The PDZ Domains of Zonula Occludens-1 Induce an Epithelial to Mesenchymal Transition of Madin-Darby Canine Kidney I Cells

EVIDENCE FOR A ROLE OF β-CATENIN/Tcf/Lef SIGNALING*

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The integrity of cell-cell contacts such as adherens junctions (AJ) and tight junctions (TJ) is essential for the function of epithelia. During carcinogenesis, the increased motility and invasiveness of tumor cells reflect the loss of characteristic epithelial features, including cell adhesion. While β-catenin, a component of AJ, plays a well-characterized dual role in cell adhesion and signal transduction leading to epithelial cell transformation, little is known about possible roles of tight junction components in signaling processes. Here we show that variants of the TJ protein zonula occludens protein-1 (ZO-1), which encode the PDZ domains (ZO-1 PDZ) but no longer localize at the plasma membrane, induce a dramatic epithelial to mesenchymal transition (EMT) of Madin-Darby canine kidney I (MDCKI) cells. The observed EMT of these MDCK-PDZ cells is characterized by a repression of epithelial marker genes, a restricted differentiation potential and a significantly induced tumorigenicity. Intriguingly, the β-catenin signaling pathway is activated in the cells expressing the ZO-1 PDZ protein. Ectopic expression of the adenosomatous polyposis coli tumor suppressor gene, known to down-regulate activated β-catenin signaling, reverts the transformed fibroblastoid phenotype of MDCK-PDZ cells. Thus, cytoplasmic localization of the ZO-1 PDZ domains induces an EMT in MDCKI cells, most likely by modulating β-catenin signaling.

In addition to mediating cell-cell adhesion, TJ regulate the paracellular diffusion across epithelial monolayers and the maintenance of the asymmetric distribution of proteins and lipids to the apical and basolateral plasma membrane domains of epithelial cells (1–3). The tight junction protein zonula occludens protein 1 (ZO-1) is part of a multi-protein complex and binds directly to the integral TJ proteins occludin and to members of the claudin family (4), thereby linking the TJ to the cytoskeleton via a direct or indirect interaction with actin (5). ZO-1 belongs to the membrane-associated guanylate kinase (MAGUK) protein family and contains three PDZ domains (6), an Src homology 3 (SH3) domain, a guanylate kinase (GUK) homology domain, and a proline rich C-terminal region (see Fig. 1). Since occludin lacks PDZ-binding motifs, binding between occludin and ZO-1 probably does not involve the PDZ domains. The function of the GUK domain, which lacks kinase activity in the MAGUK proteins analyzed so far, is not known but has been suggested to be important for binding of ZO-1 to occludin (5). ZO-2 and ZO-3, two additional members of the MAGUK protein family present in TJ, show extensive homology to each other and to ZO-1. ZO-3 interacts with ZO-1 and the cytoplasmic C-terminal tail of occludin, but does not bind ZO-2 (7). ZO-2 binds directly to ZO-1 and occludin. Actin cosedimentation studies showed that ZO-2, ZO-3, and occludin all interact directly with F-actin in vitro and colocalize with actin aggregates at cell boarders in cytochalasin D-treated MDCK cells. The suggested model at the moment is that two independent complexes comprising ZO-1-ZO-2 and ZO-1-ZO-3 exist (rather than a three-member complex, ZO-1-ZO-2-ZO-3), and that these complexes link the tight junction to the actin cytoskeleton (8). Several other proteins have been described to interact with ZO-1, but the domains involved in binding or the physiological relevance of the interactions are, in most cases, unknown.

Carcinogenesis is a multistep process well characterized in human colon cancer. Mutations in the adenomatous polyposis coli (APC) gene are thought to initiate the process, leading to aberrant crypt foci that develop into areas of benign epithelial hyperplasia or dysplasia and adenomas. Progression of these areas to carcinomas in situ and malignant tumors depends on the construct encoding the PDZ and SH3 domains; MDCK-FSG, MDCKI cells expressing a ZO-1 construct encoding the PDZ, SH3, and GUK domains; PDZ, PSD-95/disk large tumor suppressor protein Dlg/ZO-1; SH3, Src homology domain; Tcf/Lef, T cell factor/lymphocyte enhancer factor; TER, transepithelial electrical resistance; TJ, tight junction; ZO, zonula occludens; PCR, polymerase chain reaction; RT, reverse transcription; DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; bp, base pairs; BES, 2-bis(2-hydroxyethyl)aminomethanesulfonic acid; PBS, phosphate-buffered saline; GST, glutathione S-transferase; MAGUK, membrane-associated guanylate kinase; TOP, multimerized wild-type Lef binding sites; FOP, multimerized mutant Lef binding sites.

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The abbreviations used are: AJ, adherens junction; APC, adenomatous polyposis coli; EMT, epithelial-mesenchymal transition; GUK, guanylate kinase; HGF, hepatocyte growth factor; MDCK, Madin-Darby canine kidney cells; MDCKI, MDCK type I cells; MDCK-ZO-1, MDCKI cells expressing ZO-1; MDCK-PDZ, MDCKI cells expressing the PDZ domains of ZO-1; MDCK-FS, MDCKI cells expressing a ZO-1...
further changes in the transformed cells such as mutations in p21 Ras and p53 and the gradual loss of a number of characteristic features of differentiated epithelial cells. This process, also known as epithelial to mesenchymal transition (EMT), includes the disruption of apical-basolateral polarity, the disassembly of AJ and TJ, and the ability of the cells to degrade the basement membrane, to migrate, and to form metastases at distant sites.

