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Contribution of claudin-5 to barrier properties in tight junctions of epithelial cells

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Abstract Claudin-5 is a transmembrane protein reported to be primarily present in tight junctions of endothelia. Unexpectedly, we found expression of claudin-5 in HT-29/B6 cells, an epithelial cell line derived from human colon. Confocal microscopy showed colocalization of claudin-5 with occludin, indicating its presence in the tight junctions. By contrast, claudin-5 was absent in the human colonic cell line Caco-2 and in Madin–Darby canine kidney cells (MDCK sub-clones C7 and C11), an epithelial cell line derived from the collecting duct. To determine the contribution of claudin-5 to tight junctional permeability in cells of human origin, stable transfection of Caco-2 with FLAG-claudin-5 cDNA was performed. In addition, clone MDCK-C7 was transfected. Synthesis of the exogenous FLAG-claudin-5 was verified by Western blot analysis and confocal fluorescent imaging by employing FLAG-specific antibody. FLAG-claudin-5 was detected in transfected cells in colocalization with occludin, whereas cells transfected with the vector alone did not exhibit specific signals. Re-

sistance measurements and mannitol fluxes after stable transfection with claudin-5 cDNA revealed a marked increase of barrier function in cells of low genuine transepithelial resistance (Caco-2). By contrast, no changes of barrier properties were detected in cells with a high transepithelial resistance (MDCK-C7) after stable transfection with claudin-5 cDNA. We conclude that claudin-5 is present in epithelial cells of colonic origin and that it contributes to some extent to the paracellular seal. Claudin-5 may thus be classified as a tight-junctional protein capable of contributing to the “sealing” of the tight junction.

Keywords Zonula occludens · Barrier function · Stable transfection · Claudin-5 · Human colonic cell line · Madin–Darby canine kidney cells

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Introduction

Three types of transmembrane proteins located in epithelial and endothelial tight junctions have been identified: occludin (Furuse et al. 1993), the junctional adhesion molecule (first reported by Martin-Padura et al. 1998; for a review, see: Bazzoni 2003), and the claudin family (Furuse et al. 1998; Morita et al. 1999a,b). The largest group among them, the claudin family, currently comprises 24 known proteins, numbered from 1 to 24 (Tsukita et al. 2001). Recent research has demonstrated that tight junction assembly is a dynamic process (Sasaki et al. 2002; Matsuda et al. 2004). Furthermore, the significant physiological relevance of this family has been highlighted by the finding of hereditary diseases linked to mutations in claudin genes (first reported by Simon et al. 1999; for a review, see Sawada et al. 2003).

Claudin-1 is a major component of tight junctions of tight epithelia, e.g. of the epidermis (Tebbe et al. 2002; Langbein et al. 2002; Brandner et al. 2002). Its functional relevance has been recently demonstrated as mice lacking claudin-1 die within hours after birth because of dehydration (Furuse et al. 2002). Claudin-2 is responsible for the formation of a paracellular pore (Furuse et al. 2001;

Amasheh et al. 2002). Claudin-3 appears to have minor influence on permeability properties (Furuse et al. 2001), but its functional relevance has been revealed in a report concerning the specific binding of *Clostridium perfringens* enterotoxin to claudin-3 and -4 (Sonoda et al. 1999). Claudin-4 decreases epithelial cation permeability (Van Itallie et al. 2001). The characterization of claudin-2, -4, and -15 by mutagenesis studies has shown that variations in the amino acids in the extracellular loop of claudins determine their permeability properties (Colegio et al. 2002, 2003).

Claudin-5 has been cloned in correlation with the identification of the hereditary disease velo-cardio-facial syndrome (Sirotkin et al. 1997). It constitutes tight junction strands in endothelial cells (Morita et al. 1999a,b; Kiuchi-Saishin et al. 2002; Morita et al. 2003) and is a component of the blood-brain barrier (Nitta et al. 2003) and the blood-testis barrier (Kamimura et al. 2002). Claudin-5 has been detected in tight junctions of pancreatic acinar cells (Rahner et al. 2001), in autotypic tight junctions of Schwann cells (Poliak et al. 2002), during the embryonic development of chick retinal pigment epithelium (Kojima et al. 2002), and in alveolar epithelial cells (Wang et al. 2003).

In claudin-5-deficient mice, the blood-brain barrier against small molecules (<800 Da), but not larger molecules, is perturbed (Nitta et al. 2003). Furthermore, serum-derived factors have been reported to decrease both transepithelial resistance (R^t) and the presence of claudin-5 molecules in tight junctions of cultured capillary endothelial cells from porcine brain (Nitz et al. 2003), supporting the notion of a “sealing” potential of claudin-5. By contrast, synthesis of claudin-5 in a human cystic fibrosis airway epithelia cell line, IB3.1, results in an increase of permeability (Coyne et al. 2003).

