hScrib interacts with ZO-2 at the cell–cell junctions of epithelial cells

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Dedicated to the memory of Jean-Pierre Perichet

Abstract **In *Drosophila*, the tumor suppressor Scribble is localized at the septate junctions of epithelial cells. Its mammalian homologue, hScrib, is a basolateral protein likely associated to proteins of the cell–cell junctions. We report the direct interaction between hScrib and ZO-2, a junction-associated protein. This interaction relies on two PDZ domains of hScrib and on the C-terminal motif of ZO-2. Both proteins localise at cell–cell junctions of epithelial cells. A point mutation in the LRR of hScrib delocalises the protein from the plasma membrane and abrogates the interaction with ZO-2 but not with βPIX. Tyrosine phosphorylation of hScrib does not impair the interaction with ZO-2. We show a direct link between two junctional proteins that are down-regulated during cancer progression.**

Keywords: hScrib; ZO-2; Epithelium; Tumor suppressor; PDZ domain

1. Introduction

The differentiation of epithelial cells is characterized by the establishment of apico-basal polarity. In these cells, the apical surface is in contact with fluids while the lateral and basal membranes are in contact with the neighbouring cells and the extracellular matrix, respectively. One of the hallmarks of polarized epithelial cells is the presence of specialized junctions that tightly control epithelial homeostasis. Tight junctions (TJs) separate apical and lateral compartments and act as a fence and a barrier to prevent the diffusion of proteins and lipids, and regulate the paracellular movement of molecules [1]. TJs are lying apical to the adherens junctions, and comprise transmembrane (claudins, occludins, crumbs, etc.) and cytoplasmic (ZO-1, 2 and 3, AF-6/Afadin, etc.) molecules important for cell differentiation.

ZO-2 belongs to the membrane-associated guanylate kinase (MAGUK) protein family and has been first described as a TJ-associated cytoplasmic protein [2,3]. Like other MAGUKs, ZO-2 contains three PDZ domains, a SH3 domain, and a GUK domain devoided of enzymatic activity [4]. ZO-2 interacts with several components of the TJs, including ZO-1 [2,5], 4.1R protein [6], cingulin [7], occludin [5], claudins [8] and actin [9]. Association between ZO-2 and α-catenin [5], an adherens junction-associated protein, suggests the distribution of this protein at the lateral membrane. Like ZO-1 [10,11] and LIN-2 [12] MAGUKs, ZO-2 localises in the nucleus of sparse epithelial cells [13,14] where it regulates gene transcription through its association with transcription factors such as JUN, FOS, and C/EBP [15]. Implication of ZO-2 in cancer is supported by several studies. For instance, one of ZO-2 isoforms is lacking in pancreatic adenocarcinoma [16]. Furthermore, overexpression of ZO-2 inhibits cell transformation induced by the E4 oncogenic determinant of type 9 adenovirus, polyomavirus middle T proteins and activated RasV12 [17]. Studies in *Drosophila* have characterized homologues of ZO-2 as potent tumor suppressors [18].

Members of the LRR and PDZ (LAP) protein family are regulators of epithelial cell polarity [19–22]. Among this family, hScrib has similar functions to its *Drosophila* homologue [23–25]. Disruption of hScrib in mice provokes severe neural tube defects [26,27] as well as defects of the orientation of hair cell stereociliary bundles within the cochlea, due to impaired planar cell polarity [28]. In humans, the expression of hScrib decreases during the progression of uterine cervix cancers and in lobular breast cancers [29,30]. Therefore, like ZO-2, hScrib is believed to participate to tumorigenic processes.

We recently characterized a βPIX-GIT1 complex bound to the PDZ domains of hScrib [31]. hScrib has four PDZ domains that interact with multiple partners [32]. We used these domains to pull-down novel interactors and identified ZO-2 as a new partner for hScrib by mass spectrometry analysis. We describe here the interaction between these two potential tumor suppressors in epithelial cells.

2. Materials and methods

2.1. Cell culture

COS-7 and Madin Darby canine kidney II (MDCKII) cells were grown in DMEM containing 10% FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin sulfate. MCF10.2A were grown in Ham's F12/DMEM containing 5% horse serum, 2 mM glutamine, 10 μg/ml insulin, 20 ng/ml EGF, 100 ng/ml Cholera toxin, 500 ng/ml hydrocortisone, 100 U/ml penicillin and 100 mg/ml streptomycin sulfate. Cell transfections were performed using polyFECT (Qiagen) reagent according to the manufacturer's recommendations. MDCKII cells stably expressing GFP-hScrib fusions were previously described [19]. Cells were starved in DMEM/0.5% FCS for 5 h prior to activation with 200 μM sodium pervanadate in complete medium.