A reduced intercellular adhesion is a requisite for the higher motility and invasiveness of tumor cells and the expression or integrity of several components of AJ (i.e. α-catenin, β-catenin, γ-catenin/plakoglobin, E-cadherin) is altered or lost in different types of carcinoma (9). β-Catenin plays a dual role as a structural component of AJ (10) and as a signaling molecule in the Wnt signaling pathway (11). In the absence of Wnt glycoproteins, the Ser/Thr-specific glycogen synthase kinase 3β phosphorylates β-catenin, APC, and axin/conductin (12, 13), which are present as a multi-protein complex in the cytosol. Phosphorylated β-catenin is rapidly ubiquitinated and degraded by the proteosomal pathway (14). Binding of Wnt glycoproteins to the Frizzled family of receptors results in the inactivation of glycogen synthase kinase 3β and thereby to an enhanced stability of β-catenin. Stabilized β-catenin can translocate into the nucleus where, in association with members of the Tcf/Lef transcription factor family, it regulates gene expression (15, 16), probably by recruiting the basal transcription machinery to promoter regions of Wnt target genes such as cyclin D1 (17, 18).

Oncogenic transformation of mammalian cells is closely linked to the signaling function of β-catenin (19). Intestinal cells carrying mutations in APC that activate the β-catenin/Tcf/Lef signaling pathway develop into adenomas and adenocarcinomas (20). In addition, human colorectal neoplasms expressing a wild-type APC often show mutations in β-catenin that activate its signaling capacity (9). Furthermore, mice expressing a dominant allele of the β-catenin gene develop adenomatous intestinal polyps and nascent microadenomas, providing further evidence that activated β-catenin signaling contributes to cancer development (21).

A few observations suggested that TJ components, in addition to their structural role, may also be involved in signaling events. ZO-1 is related to the Drosophila discs-large tumor suppressor (Dlg-A), a component of septate junctions in Drosophila implicated in signaling during mitosis. Dlg proteins with mutations in the PDZ and SH3 domains cause neoplastic overgrowth of larval imaginal disc epithelial cells (22). The Drosophila orthologue of ZO-1, tamou, has been implicated in regulating the expression of extramacrophaetase (23), the fly orthologue of the inhibitor of differentiation protein. ZO-1 itself has been found in the nucleus of migrating epithelial cells at the edge of wounded monolayers or in epithelial cells induced to migrate by HGF (24).

To explore possible additional functions of ZO-1 besides its role as a structural component of TJ, we expressed progressive C-terminal deletion mutants of the protein in epithelial MDCKI cells. Surprisingly, mutants encoding only the N terminus including the PDZ domains no longer localized at the plasma membrane and induced a dramatic loss of the epithelial phenotype of MDCKI cells. This EMT included changes in the differentiation potential and tumorigenicity of the cells, together with a repression of epithelial (e.g. E-cadherin) and an induction of mesenchymal (e.g. fibronectin) marker genes. Interestingly, β-catenin/Tcf/Lef signaling was activated in MDCK-PDZ cells, indicating an involvement of β-catenin/Tcf/Lef signaling in the induction of the observed EMT. Thus, our results show that the cytosolic localization of the PDZ domains of ZO-1 leads to the transformation of MDCKI cells, most likely through a direct or indirect modulation of the β-catenin/Tcf/Lef signaling pathway.

MATERIALS AND METHODS

Plasmids—Human ZO-1 cDNA (GenBank™ accession no. L14837) was kindly provided by J. Anderson. The following deletion mutants (see Fig. 1) were created by the PCR technique using Pwo polymerase (Roche): ZO-1-PDZ (amino acids 1–588), ZO-1-P5 (amino acids 1–759), ZO-1-P59 (amino acids 1–788), and wild-type ZO-1. A FLAG epitope tag (5′-gattacaagggtaggtagaatg-3′) was introduced into each 5′ oligonucleotide to generate a ZO-1 fusion protein with the FLAG tag at the N terminus. The different PCR products were cloned into the pcRBlunt vector (Invitrogen), cut out with EcoRV, and cloned into the eukaryotic retrovector expression vector pLNCX (CLONTECH), cut with HpaI. Full-length human APC cDNA (GenBank™ accession no. M74088) was kindly provided by B. Vogelstein, and a mye tag (5′-gaaatcttcgatgaaggtctgcataagtcgacagatgacctg-3′) was introduced at the 3′ end by PCR. The neomycin resistance gene in the retroviral pLNCX vector was replaced by a hygromycin-thymidine kinase fusion cDNA (kindly provided by C. Karreman) to yield the pHygokTNCX vector, into which the mye-tagged APC cDNA was cloned. The plasmid coding for the GST-E-cadherin cytoplasmic fusion protein was kindly provided by A. Ullrich and described elsewhere in detail (25).

