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Claudin-3 acts as a sealing component of the tight junction for ions of either charge and uncharged solutes

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ABSTRACT

The paracellular barrier of epithelia and endothelia is established by several tight junction proteins including claudin-3. Although claudin-3 is present in many epithelia including skin, lung, kidney, and intestine and in endothelia, its function is unresolved as yet. We therefore characterized claudin-3 by stable transfection of MDCK II kidney tubule cells with human claudin-3 cDNA. Two clone systems were analyzed, exhibiting high or low claudin-2 expression, respectively. Expression of other claudins was unchanged. Ultrastructurally, tight junction strands were changed toward uninterrupted and rounded meshwork loops. Functionally, the paracellular resistance of claudin-3-transfected monolayers was strongly elevated, causing an increase in transepithelial resistance compared to vector controls. Permeabilities for mono- and divalent cations and for anions were decreased. In the high-claudin-2 system, claudin-3 reduced claudin-2-induced cation selectivity, while in the low-claudin-2 system no charge preference was observed, the latter thus reflecting the "intrinsic" action of claudin-3. Furthermore, the passage of the paracellular tracers fluorescein (332 Da) and FD-4 (4 kDa) was decreased, whereas the permeability to water was not affected. We demonstrate that claudin-3 alters the tight junction meshwork and seals the paracellular pathway against the passage of small ions of either charge and uncharged solutes. Thus, in a kidney model epithelium, claudin-3 acts as a general barrier-forming protein.

1. Introduction

Tight junctions (TJs) are the apical-most components of borders between adjacent cells, regulating the passage of ions and solutes via the paracellular pathway. Four types of transmembranal proteins located within the TJ have been identified: junctional adhesion molecule (JAM) [1], occludin [2], tricellulin [3], and the group of claudins [4]. The claudin family represents the main component of TJ strands, creating an ion-selective border between apical and basolateral compartments. To date, 24 mammalian claudins have been discovered. Several claudins have been shown to strengthen the paracellular barrier, e.g. claudin-1 [5], claudin-4 [6], claudin-5 [7,8], and claudin-8 [9–11]. On the other hand, some claudins exhibit properties of selective pores, e.g. claudin-2 [12,13], claudin-10a and claudin-10b [14,15]. While most claudins show a highly tissue-specific expression pattern, claudin-3 is a ubiquitous TJ protein, expressed in epithelia of intestine, kidney, liver, skin, lung, and in endothelia. In the blood–brain barrier (BBB), it is assumed to be a central component determining the integrity of the TJ [16]. Due to its omnipresence in different tissues and organs, it appears as a part of the basic equipment of TJ strands.

Claudin-3, formerly named rat ventral prostate protein 1 (RVP1), became generally known as functional receptor for the *Clostridium perfringens* enterotoxin (CPE) [17–20]. In numerous studies, it has been shown to be regulated during tumor genesis in various organs and tissues, e.g. in breast [21,22], ovaries [23–27], uterus [28], prostate [29], and esophagus [30].

Thus, claudin-3 is considered as an emerging target for cancer detection and therapy [31,32], although its functional role in cancer progression is unknown as yet [33], last not least because of its unclear role in barrier formation.

Within tubular structures like intestine and nephron, claudin-3 has been shown to be expressed stronger in distal than in proximal segments [34,35]. Recently, it has been shown that expression of claudin-3 along segments of rat intestine correlates with barrier properties measured by means of impedance spectroscopy [36]. Considering the distinct barrier function of these tissues, a tightening role of claudin-3 can be cautiously assumed. This concept is supported by previous studies: Coyne et al. transfected mouse fibroblast cells

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(NIH/3T3) and the human airway epithelial cell line IB3.1 with human claudin-3 cDNA and observed a trend toward an increase in transepithelial resistance which did not, however, reach significance in either cell line. Overexpression of claudin-3 in NIH/3T3 cells had no effect on permeability to 10, 70, and 2000 kDa dextrans, while overexpression in IB3.1 cells caused a decrease of permeability [37]. Moreover, knockdown of claudin-3 resulted in a marginal (−16%) or a marked (−70%) decrease of R^t in Madin–Darby canine kidney (MDCK) II cells, or human gastric adenocarcinoma cells (MKN28), respectively [38,39].

Taken together, previous studies imply a sealing effect of claudin-3, although its precise function is experimentally unsolved. In order to obtain deeper insights into the function of claudin-3, we overexpressed human claudin-3 in MDCK cells, subclone II, which represents a well established cell line with paracellular characteristics resembling the proximal nephron. To this end, we demonstrate that claudin-3 acts as a sealing component of the TJ for mono- and divalent ions and for molecules of 332 Da and 4 kDa.

2. Materials and methods

2.1. Cell culture

Madin–Darby canine kidney cells, subclone II (MDCK II) were grown in culture flasks containing MEM (PAA Laboratories, Pasching, Austria), supplemented with 10% fetal bovine serum (Biochrom, Berlin, Germany) and 1% penicillin/streptomycin (PAA Laboratories). Cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere. For all experiments, with the exception of transfection and co-immunoprecipitation, cells were grown on permeable polycarbonate culture plate inserts (Millicell HA, 0.6 cm², pore size 0.45 μm Millipore, Bedford, MA, USA).

