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

Jean-Yves Métais, Christel Navarro, Marie-Josée Santoni, Stéphane Audebert, Jean-Paul Borg*

Molecular Pharmacology, UMR 599 Inserm-Institut Paoli-Calmettes, 27 boulevard Let Roue, 13009 Marseille, France

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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 destabilizes 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.© 2005 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

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 localizes 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 µg/ml penicillin, and 100 µg/ml streptomycin sulfate. MCF10.2A were grown in Ham’s F12/DMEM containing 5% horse serum, 2 mM glutamine, 10 µg/ml insulin, 20 µg/ml EGF, 100 µg/ml Cholera toxin, 500 ng/ml hydrocortisone, 100 U/ml penicillin and 100 µg/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.

Abbreviations: TJs, tight junctions; MAGUK, membrane-associated guanylate kinase; LRR, leucine rich repeat; LAP, LRR and PDZ; LAPS7, LAP specific domain; MDCK, Madin Darby canine kidney

*Corresponding author. Fax: +33 491 260 364.
E-mail address: borg@marseille.inserm.fr (J.-P. Borg).

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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-TEL 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 MgCl₂, 10% glycerol, 1% Triton X-100, supplemented with 1 mM PMSF, 1 mM orthovanadate, 10 nM aprotinin, 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 Ca²⁺/1 mM Mg²⁺, 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 MCF10A 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. 2B). 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.

Fig. 1. ZO-2 is a binding partner for the PDZ domains of hScrib. (A) Silver-stained protein gel after pull-down assays on MCF10A 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).
3.2. hScrib and ZO-2 interact in living cells

Interaction was confirmed using MDCKII cells expressing GFP or GFP-hScrib PDZ. Proteins were immunoprecipitated with anti-GFP and detected with anti-ZO-2 antibody (Fig. 2C). ZO-2 co-immunoprecipitated with GFP-hScrib but not with GFP. As expected [31], the βPIX/GIT1 complex was also precipitated by GFP-hScrib PDZ. Then, COS-7 cells were co-transfected with HA-ZO-2 and GFP-hScrib. GFP-hScrib was immunoprecipitated with anti-hScrib antibody and bound proteins were recognized with anti-HA antibody (Fig. 3). HA-ZO-2 (lane 1), but not HA-Lano (lane 2) [20], was associated with GFP-hScrib.

3.3. Interaction between hScrib and ZO-2 is mediated by the PDZ domains of hScrib and the C-terminal motif of ZO-2

The mapping of the interaction was carried out by co-expressing mutants of GFP-hScrib and HA-ZO-2 in COS-7 cells. We focused on the PDZ binding site present at the carboxy-terminus of ZO-2 (TEL motif) likely involved in the interaction with the PDZ domain of hScrib [32]. HA-ZO-2 or HA-ZO-2 lacking the PDZ binding site (HA-ZO-2.ΔTEL) were co-expressed with GFP-tagged hScrib, hScrib.ΔPDZ, hScribPⅠ, hScrib1-724, or hScrib717-1630 (Fig. 2A and Fig. 4A). Immunoprecipitations were performed using anti-GFP antibody and bound proteins were detected using anti-ZO-2 antibody. HA-ZO-2 and HA-ZO-2.ΔTEL were equally expressed (data not shown). As shown in Fig. 4A, removal of the PDZ domains (GFP-hScrib.ΔPDZ) abrogated the interaction with ZO-2 (lane 2). Furthermore, the amino-terminus of hScrib did not interact with ZO-2 (lane 4) in contrast to the carboxy-terminus (lane 5). HA-ZO-2.ΔTEL was not precipitated by GFP-hScrib confirming that the TEL motif is required for the interaction (lane 6). Mutation of proline 305 in the leucine rich repeat (LRR) to leucine (PL mutant) precludes the retention of hScrib at the plasma membrane [31,35]. We observed a decreased interaction between GFP-hScribPⅠ and HA-ZO-2, while the binding between GFP-hScribPⅠ and βPIX was not affected (Fig. 4A, lane 3 and Fig. 5B, lane 4). A yeast two-hybrid assay confirmed that the interaction between hScrib and ZO-2 is direct and mediated by the PDZ domains and the TEL motif (Fig. 4B).