Cell Culture—MDCKI cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS) (Life Technologies, Inc.), 100 units/ml penicillin and streptomycin, and 2 mM glutamine. MDCKI cells were plated at a density of 1 × 10⁶ cells/cm² on 10-cm plates (Nunc) the day before transfection using the calcium phosphate technique as described (26). Briefly, 16 μl of plasmid DNA (Nucleobond purified) were mixed with 40 μl of 2.5 mM CaCl₂ to a final volume of 400 μl. An equal volume of 2 × BBS (50 mM BES (Sigma), 280 mM NaCl, 1.5 mM Na₂HPO₄, pH 6.5) was added, and after 10 min at room temperature the mixture was added to the cells. The cells were incubated overnight at 3% CO₂, washed twice with PBS, and incubated at 5% CO₂. For stable transfections, 750 μg/ml G418 or 350 μg/ml hygromycin, respectively (Life Technologies, Inc.), were added 24 h after transfection. Single clones were established using either limited dilution or cloning rings.

Transepithelial electrical resistance (TER) was measured 3 days after 5 × 10⁴ cells/cm² had been plated on Transwell filters (Costar). TER was determined by applying an AC square wave current of 620 mA at 12.5 Hz across a cell monolayer plated on a 6.5-mm diameter Transwell filter. The voltage deflection was measured with a pair of Ag/AgCl von Instruments). TER values were calculated by subtracting the blank values from the filter and were normalized to the area of the monolayer (filter).

Indirect Immunofluorescence—5 × 10⁴ cells/cm² were grown on coverslips to confluence, washed twice with PBS, and fixed with 3% freshly prepared paraformaldehyde for 25 min at room temperature. The cells were permeabilized and permeabilized with 0.5% Triton X-100 at room temperature. Unspecific binding was blocked with 10% goat serum in PBS for 1 h at room temperature, and primary antibodies (M2 monoclonal α-FLAG antibody; Eastman Kodak Co.) or fluorescein isothiocyanate-labeled phalloidin (Sigma; diluted 1:100 in 10% goat serum) were added for 2 h at room temperature. After washing the cells several times with PBS, bound primary antibodies were detected with fluorescein isothiocyanate-coupled isotype-specific secondary antibodies (Alexa). Coverslips were mounted (16.7% Mowiol, 33% glycerol in 120 mM Tris-HCl, pH 8.5) and viewed with a conventional fluorescence microscope (Leica).

Cell Culture in Collagen Gels—Cells were trypsinized, and 2 × 10⁴ cells were added to 800 μg of collagen type I (Promocell) in 10× DMEM to yield a final volume of 2 ml in 1× DMEM, 10% FCS and plated in 24-well plates (Costar). The plates were incubated 10 min at room temperature and 45 min at 37 °C before 1 ml of DMEM containing 10% FCS was added. Where indicated, HGF (40 ng/ml, kindly provided by W. Birchmeier) was added to the medium and incubation was continued until a duct-like morphology became visible. Cells were fixed with 3% freshly prepared paraformaldehyde, permeabilized with 0.5% Triton X-100, and stained with 0.2% Carmin-Hemalaun in H₂O overnight.

Tumor Formation in Nude Mice—Cells were grown in DMEM containing 10% FCS to yield a cell density of 2 × 10⁶ cells in 50 μl of DMEM and injected subcutaneously into the flank region of Swiss nude mice (IGR Villejuif, Paris, France) using five animals per cell line. Tumor formation was monitored by measuring the width (W) and length (L) of the tumors with W < L. The tumor volume was calculated according to the formula (W² × L × π/6).

Reporter Gene Transcription Assays—Cells were plated at a density of 1 × 10⁶ cells/cm² in six-well plates (Costar) the day before transfection.
tion. Transfection was performed using the calcium-phosphate technique as described above with 4 μg of luciferase reporter constructs containing either multimerized wild-type (TOP-FLASH) or mutant (TOP-FLASH) Tcf/Lef binding sites (kindly provided by H. Clevers). As a control for transfection efficiency, 1 μg of β-galactosidase construct under control of the same 4X promoter was included in each transfection. Cells were washed 24 h after transfection, and extracts were prepared in 400 μl of reporter lysis buffer (Promega). Luciferase and β-galactosidase activity were assayed according to the manufacturer’s protocol using the luciferase assay kit from Promega. The relative luciferase units corresponding to the enzymatic luciferase activities obtained from the TOP or PO reporter gene transcription, respectively, were normalized to the relative β-galactosidase activity. To allow easier comparison of the transcriptional activities, the background transcriptional activity represented by the TOP values was subtracted from the TOP values. Each transfection was done in triplicate, and the luciferase and β-galactosidase activities of each sample were measured in triplicate. The assay was performed in three independent experiments.