2.2. Stable transfection

Human claudin-3 cDNA was obtained by isolation of total RNA from the human colon carcinoma cell line Caco-2, followed by reverse transcription and specific amplification by means of polymerase chain reaction (PCR). After verification of the PCR product by DNA sequence analysis, it was inserted into the eukaryotic expression vector pFLAG-CMV-10 (Sigma Aldrich, Taufkirchen, Germany). MDCK II cells were stably transfected with the pFLAG-CMV-10-claudin-3 construct, further referred to as pFLAG-CLD3, employing Lipofectamine Plus™ reagent (Invitrogen, Carlsbad, CA, USA). In parallel, another set of cells was transfected with the empty vector to serve as control. Transfected cells were treated with the antibiotic G418 (PAA Laboratories, Pasching, Austria) to select stably transfected clones, containing either pFLAG-CLD3 or the empty vector, respectively. All clones were screened for expression of claudin-3 and other claudins by Western blot analysis.

2.3. Western blotting

Cells were scraped from culture plate inserts and homogenized in lysis buffer (20 mM TRIS, 5 mM $MgCl_2$, 1 mM EDTA, 0.3 mM EGTA) containing protease inhibitors (Complete, Boehringer, Mannheim, Germany). Membrane fractions were obtained by passing through a 26 G × 3/8" needle, followed by a centrifugation at 200*g* for 5 min and subsequent centrifugation of the remaining supernatant at 43,000*g* for 30 min. Extracted protein was diluted in lysis buffer and quantified using the BCA Protein assay reagent (Pierce, Rockford, IL, USA) and a plate reader (Tecan, Grödig, Austria). Aliquots of protein were mixed with sodium dodecyl sulfate (SDS) containing buffer (Laemmli), denatured at 95 °C for 5 min, fractionated on SDS polyacrylamide gels and subsequently blotted onto PVDF membranes (PerkinElmer, Boston, MA, USA). Proteins were detected using specific antibodies against TJ proteins (Invitrogen, Carlsbad, CA, USA) or the FLAG sequence (Sigma Aldrich, Taufkirchen, Germany) respectively, and visualized by luminescence imaging (LAS-1000, Fujifilm, Tokyo, Japan). Signals were quantified by densitometry employing the AIDA application (Raytest, Straubenhardt, Germany).

For Triton X-100 (TX-100) fractionation, cells were homogenized in ice-cold extraction buffer (150 mM NaCl, 15 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM $CaCl_2$) containing 0.5% TX-100. Cells were incubated on ice for 20 min and subsequently centrifuged at 15,000*g* for 15 min to separate TX-100-soluble from -insoluble material. The TX-100-insoluble pellet was resuspended in an equal volume of extraction buffer. Both fractions were sonicated for 3 × 10 s at an amplitude of 40%. Protein quantification, blotting, detection, and visualization were performed as described above.

2.4. Co-immunoprecipitation

Cells were scraped from flasks and homogenized in lysis buffer (150 mM NaCl, 25 mM TRIS, 5 mM EDTA, 0.1% SDS, 1% Nonidet P40, 1% sodium deoxycholate) containing protease inhibitors (Complete, Boehringer, Mannheim, Germany). Whole cell lysates were obtained by centrifugation at 15,000*g* for 15 min. 250 μg of total protein was mixed with 4 μg antibody and incubated over night at 4 °C. Protein A agarose beads (Upstate, Lake Placid, NY, USA) were washed with lysis buffer and incubated with the protein/ antibody mixture for 1 h. Agarose beads were isolated by centrifugation (1000*g*; 1 min), washed with lysis buffer and denatured in Laemmli buffer at 95 °C for 20 min. Beads were pelleted (14,000*g*) and supernatant was fractionated on SDS polyacrylamide gels. Protein blotting, detection and visualization were performed as already described.

2.5. Immunofluorescence microscopy

Cells were grown to confluence on permeable culture plate inserts and fixed with absolute ethanol for 10 min at −20 °C. After permeabilization with phosphate-buffered saline (PBS) containing 0.5% Triton X-100 for 7 min, cell layers were preincubated with a blocking solution (PBS containing 5% goat serum) for 15 min at room temperature to avoid unspecific binding. For subsequent immunostaining of TJ proteins, cells were incubated for 60 min with specific antibodies, diluted 1:100 (anti-claudin and anti-occludin antibodies) or 1:600 (anti-FLAG antibody) in blocking solution. After washing, cell layers were exposed to secondary antibodies (Alexa Fluor goat anti-rabbit IgG or Alexa Fluor goat anti-mouse IgG with wavelengths of 488 or 594 nm, Molecular Probes, USA, diluted 1:500 in blocking solution) for 60 min. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, 461 nm, diluted 1:1,000 in blocking solution). Finally, cells were mounted onto microscope slides in ProTags MountFluor (Biocyc, Luckenwalde, Germany) and fluorescence images were obtained with a confocal laser-scanning microscope (Zeiss LSM 510 Meta, Zeiss, Germany).

2.6. Freeze fracture electron microscopy

Cells grown on permeable culture plate inserts were fixed with phosphate-buffered 2.5% glutaraldehyde and equilibrated in 10% and 30% glycerol. After freezing in liquid nitrogen-cooled Freon 22, preparations were fractured at −100 °C and shadowed with platinum and carbon in a vacuum evaporator (Denton DV-502, Cherry Hill, NJ, USA). Replicas were bleached with sodium hypochloride, picked up on grids (Ted Pella Inc., Tustin, CA, USA), and examined with a video-equipped Zeiss 902 electron microscope (Carl Zeiss AG, Oberkochen, Germany; Olympus iTEM Veleta, Münster, Germany). TJs were morphometrically analyzed at a final magnification of 51,000×. Perpendicular to the most apical TJ strand, vertical grid lines were layered at 200-nm intervals [40]. The number of horizontally oriented