3.4. Tyrosine phosphorylation of hScrib does not impair the interaction with ZO-2

Examination of the carboxy-terminus of ZO-2 shows the presence of a tyrosine residue at position -5 from the carboxy-terminal leucine. Transfected COS-7 cells were stimulated with sodium pervanadate and co-immunoprecipitations were performed with anti-GFP antibody. We observed a strong tyrosine phosphorylation of GFP-hScrib proteins in these lysates (red dots in right panel of Fig. 5A) while ZO-2 was very weakly phosphorylated. GFP alone was not tyrosine phosphorylated.
Interaction between hScrib and ZO-2 was conserved upon tyrosine kinase activation (Fig. 5B). GIT1, but not βPIX, was strongly tyrosine phosphorylated and still interacted with hScrib (Fig. 5A and data not shown). Therefore, the hScrib-ZO-2 interaction likely takes place at the cell–cell junctions before segregation of ZO-2 to the TJs.

3.5. hScrib and ZO-2 partially co-localise in MDCKII cells

We looked for the subcellular localisation of ZO-2 and hScrib in epithelial cells. We performed immunostaining and confocal analysis of unpolarized and polarized MDCKII cells and detected a partial co-localisation between GFP-hScrib and ZO-2 (or ZO-1) at the cell–cell junctions of unpolarized cells (Fig. 6A). hScrib did not co-localise with the TJ markers ZO-1 or ZO-2 in polarized MDCK (Fig. 6B) cells whereas it co-localized with β-catenin, a marker of adherens junctions (data not shown) [19]. Therefore, the hScrib-ZO-2 interaction likely takes place at the cell–cell junctions before segregation of ZO-2 to the TJs.
4. Discussion

Genetic studies carried out in Drosophila identified scribble anddlg as potent neoplastic tumor suppressor genes [18,23]. In humans, loss of their homologues has been associated to progression of mammary, uterine and pancreatic cancers [16,17,30]. We previously demonstrated that hScrib belongs to a signalling complex comprising βPIX and GIT1, two regulators of small GTPase activities [31]. Furthermore, hScrib is associated to the basolateral membrane of epithelial cells by its LRR domain, suggesting an association with junctional proteins [19].

Here, we identify ZO-2 as a protein associated to hScrib at the cell–cell junctions. We show that this interaction relies on the PDZ3 and PDZ4 domains of hScrib and on the carboxyterminus of ZO-2. Interestingly, PDZ1 and PDZ2 have the ability to interact with βPIX, but not with ZO-2, demonstrating that the PDZ domains of hScrib have different binding specificities (Fig. 2B).

We could recover the interaction between epitope-tagged proteins in living cells and demonstrated that it takes place at the cell–cell junctions of unpolarized epithelial cells (Fig. 6). We nevertheless failed to obtain convincing co-immunoprecipitation results with endogenous proteins. Interestingly, a point mutation in the LRR of hScrib (hScribPL mutant), which delocalises the protein from the plasma membrane [19,31,35], impaired the interaction with ZO-2 but not with βPIX (Fig. 4A and Fig. 5B). Accordingly, βPIX co-immunoprecipitates with hScrib in the cytosolic fraction of epithelial cells (data not shown). From these results, we infer that the localisation of hScrib at the cell periphery is important for the interaction with ZO-2.

Tyrosine phosphorylation of hScrib did not modify its ability to interact with ZO-2 or βPIX (Fig. 5). While hScrib and GIT1 are heavily phosphorylated on tyrosine residues, we observed a very poor tyrosine phosphorylation of ZO-2. Therefore, it remains unknown if the carboxy-terminal tyrosine residue of ZO-2 is phosphorylated. Accordingly, previous studies have described ZO-2 as a target for serine and threonine, but not for tyrosine kinases [36,37].

Delocalisation of ZO-2 from the cell–cell junctions alters the integrity of epithelial monolayers [36,37]. Overexpression of wild type or mutant hScrib or knock-down of hScrib by siRNA did not affect the subcellular localisation of ZO-2 and had no incidence on the morphology of MDCKII cells (data not shown). Functional redundancy within the LAP and ZO families may explain this result.

We have recently observed that, in breast cancers, most lobular tumors do not express hScrib and E-cadherin while ductal tumors have a less frequent down-regulation of hScrib [19]. It will be interesting to evaluate the expression of ZO-2 in these tumors to see if it correlates with the expression pattern of hScrib. Additional functional assays will also analyze the role of hScrib-ZO-2 complex with regards to cell proliferation and transformation.

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References