Cellular Extracts—To analyze the expression of proteins or to determine the free pool of β-catenin, cells were washed with ice-cold PBS and scraped from the plate in ice-cold lysis buffer (20 mM imidazole-HCl, pH 6.8, 100 mM KCl, 2 mM MgCl₂ 20 mM EDTA, 300 mM sucrose, 0.1 mM sodium orthovanadate; bound complexes were separated by SDS-PAGE and transferred onto nitrocellulose membranes, and the mRNA was separated on a denaturing formaldehyde-agarose gel to visualize the integrity of the RNA. For semiquantitative analysis, 0.5 μg of total RNA was used for RT-PCR using the TITAN One-Tube RT-PCR kit (Roche Molecular Biochemicals) according to the manufacturer’s protocol. The reverse transcriptase reaction was performed for 45 min at 50 °C or 40 °C. PCR cycles used to amplify the cDNA were as follows: denaturing step: 45 s at 94 °C, annealing step: 45 s at 40–50 °C, and elongation step: 68 °C, 1 min per 1000 bp to be amplified; 25 cycles were used. RT-PCR products were analyzed on 0.5–2% agarose gels. The following primer pairs were used: gapdh, 5'-gcttggaaac-3' and 5'-ggtcttcgctttactagctg-3'; ZO-1, 5'-ggttcacctggaagaggtga-3' and 5'-ggtcttggcttgtctg-3'; E-cadherin, 5'-gcagattctgtaaag-3' and 5'-ggaagactgtctgtcgcggc-3'; occludin, 5'-gggggaggtcagacagcat-3' and 5'-gggggaggtcagacagcat-3'; fibronectin, 5'-ggtctgtaggtgtctgtcgc-3' and 5'-ggtctgtaggtgtc-3'; vimentin, 5'-ggggctgcctgctgctgctg-3' and 5'-ggggctgcctgctgctgctg-3'.

RESULTS

Generation of MDCKI Cells Expressing Epitope-tagged ZO-1 Constructs—ZO-1 wild-type and deletion mutants encoding the PDZ domains (PDZ), the PDZ and the SH3 domains (PSG), or the PDZ, SH3 and GUK domains (PSG) were constructed, each carrying a C-terminal FLAG epitope tag (Fig. 1). The different cDNAs were subcloned into the pLNCX expression vector and transfected into MDCKI epithelial cells. These cells possess characteristics similar to the principal cells of the collecting systems in the kidney and polarize under appropriate conditions. Compared with the MDCKII cell line, MDCKI cells show a significantly increased TER (28, 29), indicating the formation of well established TJ. After transfection, G418-resistant cells were selected, pooled, and used for further analysis. Immunofluorescence experiments using M2 anti-Flag antibodies showed that the cells in a given pooled population of G418-resistant cells homogeneously expressed the different ZO-1 proteins (data not shown, see below).

ZO-1 Mutants That No Longer Localize at the Plasma Membrane Induce an EMT—To characterize the subcellular distribution of the different ZO-1 proteins, cells were grown to confluence and the localization of the tagged proteins was detected by indirect immunofluorescence (Fig. 2a). In MDCK-ZO-1 cells, the transfected FLAG-tagged wild-type ZO-1 localized at the plasma membrane to regions of cell-cell contact (Fig. 2a, panel A). After transfection, G418-resistant cells were selected, pooled, and used for further analysis. Immunofluorescence experiments using M2 anti-Flag antibodies showed that the cells in a given pooled population of G418-resistant cells homogeneously expressed the different ZO-1 proteins (data not shown, see below).

ZO-1-PDZ-SH3 (ZO-1, PS; bp 1227–3503), and ZO-1-PDZ (bp 258–1227).

Immunoblot Analysis—Equal amounts of proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane (BDH Laboratories) using a semidrying blotting apparatus (Bio-Rad). In specific antibody binding was blocked with 10% nonfat milk powder in PBS. The membrane was probed with primary antibodies (anti-Flag M2 (Kodak), anti-β-catenin, anti-plakoglobin (Transduction Laboratories), anti-E-cadherin (kindly provided by Axel Ullrich), anti-ZO-1 (Zymed Laboratories Inc.), or anti-pan-cytokeratins (Sigma) overnight at 4 °C. The membranes were washed several times with PBS, 0.5% Tween20 (Sigma), and immunoreactive bands were visualized with the enhanced chemiluminescence detection system (Ferrett) using horseradish peroxidase-coupled secondary antibodies (Jackson).

Affinity Precipitation—Equal amounts of cell lysates were preclared with glutathione-Sepharose for 30 min at 4 °C and incubated with 5 μg of purified GST-E-cadherin cytoplasmic fusion protein or a 3-fold molar excess of GST immobilized on glutathione-Sepharose (Amersham Pharmacia Biotech). The resulting complexes were washed three times with 20 mM HEPS, pH 7.5, 150 mM NaCl, 10 mM pyrophosphate, 10 mM NaF, 0.2 mM ammonium molybdate, 10% glycerol, 0.1% Triton X-100, 2 mM sodium orthovanadate; bound complexes were separated by SDS-PAGE and transferred onto nitrocellulose membranes, and β-catenin was visualized by immunodetection.

Preparation of Total RNA and RT-PCR Analysis—Total RNA was prepared as described previously (27). Briefly, the cells were washed with 4 ml of PBS and lysed in 4 ml guanidinum thiocyanate (Pierce 100 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1% β-mercaptoethanol (Sigma)). After the lysis had been passed several times through a sterile needle, an equal volume of water-saturated phenol:chloroform (1:1) was added and vortexed for at least 10 s. After centrifugation, the total cellular RNA of the aqueous phase was precipitated with an equal volume of isopropanol. The RNA was recovered by centrifugation, the pellet was resuspended in sterile water, and the RNA was separated on a denaturing formaldehyde-agarose gel to visualize the integrity of the RNA. For semiquantitative analysis, 0.5 μg of total RNA was used for RT-PCR using the TITAN One-Tube RT-PCR kit (Roche Molecular Biochemicals) according to the manufacturer’s protocol. The reverse transcriptase reaction was performed for 45 min at 50 °C or 40 °C. PCR cycles used to amplify the cDNA were as follows: denaturing step: 45 s at 94 °C, annealing step: 45 s at 40–50 °C, and elongation step: 68 °C, 1 min per 1000 bp to be amplified; 25 cycles were used. RT-PCR products were analyzed on 0.5–2% agarose gels. The following primer pairs were used: gapdh, 5'-gcttggaaac-3' and 5'-ggtcttcgctttactagctg-3'; ZO-1, 5'-ggttcacctggaagaggtga-3' and 5'-ggtcttggcttgtctg-3'; E-cadherin, 5'-gcagattctgtaaag-3' and 5'-ggaagactgtctgtcgcggc-3'; occludin, 5'-gggggaggtcagacagcat-3' and 5'-gggggaggtcagacagcat-3'; fibronectin, 5'-ggtctgtaggtgtctgtcgc-3' and 5'-ggtctgtaggtgtc-3'; vimentin, 5'-ggggctgcctgctgctgctg-3' and 5'-ggggctgcctgctgctgctg-3'.

