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ARF6-dependent interaction of the TWIK1 K^+ channel with EFA6, a GDP/GTP exchange factor for ARF6

Sonia Decressac[1], Michel Franco[1], Said Bendahhou[1], Richard Warth[2], Sebastian Knauer[2], Jacques Barhanin[1], Michel Lazdunski[1+] & Florian Lesage[1,3]

[1]Institut de Pharmacologie Moléculaire et Cellulaire, CNRS UMR6097/UNSA, Institut Paul Hamel, Valbonne, France, [2]Institute of Physiology, Regensburg, Germany, and [3]Service de Neurologie, Hôpital Pasteur, Centre Hospitalo-Universitaire de Nice, Nice, France

TWIK1 belongs to a family of K^+ channels involved in neuronal excitability and cell volume regulation. Its tissue distribution suggests a role in epithelial potassium transport. Here we show that TWIK1 is expressed in a subapical compartment in renal proximal tubules and in polarized MDCK cells. In nonpolarized cells, this compartment corresponds to pericentriolar recycling endosomes. We identified EFA6, an exchange factor for the small G protein ADP-ribosylation factor 6 (ARF6), as a protein binding to TWIK1. EFA6 interacts with TWIK1 only when it is bound to ARF6. Because ARF6 modulates endocytosis at the apical surface of epithelial cells, the ARF6/EFA6/TWIK1 association is probably important for channel internalization and recycling.

Keywords: ion channel; small G protein; trafficking; recycling endosomes; apical compartment

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INTRODUCTION

TWIK1 has been originally cloned from human brain (Lesage *et al*, 1996a,b). By homology, 14 related subunits have been further isolated. They share a similar structure with four membrane-spanning segments (M1–M4) and two pore (P) domains involved in the formation of the selectivity filter. These two P-domain K^+ (K_{2P}) channels produce almost time- and voltage-independent currents that are modulated by a wide spectrum of chemical and physical stimuli including pH, temperature, unsaturated fatty acids and membrane stretch (Patel & Honore, 2001; Lesage, 2003). Some of the physiological functions of these channels have been recently elucidated. Acid-sensitive TASK1 and TASK3 (Duprat *et al*, 1997; Bayliss *et al*, 2003; Chemin *et al*, 2003; Lesage, 2003) and arachidonic acid-activated and mechano-gated TREK1 (Patel *et al*, 1998; Chemin *et al*, 2003; Heurteaux *et al*, 2004) channels control neuronal excitability, whereas TASK2 (Reyes *et al*, 1998) is important for cell volume regulation (Barriere *et al*, 2003) and bicarbonate transport (Warth *et al*, 2004) in the kidney. No general or specific functions have yet been assigned to TWIK1. In this study, we show that TWIK1 is mainly localized in the recycling endosomal compartment in polarized and nonpolarized epithelial cells. In an attempt to identify a mechanism responsible for this specific location, we identified EFA6 as a protein able to interact with TWIK1. This association occurs only when the small G protein ADP-ribosylation factor 6 (ARF6), a modulator of endocytosis, is bound to EFA6. We also show that TWIK1 overexpression slows down the regulated endocytosis of transferrin receptor.

RESULTS AND DISCUSSION

In adult mouse kidney, an intense TWIK1 immunoreactivity was detected in proximal tubules (Fig 1A). The labelling was mainly restricted to a subapical cellular domain next to the α-actin-rich microvilli that line the lumen (Fig 1B). No signal was seen in the basolateral membranes. The subcellular localization of TWIK1 was then characterized in Mabin–Darby canine kidney (MDCK) cells. MDCK cells are cultured epithelial cells of nephric tubule origin that form confluent monolayers of polarized cells on porous membranes. When stably expressed in MDCK, TWIK1 is mainly present in a subapical compartment in polarized cells (Fig 1B), and in a perinuclear and vesiculo-tubular compartment in nonpolarized cells (Fig 1C). This compartment is dependent on intact microtubules for its integrity. A treatment with the tubulin-depolymerizing agent nocodazole dispersed the TWIK1-containing vesicles (Fig 1C). Taken together, these data suggested that TWIK1 was expressed in an endosomal compartment called the pericentriolar recycling compartment in nonpolarized cells and the apical recycling compartment in polarized cells

