 (a gift from B. Goud and S. Monier, UMR144 Institut Curie) was used as negative control. After washing, the extent of PKA binding was determined by SDS-PAGE and Western blotting using polyclonal anti-PKA α cat or anti-PKC ζ antibodies.

In vitro kinase assay

10 U of purified PKA catalytic subunit was incubated with VASP immunoprecipitate and either 1 μ g of protein kinase inhibitor peptide, PKI (Sigma-Aldrich), and 10 μ g of purified GST-Rab13 loaded with GTP γ S or GST in kinase buffer (100 mM NaCl, 20 mM Tris, pH 7.5, 10 mM MgCl₂, 1 mM DTT, 1 mM ATP) for 30 min at 30°C. The reaction was stopped by adding 3 \times SDS sample buffer, separated on SDS-PAGE, and VASP phosphorylation determined by Western blot using the anti-VASP antibody.

Online supplemental material

The online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200312118/DC1>. Fig. S1 shows that Rab13 mutants do neither alter the recruitment of adherens junction proteins such as (A) β -catenin or (B) afadin nor (C) cortical actin in a calcium switch experiment. Anti- β -catenin and anti-afadin antibodies were purchased from Sigma-Aldrich and BD Transduction Laboratories, respectively. Phalloidin was purchased from Sigma-Aldrich. Supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.jcb200312118/DC1>.

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