ZO-1 and β-Catenin Signaling

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Fig. 1. Schematic representation of the different ZO-1 constructs encoding a C-terminal Flag epitope tag: ZO-1 wild-type (bp 1227–6437), ZO-1-PDZ-SH3-GUK (ZO-1-PSG; bp 1227–3621), ZO-1-PDZ-SH3 (ZO-1, PS; bp 1227–3503), and ZO-1-PDZ (bp 1227–2929).

2 M. Reichert and W. Hunziker, unpublished observations.
sively detected in the membrane fraction. In contrast, the ZO-1 PDZ protein was mostly recovered in the cytosolic fraction. This experiment thus confirms the immunofluorescence data and shows that the ZO-1 PDZ protein no longer localizes at the plasma membrane. These results are consistent with previous data (5) indicating that the GUK domain may be critical for the localization of ZO-1 at the plasma membrane, presumably by binding to occludin.

Neither MDCKI cells expressing the wild-type ZO-1 nor cells transfected with the empty vector (MDCK-pLNCX) showed apparent changes in morphology when compared with the parental cell line, and the morphology of MDCK-PSG cells was also not altered. Surprisingly, however, the expression of proteins encoding the PDZ domains but lacking the GUK domain led to a dramatic change in the morphology of MDCK-PDZ and MDCK-PS cells (panel B and data not shown). The cells lost their epithelial phenotype and instead displayed a fibroblast-like morphology with long lamellipodia. Alterations in the morphology of MDCK-PDZ cells correlated with changes in the organization of the cytoskeleton. The typical cortical actin ring of polarized epithelial cells was observed in control MDCK-pLNCX cells (Fig. 2a, panel F). In contrast, MDCK-PDZ cells displayed actin stress fibers normally absent from polarized epithelial cells but characteristic for fibroblastoid or migrating epithelial cells (panel E) as well as ducts (arrowheads) only observed in control cells.

**Fig. 2.** Cellular localization of the different ZO-1 proteins in MDCKI cells and epithelial to mesenchymal transformation of MDCK-PDZ cells. a, MDCKI cells expressing ZO-1 (panel A), the ZO-1 PDZ protein (MDCK-PDZ, panel B), the ZO-1-PSG protein (MDCK-PSG, panel C), or control cells transfected with the empty vector (MDCK-pLNCX, panel D) were grown on coverslips, fixed, and stained with the anti-Flag monoclonal antibody M2 to visualize the localization of the ZO-1 proteins. To visualize the actin cytoskeleton, MDCK-PDZ (panel E) or MDCK-pLNCX (panel F) cells were stained with fluorescein isothiocyanate-phalloidin. b, localization of ZO-1-PSG and ZO-PDZ to the membrane and cytosolic fraction of MDCKI cells. Cells were homogenized in the absence of detergent to obtain cytosol and membrane fractions as described under "Materials and Methods," and the ZO-1 proteins were detected by Western blot using the anti-Flag antibody.

**Fig. 3.** MDCK-PDZ cells fail to form tight monolayers and do not differentiate in collagen type I gels. a, cells (5 × 10⁴ cells/cm²) were plated on Transwell filters and incubated for 3 days. TER was measured, and values were calculated by subtracting the blank values from a filter with medium alone and normalized to the area of the monolayer (filter). Each experiment was done in triplicate. b, MDCK-pLNCX (panels A and C) or MDCK-PDZ (panels B and D) cells were plated in 24 well plates in 2 ml of complete medium containing 800 μg of collagen I. Where indicated (panels C and D), HGF (40 ng/ml) was added to the medium and incubation was continued until differentiation morphology became visible. Cells were fixed with 3% paraformaldehyde, permeabilized with 0.5% Triton X-100, and stained with Carmin-Hemalaun. The formation of cysts and budlike structures (arrows) as well as ducts (arrowheads) was only observed in control cells.

Thus, MDCKI cells expressing the PDZ domains of ZO-1 in the cytosol (i.e. ZO-1 PDZ or ZO-PS) underwent profound changes in morphology and cytoskeletal organization. This loss of epithelial polarity and gain of mesenchymal properties was characterized in more detail using MDCK-PDZ cells.