[1]Institut de Pharmacologie Moléculaire et Cellulaire, CNRS UMR6097/UNSA, Institut Paul Hamel, 660, route des lucioles, 06560 Valbonne, France
[2]Institute of Physiology, Universitätsstrasse 31, 93053 Regensburg, Germany
[3]Service de Neurologie, Hôpital Pasteur, Centre Hospitalo-Universitaire de Nice, 30, avenue de la voie romaine, BP 69, 06002 Nice cedex 01, France
[+]Corresponding author. Tel: +33 4 93 95 77 01; Fax: 33 4 93 95 77 04; E-mail: ipmc@ipmc.cnrs.fr

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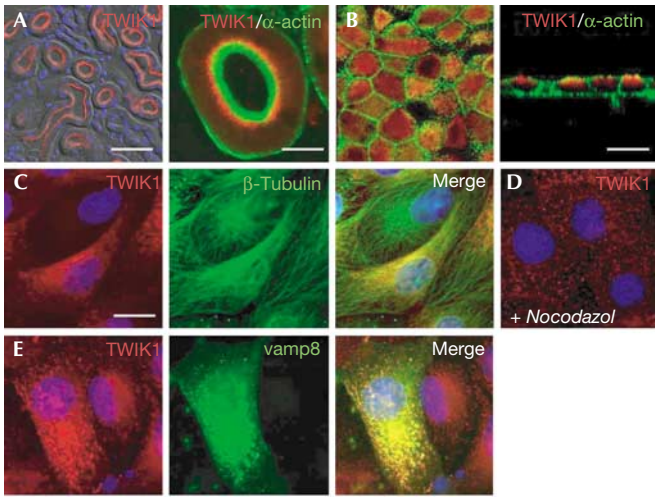

Fig 1 | Immunolocalization of TWIK1 in proximal tubules in adult mouse kidney (**A**) and in stably transfected MDCK cells (**B–E**). TWIK1 is preferentially localized in a subapical domain facing the luminal medium in polarized cells (**A,B**). (**B**) Left panel: *xy* confocal view; right panel: *xz* confocal view. In nonpolarized MDCK cells, TWIK1 immunoreactivity is associated with a perinuclear and vesiculotubular compartment (**C,D**). This compartment is β-tubulin dependent as shown by nocodazole treatment (**C**) and colocalizes with vamp8-EGFP (**D**). The colour of each labelling is indicated. The cell nuclei are in blue. Overlapping red and green labellings are in yellow. Scale bars: (**A**) left, 56 μm; right, 200 μm; (**B**) 30 μm; (**C,D**) 10 μM.

(Altschuler *et al*, 1999; Apodaca, 2001; Hoekstra *et al*, 2004). This particular endosomal compartment is located distal to early endosomes and is not directly related to the late endosomal/lysosomal pathway.

To confirm the specific localization of TWIK1 in recycling endosomes, different markers of both biosynthetic/exocytic and endocytic pathways were expressed by transfecting subcellular localization vectors. No significant overlapping distributions were seen between TWIK1 and transiently transfected ER-EGFP (enhanced green fluorescent protein targeted to endoplasmic reticulum), Golgi-EGFP (Golgi), peroxi-EGFP (peroxysomes), endo-EGFP (early endosomes), and Rab7-EGFP and Rab9-EGFP (late endosomes) (data not shown). But, as expected, TWIK1 colocalized with vamp8-EGFP (Fig 1D). Vamp8, also called endobrevin, is a vesicle-associated protein that localizes to the pericentriolar recycling compartment in MDCK cells and to the apical recycling compartment in nephric tubule epithelium (Steegmaier *et al*, 2000). We confirmed the presence of TWIK1 in recycling endosomes in transfected baby hamster kidney (BHK) cells by double immunolabelling using antibodies directed against proteins resident in different cellular compartments. TWIK1 was detected only in vesicles expressing vamp8-EGFP or Rab11-EGFP, another marker of recycling endosomes (Casanova *et al*, 1999) (not shown).