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Abbreviations: TJs, tight junctions; MAGUK, membrane-associated guanylate kinase; LRR, leucine rich repeat; LAP, LRR and PDZ; LAPSD, LAP specific domain; MDCK, Madin Darby canine kidney

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2.2. Expression vectors

HA-ZO-2 was expressed in mammalian cells using the pGW1-CMV vector kindly provided by Dr. Ronald T. Javier (Baylor College of Medicine, Houston, Texas). HA-Lano was expressed using pCDNA3-HA vector. pGW1-CMV-HA-ZO-2.ΔTEL was obtained using the QuickChange™ mutagenesis kit (Stratagene). The GFP-hScrib constructs were previously described [31].

2.3. Biochemical procedures

Cells were rinsed twice in cold PBS, and lysed in buffer containing 50 mM HEPES, pH 7.5, 1 mM EGTA, 150 mM NaCl, 1.5 mM $MgCl_2$, 10% glycerol, 1%Triton X-100, supplemented with 1 mM PMSF, 1 mM orthovanadate, 10 nM aprotinine, and 1 μM leupeptine. Triton-soluble proteins were recovered in the supernatant of a 20 min centrifugation at 13 000 × *g* and 4 °C. GST pull-down, two-hybrid assays, immunoprecipitations and Western blots were performed as previously described [20].

2.4. Mass spectrometry

For mass spectrometry analyses, protein complexes were separated in a Xcell sure lock™ electrophoresis unit with 4–12% bis–tris gradient pre-cast NuPAGE™ gels in MOPS buffer according to the manufacturer's instructions (Invitrogen). Silver stained samples were prepared and digested according to Shevchenko et al. [33]. Mass spectrometry analyses were performed using MALDI-TOF by Ultraflex Bruker instrument (Bruker Daltonique). Protein identification was carried out with Mascot software (Matrixscience Inc.).

2.5. Immunofluorescence and confocal microscopy

MDCKII cells were grown on Transwell™ filters, washed twice in PBS 0.1 mM Ca^{2+}/1 mM Mg^{2+}, and fixed for 20 min in 4% paraformaldehyde at 4 °C. Cells were permeabilized during 5 min with 0.5% Triton X-100 at RT, and blocked in 0.25% gelatine for 1 h at RT. Antibodies diluted in the blocking buffer were incubated overnight at 4 °C. After 4 washes of 15 min cells were incubated for 1 h at RT with secondary antibodies coupled to fluorescent probes. Cells were washed and filters were mounted in Dako (Jackson laboratories) for confocal microscopy analyses.

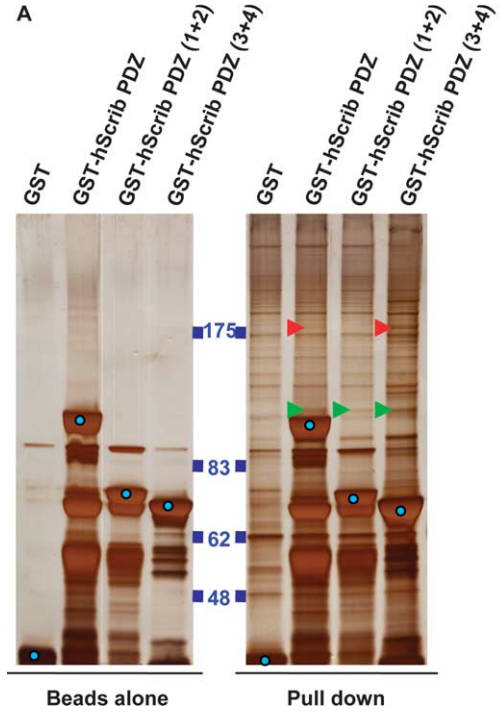
2.6. Antibodies

Polyclonal anti-ZO-2 antibody is from Zymed laboratories. Polyclonal anti-βPIX antibody is from Chemicon. Monoclonal anti-GFP and anti-HA antibodies are from Roche. Monoclonal anti-β-catenin antibody is from BD Bioscience. Monoclonal antibody directed against hScrib and Lano (8.1.1) was previously described [20]. Polyclonal-anti-GIT1 antibody was kindly provided by Dr. Richard T. Premont [34]. Monoclonal anti-phosphotyrosine antibody is from Upstate Biotechnology. Secondary antibodies coupled to horseradish peroxidase used for Western blotting are from DakoCytomation and Jackson laboratories. Secondary antibodies coupled to alexa fluorophores for immunofluorescent experiments are from Molecular Probes.