**MDCK-PDZ Cells Do Not Form Polarized Monolayers and Have Lost Their Potential to Differentiate—**To determine whether MDCK-PDZ cells were still able to form monolayers with functional tight junctions, cells were grown on permeable filter supports. As shown in Fig. 3a, control MDCK-pLNCX cells formed tight monolayers as evidenced by the establishment of a TER characteristic for MDCKI cells. MDCK-ZO-1 and MDCK-PSG cells expressing mutant forms of ZO-1, which still localized at the plasma membrane, showed a slight increase in the TER compared with vector-transfected control cells. These data are consistent with assembly of the different ZO-1 constructs into functional TJ and provide evidence for a role of ZO-1 in the regulation of TJ permeability. In contrast, no
The expression of epithelial and mesenchymal marker proteins in MDCK-PDZ cells. a, cell extracts containing Triton X-100-soluble and -insoluble material were prepared as described under "Materials and Methods" and analyzed by Western blot using the indicated antibodies. b, 2.5 μg of total RNA was analyzed by semi-quantitative RT-PCR using specific primer pairs for the indicated genes and the amplified PCR fragments were separated on 0.5–2% agarose gels. c, MDCK-pLNCX and MDCK-PSG cells were lysed in RIPA buffer and the lysates analyzed by Western blot using antibodies against E-cadherin.

Changes in mRNA levels were analyzed by semi-quantitative RT-PCR.

As shown in Fig. 5a, the amount of typical epithelial marker proteins like cytokeratins or E-cadherin was significantly reduced in MDCK-PDZ cells. Semi-quantitative RT-PCR furthermore showed decreased mRNA levels for E-cadherin, occludin, and endogenous ZO-1 in MDCK-PDZ cells (Fig. 5b), indicating that the loss of at least some epithelial marker proteins reflected changes in gene expression and/or mRNA stability. Interestingly, while the endogenous ZO-1 (α+) transcript was reduced in MDCK-PDZ cells, a new transcript, identified by sequencing the PCR product as the alternatively spliced ZO-1 (β−) splice form is often found in cells that show an increased plasticity in cell-cell contacts and junction stability (31, 32).

In addition, the amounts of α, β- and γ-catenin/plakoglobin protein were reduced in MDCK-PDZ cells (Fig. 5a). In particular, the amount of the different catenins present in the Triton X-100-soluble fraction was reduced. Catenin mRNA levels were either not significantly altered or slightly elevated in MDCK-PDZ cells (data not shown), indicating that the lower protein levels in MDCK-PDZ cells were not due to reduced mRNA levels. Additionally, the amount of APC protein in the different fractions was reduced in MDCK-PDZ cells and a redistribution of APC from a detergent-soluble into a more detergent-resistant fraction was observed (Fig. 5a). Immunofluorescence analysis revealed that APC was present in clusters at the end of lamellipodia (data not shown), possibly correlating with the localization of APC observed during migration processes (33).
The endogenous ZO-1 protein is mainly detected in the Triton-insoluble fraction in MDCK-pLNCX and MDCK ZO-1 cells, and immunofluorescence analysis revealed that ZO-1 is localized at the plasma membrane (data not shown). Consistent with the RT-PCR analysis, the amount of endogenous ZO-1 in MDCK-PDZ cells is strongly reduced (Fig. 5a). Concomitant with the repression of epithelial markers, mRNA levels of the mesenchymal marker genes fibronectin and vimentin were increased in MDCK-PDZ cells (Fig. 5b).

Importantly, and in contrast to MDCK-PDZ cells, E-cadherin was still expressed in MDCK-ZO-1 and MDCK-PSG cells (Fig. 5, a and c), which retained the polarized epithelial phenotype, excluding that the expression of exogenous ZO-1 per se alters the expression of the analyzed marker genes. These results thus further confirm the inverse correlation between plasma membrane association of the PDZ domains and the induction of an EMT.

In conclusion, the profound changes in MDCK-PDZ cells were paralleled by the repression of epithelial and the induction of mesenchymal marker genes, respectively.

**β-Catenin/Tcf/Lef Signaling Is Constitutively Activated in MDCK-PDZ Cells**—It has been suggested that β-catenin/Tcf/Lef signaling may negatively regulate E-cadherin and cytokeratin expression due to activated Tcf/Lef binding sites within their promoter regions (34, 35). This prompted us to investigate whether this signaling pathway was activated in MDCK-PDZ cells. Free cytosolic β-catenin can act as a regulator of transcription after translocation to the nucleus and interaction with members of the Tcf/Lef family of transcription factors (36, 37). To determine whether the free pool of β-catenin was elevated in MDCK-PDZ cells, the amount of β-catenin in cell lysates from control and MDCK-PDZ cells that is able to bind to a GST fusion protein comprising the cytoplasmic tail of E-cadherin was determined (25, 38). As shown in Fig. 6a, slightly less free monomeric β-catenin from MDCK-PDZ cell lysates bound to the immobilized E-cadherin tail fusion protein as compared with control cells, indicating that the cytosolic free pool of β-catenin was not increased in MDCK-PDZ cells. This observation is consistent with the lower amounts of β-catenin found in the Triton-soluble pool of cell lysates of MDCK-PDZ cells (Fig. 5a).