During the initial phase of this work, we expressed a TWIK1 channel fused to *Heteractis crispa* red (HcRed) fluorescent protein in MDCK cells. The fusion protein was localized mainly in pericentriolar recycling compartment as wild-type TWIK1. However, red fluorescence was also seen at the junction between adherent cells, indicating that a fraction of HcRed-TWIK1 was present at the cell surface (supplementary Fig 1A online). That was further confirmed by electrophysiology. In HcRed-TWIK1-expressing MDCK cells, a TWIK1 current was recorded that was not present in MDCK cells expressing the wild-type channel (supplementary Fig 1B online). In polarized cells, HcRed-TWIK1 had the same apical/subapical localization as TWIK1 (not shown). Taken together, these data suggest that the fusion of HcRed on the intracellular side of the channel partly masks a signal or prevents interaction with a protein involved in TWIK1 trafficking between plasma membrane and recycling endosomes.

In transfected HeLa (Henrietta Lacks), CHO and COS cells, TWIK1 was systematically detected in a compartment structurally similar to the pericentriolar recycling compartment of MDCK and BHK cells (not shown). This mainly intracellular localization could explain the small amplitude of the TWIK1 currents heterogenously expressed in *Xenopus* oocytes and COS cells (Lesage *et al*, 1996a; Lesage & Lazdunski, 1999).

To identify proteins interacting with TWIK1 and potentially regulating its trafficking between plasma membrane and recycling endosomes, we carried out two-hybrid screening in the bacteria. The entire post-M4 carboxy-terminal part of TWIK1 was used as bait for screening a mouse brain complementary DNA library. From 2×10^6 cDNAs, we obtained three independent positive clones, each of which encoded a C-terminal fragment of EFA6A (Fig 2). EFA6A, an exchange factor for the small G protein ARF6, regulates membrane recycling and actin skeleton organization (Franco *et al*, 1999). Four EFA6 isoforms (EFA6A to EFA6D) have been identified. Three of them are expressed mainly in the nervous system, whereas the last one (EFA6B) is expressed ubiquitously in the other tissues (Perletti *et al*, 1997; Franco *et al*, 1999; Derrien *et al*, 2002). In polarized MDCK cells, EFA6 localizes to the apical pole including the plasma membrane (Luton *et al*, 2004). For these different reasons, EFA6 was an excellent candidate as a protein physiologically interacting with TWIK1. Its binding to TWIK1 was verified *in vitro* and in transfected cells. Fig 2B shows that EFA6 can be pulled down by a glutathione-*S*-transferase (GST) fusion protein containing the C-terminal part of TWIK1. Interaction between the two full-length proteins was verified by co-immunoprecipitation from transfected BHK cells (Fig 2C). However, in this case, TWIK1 and EFA6 were co-precipitated only when ARF6T27N was coexpressed. ARF6T27N is a dominant-negative mutant that forms a high-affinity complex with EFA6 (Macia *et al*, 2004; Peters *et al*, 1995), in comparison with wild-type ARF6 that binds to EFA6 only under its GDP-bound form, and ARF6Q67L, a constitutively activated form of ARF6 locked in the GTP-bound form that does not bind to EFA6 (D'Souza-Schorey *et al*, 1995; Peters *et al*, 1995). The ARF6 dependence of TWIK1/EFA6 interaction was also observed by immunocytochemistry (Fig 2D,E). In BHK cells, EFA6 is located at the cell surface, mainly in actin-rich membrane ruffles (Fig 2D; Franco *et al*, 1999; Macia *et al*, 2004). In the absence of ARF6T27N, TWIK1 shows its typical distribution in pericentriolar recycling compartment (Fig 2D). But in the presence of ARF6T27N, the three proteins have the same localization as EFA6 expressed alone (Fig 2E,F). In the absence of EFA6, there is no overlapping distribution of TWIK1 and ARF6T27N (not shown). ARF6Q67L was unable to associate with EFA6 and to promote binding of EFA6 to TWIK1 (supplementary Fig 2 online). Only a partial association between TWIK1, EFA6