3. Results

3.1. Identification of ZO-2 as a new partner of the PDZ domains of hScrib

To look for new binding partners of the PDZ domains of hScrib (Fig. 1A), we carried out GST pull-down experiments using non-transformed mammary epithelial MCF10.2A cell extracts and purified GST-hScrib PDZ proteins. Bound proteins to the GST were separated on SDS–PAGE, silver stained and identified by mass spectrometry. GST-hScrib PDZ and GST-hScrib (3 + 4) domains reproducibly precipitated a 175 kDa protein (red arrowheads). We identified this band as ZO-2 (Fig. 1B). A 80-kDa protein (green arrowheads) was also purified by GST-hScrib PDZ, (1 + 2), and GST-hScrib (3 + 4) and was identified as βPIX, a known partner for hScrib [31]. Interaction between hScrib and ZO-2 was confirmed by GST pull-down and Western blotting using anti-ZO-2 antibody (Fig. 2B). No interaction was found with GST and GST-LIN-2 PDZ.



B

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MPVRGDRGFP PRRELSGWLR APGMEELIWE QYTVTLQKDS KRGFGIAVSG GRDNPHFENG   60
ETSIVISDVL PGGPADGLLQ ENDRVVMVNG TPMEDVLHSF AVQQLRKSGK VAAIVVKRPR  120
KVQVAALQAS PPLDQDDRAF EVMDEFDGRS FRSGYSERSR LNSHGGRSRS WEDSPERGRP  180
HERARSRERD LSRDRSRGRS LERGLDQDHA RTRDRSRGRS LERGLDHDFG PSRDRDRDRS  240
RGRSIDQDYE RAYHRAYDPD YERAYSPEYR RGARHDARSR GPRSRSREHP HSRSPSPEPR  300
GRPGPIGVLL MKSRANEEYG LRLGSQIFVK EMTRTGLATK DGNLHEGDII LKINGTVTEN  360
MSLTDARKLI EKSRGKLQLV VLRDSQQTLI NIPSLNDSDS EIEDISEIES NRSFSPEERR  420
HQYSDYDYHS SSEKLKERPS SREDTPSRLS RMGATPTPFK STGDIAGTVV PETNKEPRYQ  480
EEPPAPQPKA APRTFLRPSP EDEAIYGPNT KMVRFKKGDS VGLRLAGGND VGIFVAGIQE  540
GTSAEQEGLQ EGDQILKVNT QDFRGLVRED AVLYLLEIPK GEMVTILAQS RADVYRDILA  600
CGRGDSFFIR SHFECEKETP QSLAFTRGEV FRVVDTLYDG KLGNWLAVRI GNELEKGLIP  660
NKSRAEQMAS VQNAQRDNAG DRADFWRMRG QRSGVKKNLR KSREDLTAVV SVSTKFPAYE  720
RVLLREAGFK RPVVLFGPIA DIAMEKLANE LPDWFQTAKT EPKDAGSEKS TGVVRLNTVR  780
QIIEQDKHAL LDVTPKAVDL LNYTQWFPIV IFFNPDSRQG VKTMRQRLNP TSNKSSRKLF  840
DQANKLKKTC AHLFTATINL NSANDSWFGS LKDTIQHQQG EAVWVSEGKM EGMDDDPEDR  900
MSYLTAMGAD YLSCDSRLIS DFEDTDGEGG AYTDNELDEP AEEPLVSSIT RSSEPVQHEE  960
SIRKPSPEPR AQMRRAASSD QLRDNSPPPA FKPEPPKAKT QNKEESYDFS KSYEYKSNPS 1020
AVAGNETPGA STKGYPPPVA AKPTFGRSIL KPSTPIPPQE GEEVGESSEE QDNAPKSVLG 1080
KVKIFEKMDH KARLQRMQEL QEAQNARIEI AQKHPDIYAV PIKTHKPDPG TPQHTSSRPP 1140
EPQKAPSRPY QDTRGSYGSD AEEEEYRQQL SEHSKRGYYG QSARYRDTEL          1190
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Fig. 1. ZO-2 is a binding partner for the PDZ domains of hScrib. (A) Silver-stained protein gel after pull-down assays on MCF10.2A extracts using GST fusion proteins encompassing the PDZ domains of hScrib. (B) Peptide sequence of ZO-2 showing the peptide coverage (red sequence) from the mass spectrometry analysis. Note the canonical C-terminal PDZ binding motif at the end of ZO-2 sequence (TEL motif).