Since recent reports have shown that an increased transcriptional activity of β-catenin/Tcf/Lef does not necessarily correlate with the detection of an increased free pool of β-catenin (39, 40), we directly analyzed β-catenin/Tcf/Lef signaling in MDCK-PDZ cells using the TOP-FOP Tcf/Lef luciferase reporter constructs, a well established assay to measure transcriptional activation due to activated β-catenin signaling (20). Briefly, MDCK-pLNCX, MDCK-ZO-1, MDCK-PSG, and MDCK-PDZ cells were transfected with plasmids encoding multimerized wild-type (TOP) or mutant (FOP) LEF binding sites followed by a luciferase reporter gene (36). As a control for transfection efficiency, an SV40-driven β-galactosidase cDNA was cotransfected. Relative luciferase activities were calculated as described under “Materials and Methods.” As shown in Fig. 6b, the relative transcriptional activity of the β-catenin/Lef complex was 6 times higher in MDCK-PDZ cells as compared with vector transfected cells. Thus, despite the lack of an increased free pool of β-catenin, β-catenin/Tcf/Lef signaling is constitutively activated in MDCK-PDZ cells.

**Ectopic Expression of APC Reverts the ZO-1 PDZ-induced EMT and Abolishes β-Catenin/Tcf/Lef Signaling**—When the loss of the epithelial phenotype induced by the expression of mislocated ZO-1 PDZ domains involves an activated β-catenin/Tcf/Lef signaling, transfection of MDCK-PDZ cells with APC, a known negative regulator of β-catenin/Tcf/Lef signaling (41, 42), may affect the phenotype of MDCK-PDZ cells. We therefore stably transfected MDCK-PDZ cells with a cDNA encoding the human APC cDNA or, as a control, with the empty vector, pLHygTKCX.

While cells transfected with the empty vector retained their fibroblastoid morphology (Fig. 7a, panel A), cells transfected with the APC cDNA reverted to an epithelial phenotype (panels B and C). Several individual clones derived from cells transfected with APC were isolated and analyzed by RT-PCR to confirm the expression of the human APC cDNA. The phenotypic reversion to the epithelial morphology was found to correlate with the expression level of APC (Fig. 7b). Western blot analysis confirmed that the reverted phenotype did not result from a loss of the expression of the ZO-1 PDZ protein (Fig. 7c). Furthermore, the phenotypic conversion of MDCK-PDZ cells ectopically expressing APC was paralleled by the re-expression of E-cadherin (Fig. 7c) and the repression of the transcriptional activity of the β-catenin/Lef complex (Fig. 7d).
Thus, the ectopic expression of APC reverted the EMT of MDCK-PDZ cells and led to a repression of the $\beta$-catenin/Lef transcriptional activity, consistent with a direct or indirect effect of the ZO-1-PDZ protein on the $\beta$-catenin/Tcf/Lef signaling pathway.

**DISCUSSION**

We show that expression of ZO-1 mutants encoding the N terminus comprising the PDZ domains that localize to the cytosol induces a dramatic EMT in MDCKI cells. Localization of ZO-1 at the plasma membrane required the presence of the GUK domain, and no EMT was induced by ZO-1 mutants that were properly targeted to the plasma membrane. Uncloned polyclonal cell populations were used for the experiments to exclude effects due to clonal selection. However, similar phenotypes were observed for cell clones obtained by limited dilution (data not shown). Characterization of ZO-1 PDZ protein expression levels in individual clones indicated that the observed effect was not due to the expression of large amounts of the PDZ domains, since similar effects were observed in clones expressing the PDZ domains at levels barely detectable by Western blot (data not shown). The loss of contact inhibition of proliferation and differentiation potential, as well as an increased tumorigenicity in nude mice. These phenotypic alterations correlated with a reduced expression level of proteins characteristic for epithelial cells (i.e. E-cadherin and cytokeratins). Ectopic expression of APC in MDCK-PDZ cells reverted the transformed phenotype and led to the re-expression of E-cadherin and cell-cell contact formation. Down-regulation of E-cadherin expression correlates with increased tumor invasion, metastasis, and poor clinical prognosis (43). Furthermore, inhibition of E-cadherin function with interfering antibodies alters the morphology of MDCK cells and enables them to invade both collagen gels and embryonic chicken heart tissue (43, 44), consistent with the importance of E-cadherin in maintaining the epithelial phenotype. However, ectopic expression of full length E-cadherin was not sufficient to revert the fibroblastoid phenotype of MDCK-PDZ cells (data not shown). Since nonepithelial cadherins like N-cadherin can promote motility and invasiveness of epithelial cells regardless of the presence of E-cadherin (45), it will be interesting to determine if MDCKI cells express nonepithelial cadherins or if their expression is induced in MDCK-PDZ cells.

MDCK-PDZ cells also showed an increase in the mRNA levels of fibronectin and vimentin. Vimentin is a type III intermediate filament protein normally expressed in cells of mesenchymal origin. Using an in vitro wound healing model, it has been shown that vimentin mRNA and protein expression were exclusively induced in cells at the wound's edge that were actively migrating toward the center of the lesion. The vimentin protein disappeared when the cells became stationary after the wound closure (46). This correlates with the observed migrating phenotype of MDCK-PDZ cells. The changes at the protein level reflected, at least for some proteins, corresponding
changes in mRNA levels, indicating that the ZO-1 PDZ protein had an effect on gene expression and/or mRNA stability. Intriguingly, β-catenin/Tcf/Lef signaling was constitutively activated in MDCK-PDZ cells. β-Catenin/Tcf/Lef has been implicated in signaling events leading to EMT in vitro during embryonic development (47) and in vitro in epithelial cells (48). We observed an inverse correlation between β-catenin/Tcf/Lef transcriptional activity and the expression of E-cadherin and cytotekatins, consistent with a negative regulation of transcription by β-catenin signaling via Tcf/Lef binding sites present in the promoter regions of the E-cadherin and cytokeratin genes (34, 35, 49).