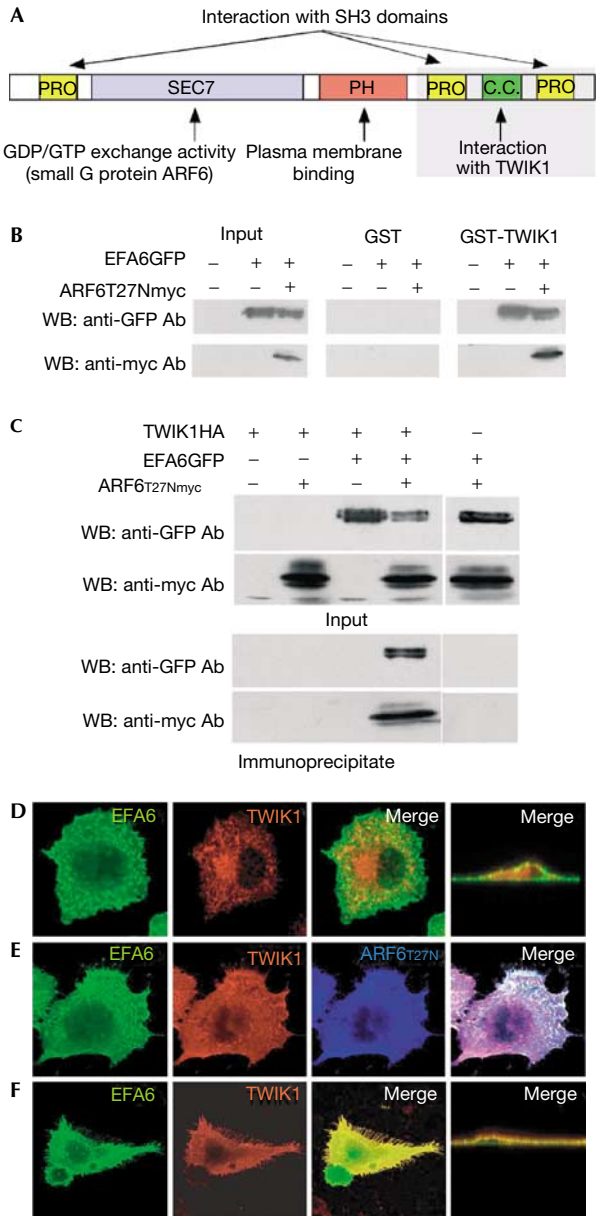

Fig 2 | EFA6A interacts with TWIK1. (**A**) EFA6A is a protein containing multiple modules of interaction. The C-terminal fragment interacting with TWIK1 in the bacterial two-hybrid system is indicated. (**B**) GST pull-down of GFP-tagged EFA6A and Myc-tagged ARF6 by the C-terminal part of TWIK1 fused to GST protein. (**C**) Co-immunoprecipitation by anti-HA antibodies of GFP-tagged EFA6A, HA-tagged TWIK1 and Myc-tagged ARF6 from transfected BHK cells. (**D**) Immunolocalization of GFP-tagged EFA6A and TWIK1 in permeabilized BHK cells. Right panel: *xz* confocal view. (**E**) Immunolocalization of GFP-tagged EFA6A, HA-tagged TWIK1 and Myc-tagged ARF6T27N in permeabilized BHK cells. Overlapping red, green and blue labellings are in white. (**F**) Localization of GFP-tagged EFA6A and immunolocalization of TWIK1 by antibodies directed against its extracellular side in nonpermeabilized BHK cells expressing Myc-tagged ARF6T27N. Right panel: *xz* confocal view. Overlapping red and green labellings are in yellow.

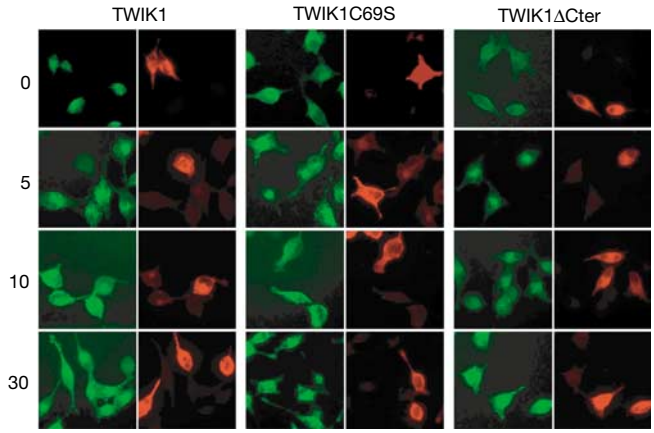

Fig 3 | Transferrin endocytosis in HeLa cells expressing TWIK1, TWIK1C69S, a nonconductive mutant, or TWIK1ΔCter, a deletion mutant lacking the C-terminal region binding to EFA6. Cells were incubated for 0, 5, 10 and 30 min at 37 °C with FITC-labelled transferrin before being fixed and processed for channel detection. For each condition, a unique field containing transfected and nontransfected cells is shown; left panel: FITC-labelled transferrin in green; right panel: TWIK1 labelling in red.

and wild-type ARF6 was seen, in agreement with the dynamic nature of ARF6/EFA6 interaction (supplementary Fig 2 online). The same results were obtained using either EFA6A (Fig 2D–F) or EFA6B (not shown).

ARF6 is located in the apical endocytic apparatus of proximal tubules in the kidney (Maranda *et al*, 2001). In MDCK, ARF6 has been shown to be an important modulator of endocytosis at the apical surface (Altschuler *et al*, 1999). This strongly suggests that the ARF6-dependent interaction between TWIK1 and EFA6 is important for trafficking of TWIK1 in polarized epithelial cells. By associating with EFA6 and ARF6 at the plasma membrane, TWIK1 would favour the recruitment of the coat mediated by ARF6 activation and its own internalization. The subapical compartment in polarized epithelial cells functions as an intracellular site where apical and basolateral proteins can be sorted and targeted. In addition, the apical recycling compartment, or some of its subcompartments, could adapt quickly to situations where a rapid addressing of plasma membrane components such as channel and transporters is required. The signal allowing the addressing of TWIK1-containing vesicles to the cell surface has yet to be identified.

Like EFA6 and ARF6, TWIK1 is expressed not only in polarized epithelial cells but also in many other cell types. What could be the physiological significance of ARF6/EFA6/TWIK1 association in nonpolarized cells? When TWIK1 is transfected in HeLa cells that express an endogenous receptor for transferrin (TfR), the endocytosis rate of FITC-labelled transferrin was strongly reduced when compared with that in untransfected cells (Fig 3, left). Endocytosis occurred but was considerably slower. The expression of TWIK1C69S, a nonconductive mutant of TWIK1 containing a single residue substitution (Lesage *et al*, 1996b), led to a decrease of transferrin internalization similar to the decrease produced by the expression of TWIK1 (Fig 3, central). This result rules out a nonspecific effect due to channel activity and variations of the

cytoplasmic K^+ concentration or electrical membrane potential. Conversely, a deletion mutant of TWIK1 lacking the EFA6-interacting C-terminal region (residues 289–336) had no more effect on transferrin endocytosis (Fig 3, right). These results suggest that TWIK1 affected the endocytic process by binding to endogenous EFA6. By interacting with EFA6 and ARF6 at the plasma membrane, TWIK1 would favour its own internalization in competition with TfR endocytosis. Another possibility is that TWIK1 acts at the level of the recycling process. ARF6 has been shown to control post-endocytic recycling, which implies that ARF6 cycles between plasma membrane and recycling endosomes (Prigent *et al*, 2003). TWIK1 could slow down that cycling by transiently binding ARF6 through EFA6. At $T=0$, there is less binding of transferrin on TWIK1-expressing cells, indicating that TWIK1 expression changes the level of TfR at the cell surface.