Free cytosolic β-catenin, upon translocation into the nucleus with Tcf/LEF family members, can activate the transcription of specific target genes. Despite a significant increase in the transcriptional activation of the TOP reporter construct by β-catenin, the free pool of β-catenin was not elevated in MDCK-PDZ cells. Transcriptional activation via β-catenin/Tcf/Lef without an increase in the free pool of β-catenin is not unprecedented and has been described for the integrin-linked kinase-mediated transformation (39). Furthermore, nuclear localization of β-catenin and Tcf/Lef may not be sufficient to activate gene expression, implicating additional cell type-specific regulatory factors (40). An example of an additional regulatory mechanism may be the interaction of the Drosophila orthologue of β-catenin, Armadillo, with Teashirt, a transcription factor involved in the establishment of trunk segment identity and whose nuclear localization depends on Wnt signaling (50).

Our results demonstrating a contribution of β-catenin/Tcf/Lef signaling in the EMT induced by the ZO-1 PDZ protein are in line with several studies implicating activated β-catenin/Tcf/Lef signaling in epithelial cell transformation (16, 51, 52). Modest overexpression of β-catenin in MDCK cells leads to cellular transformation by affecting contact inhibition of proliferation, anchorage-independent growth, anoikis, and radiation-induced cell cycle arrest (51). The ectopic expression of a constitutively active N-terminal deletion mutant of β-catenin in MDCKII cells results in a dispersed fibroblastoid morphology (48). Furthermore, an activated mutant of β-catenin induces dysplasia and adenoma in transgenic mice (52, 53). Loss of APC thereby activating β-catenin signaling also leads to dysplasia and adenoma formation (53, 54). Interestingly, during HGF-induced migration of MDCKII cells, an increase in tyrosine phosphorylation of β-catenin correlates with the translocation of ZO-1 to the cytosol and HGF inhibits the reassembly of ZO-1 at the plasma membrane (55).

The mechanism by which the ZO-1 PDZ protein activates β-catenin/Tcf/Lef signaling and induces the observed EMT is currently under investigation, and several possibilities can be considered. The constitutive cytosolic localization of the ZO-1 mutant could interfere with the proper negative regulation of a signaling function of ZO-1 at the plasma membrane, similar to the negative regulation of β-catenin/Tcf/Lef signaling by cadherins (56–59). Alternatively, the ZO-1 PDZ protein could interact with molecules of the Wnt signaling pathway or with components of adherens junctions (i.e. α-catenin; Ref. 60), thereby altering the equilibrium between free and sequestered β-catenin or interfering with the signaling pathway. Interestingly, the hDlg protein, which shows homology to ZO-1, interacts with APC (61). While it is not known whether ZO-1 binds APC, such an interaction would be consistent with our observation that the ectopic expression of APC, known to negatively regulate activated β-catenin/Tcf/Lef signaling (41), reverted the EMT in MDCK-PDZ cells. Yet another possible link could involve the interaction between ZO-1 and AF-6 (62, 63), which in turn can recruit the de-ubiquitinating enzyme FAM to sites of cell-cell adhesion (64). Since FAM also interacts with β-catenin and E-cadherin in vitro, FAM may normally stabilize cell-cell contacts by de-ubiquitinating proteins at these sites. Interestingly, p21Ras interferes with the interaction between ZO-1 and AF-6 (65), thereby contributing to the perturbation of cell-cell adhesion in p21Ras-transformed cells. Finally, because ZO-1 was shown to translocate to the nucleus of migrating epithelial cells (24), the ZO1-PDZ protein itself could be engaged in an active signaling process. Since, in contrast to in vitro model systems, mutations in APC or β-catenin that activate β-catenin/Tcf/Lef signaling in vivo normally lead to less dramatic changes of the epiblastal phenotype (i.e. induction of hyperplasia or dysplasia and adenomas; Refs. 53 and 54), the observed profound effects raise the possibility that the ZO-1 PDZ protein itself may play a more direct role in mediating the observed EMT.

In conclusion, our results show that expression of the ZO1-PDZ protein in MDCKI cells leads to a fibroblastoid, transformed phenotype of these cells in vitro and an increased tumorigenicity in vivo, paralleled by the activation of β-catenin/Tcf/Lef transcriptional activity. Thus, a dysregulation of ZO1 localization or function may contribute to an EMT and possibly to the development of tumors. Indeed, in breast carcinomas, the absence of ZO-1 at the plasma membrane correlates with tumor formation (66). The novel finding that β-catenin/Tcf/Lef signaling can be modulated by a member of the TJ, together with the established regulation by components of AJ and integrins, indicates that this signaling pathway plays a general role in integrating signals generated in response to cell-cell and cell-matrix interactions.

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The PDZ Domains of Zonula Occludens-1 Induce an Epithelial to Mesenchymal Transition of Madin-Darby Canine Kidney I Cells: EVIDENCE FOR A ROLE OF β-CATENIN/Tcf/Lef SIGNALING
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