Taken together, these results suggest that in nonpolarized cells, ARF6/EFA6/TWIK1 interaction is important for TWIK1 trafficking as in epithelial polarized cells. They suggest also that depending on the respective expression levels of TWIK1, EFA6 and ARF6, TWIK1 could interfere with the recycling of other channels, receptors and transporters in many cell types. The recycling compartment is also important for membrane domain maintenance and membrane protein mobilization. For instance, in migrating fibroblasts, recycling TfRs are concentrated in narrow tubules in the pericentriolar area through which they are subsequently routed to the plasma membrane of the leading lamella (Hopkins *et al*, 1994). In neurons, which have distinct axonal and somatodendritic plasma membrane domains, the polarized sorting of TfR in the endocytic system is probably mediated in the pericentriolar recycling compartment (West *et al*, 1997). More work will be necessary to test the hypothesis that TWIK1 not only functions as K^+ channel but may also act as a modulator of the endocytosis/recycling machinery.

METHODS

Plasmids and DNA constructs. The sequence encoding the last 72 amino acids of mouse TWIK-1 (residues 265–337) was amplified by PCR and subcloned in-frame with the lambda-C1 DNA-binding domain into pBT (Bacteriomatch™ Two-Hybrid System, Stratagene, La Jolla, CA, USA) to get pBT-TWIK1. The same sequence was also subcloned in-frame with the GST sequence into the pGEX-3a vector (Amersham Biosciences, Freiburg, Germany). Full-length DNA encoding mouse TWIK1 and the deletion mutant TWIK1ΔCter were generated by PCR and subcloned in pCMV-HA vector (Clontech, Palo Alto, CA, USA). All the constructs were verified by sequencing. The vectors for expression of EFA6GFP and ARF6T27Nmyc constructs have been described elsewhere (Derrien *et al*, 2002; Macia *et al*, 2004).

Cell culture, reagents and antibodies. Culture, cytochemistry and transferrin internalization experiments were carried out according to standard protocols (supplementary information online).

Bacterial two-hybrid screening. The Bacteriomatch™ Two-Hybrid System Reporter Strain Competent cells (Stratagene) were transformed with pBT-TWIK1 and the BacterioMatch® Mouse Brain Library (Stratagene) according to the manufacturer's protocol (Stratagene). Positive clones were selected on CTCK (chloramphenicol, tetracyclin, carbenicillin and kanamycin) LB medium. All positive clones were analysed by sequencing.

GST pull-down. The experiments were carried out according to standard protocols (supplementary information online).

Co-immunoprecipitation. BHK21 cells (2×10^6 in 100 mm tissue culture dishes) were transfected with TWIK-1HA and EFA_6-GFP or ARF_6T27Nmyc constructs or both. At 48 h after transfection, the cells were scrapped and lysed in 0.5 ml of lysis buffer (20 mM Hepes (pH 7.4), 1% NP-40, 100 mM NaCl, 1 mM $MgCl_2$, 0.25 mM PMSF and a tablet of protease inhibitors (Roche Diagnostics, Basel, Switzerland)). Lysates were clarified by centrifugation at 13,000*g* for 30 min. Aliquots of each supernatant were immunoprecipitated with 3F10 antibody (5 μg) immobilized on protein A–Sepharose 4B Fast flow (Sigma, Saint Louis, USA). After three washing steps with the lysis buffer, immunoprecipitated proteins were eluted by boiling with the SDS–polyacrylamide gel electrophoresis loading buffer, separated on 10–15% polyacrylamide gel and blotted onto PVDF membrane (Hybond-P, Amersham Biosciences). Western blot of immunoprecipitated proteins with 3F10 antibody was incubated with 9E10 (1/10,000) or anti-GFP (1/100) antibodies for 1 h at 25 °C. Primary antibodies were then removed, blots were washed three times for 10 min with a phosphate-buffered saline solution containing 0.1% Tween 20. After washing steps, peroxidase-conjugated secondary antibodies (1/10,000) were added for 1 h at 25 °C. Bands were shown with the supersignal WestPico luminescent detection system (Pierce Biotechnology, Rockford, IL, USA).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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