In the intestine, claudin-5 is reported to be strictly located in the junctions of both endothelial and epithelial cells without gradation along the crypt-to-villus surface axis in any part of the intestine (Rahner et al. 2001) and in biopsy specimens of the sigmoid colon, comprising epithelium and vascular endothelium (Bürgel et al. 2002). In parallel to the present study, the synthesis of murine claudin-5 in MDCK II cells has been demonstrated to increase transepithelial resistance, whereas paracellular flux of monosaccharides is not significantly changed when compared with controls (Wen et al. 2004). However, functional studies of the contribution of claudin-5 to the epithelial barrier are just beginning. In the present study, we have focused on the specific contribution of human claudin-5 to paracellular permeability by means of stable transfection with FLAG-claudin-5 cDNA in human colonic cell line (Caco-2) and a tight canine cell line (MDCK subtype C7).

Materials and methods

Cells and solutions Monolayers of Madin-Darby canine kidney (MDCK) subtypes C7 and C11 (Gekle et al. 1994) and of the human colon carcinoma cell lines Caco-2 and HT-29 clone B6 (Kreusel et al. 1991) were grown in 25-

cm² culture flasks containing DMEM (Biochrom, Berlin, Germany) supplemented with 10% (v/v) fetal bovine serum, 100 mg/ml streptomycin, and 100 U/ml penicillin (Biochrom). Cells were cultured at 37°C in a humidified 5% CO₂ atmosphere.

Electrophysiology For electrophysiological and molecular analyses, monolayers were grown on porous polycarbonate culture plate inserts (effective area: 0.6 cm²; Millicell-HA, Millipore, Bedford, Mass., USA). Transepithelial resistance referring to the tissue area (R^t , Ω cm²) was measured in Ussing chambers. The experimental setup was specially designed for the insertion of Millicell filter supports (Kreusel et al. 1991). Inserts were mounted in Ussing chambers, and water-jacketed gas lifts were filled with 10 ml circulating fluid on each side. Bathing Ringer solution contained: 113.6 mM NaCl, 2.4 mM Na₂HPO₄, 0.6 mM NaH₂PO₄, 21 mM NaHCO₃, 5.4 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 10 mM D(+)-glucose, 10 mM D(+)-mannose, 2.5 mM L-glutamine, and 0.5 mM β -hydroxybutyric acid. Ringer solution was gassed with 95% O₂ and 5% CO₂, to ensure a pH value of 7.4 at 37°C. Prior to each single experiment, the resistance of the bathing solution and the filter support was measured and used as the baseline.

Reverse transcription/polymerase chain reaction and molecular cloning of human claudin-5 Total RNA was obtained from MDCK subtypes C7 and C11 and from the human colon carcinoma cell lines Caco-2 and HT-29/B6 by using RNazol B reagent (WAK Chemie, Bad Soden, Germany). First-strand cDNA was synthesized by the reverse transcription (RT) reaction (M-MLV; Gibco BRL, Bethesda, Md., USA) with oligo dT primer. Sense (5' GCTGCCCATGTGGCAGGTGA 3') and antisense (5' GCCCAGCCGATGTACAGCG 3') primers were selected according to the human claudin-5 sequence (Sirotkin et al. 1997) and the canine claudin-5 sequence (*Canis familiaris*: a BLAST nucleotide homology search in the dog genome dataset at <http://www.pre.ensembl.org/> revealed the *C. familiaris* claudin-5 sequence on chromosome 26 at nucleotide positions 31,720,293–31,720,949 of the dog genome). The resulting polymerase chain reaction (PCR) product was analyzed on a 1% agarose gel and screened for the presence of a band of the expected size (430 bp).

For the cloning of the complete coding sequence of human claudin-5, sense (5' CCTCTAGCCATGGGGTCCG CA3') and antisense (5' CCAGCGCCCTCAGACGTAGTT 3') primers were synthesized according to the human claudin-5 sequence (Sirotkin et al. 1997) and used for PCR (Metabion, Martinsried, Germany) by employing HT-29/B6 cDNA synthesized by the RT reaction. The resulting 675-bp PCR product encompassing the complete claudin-5 cDNA was cloned into pCR2.1-Topo (Promega, Madison, Wis., USA). The correctness of the cDNA was verified by sequencing, and the cDNA was then subcloned into the eukaryotic expression vector pFLAG-CMV-4 (Sigma Aldrich, St. Louis, Mo., USA), further referred to as pFLAG-Cld5. MDCK-C7 and Caco-2 cells were stably transfected

with pFLAG-CLD5 by employing the Lipofectamine plus approach (Gibco BRL). G418-resistant cell clones were screened for claudin-5 synthesis by Western blot analysis. MDCK-C7 and Caco-2 cells transfected with an empty vector (pFLAG-CMV-4) served as controls. Synthesis rates of 60% were regarded as sufficient for further studies.

Western blot analysis Western blot analysis was performed as reported recently (Amasheh et al. 2002). Cells were scraped from the permeable supports and homogenized in TRIS-buffer containing 20 mM TRIS, 5 mM MgCl₂, 1 mM EDTA, 0.3 mM EGTA, and protease inhibitors (Complete; Boehringer, Mannheim, Germany). Protein was obtained by three freeze-thaw cycles and subsequent passage through a 26 G 1/2 needle. The membrane fraction was obtained by a 5-min centrifugation at 200g (4°C) and a subsequent 30-min centrifugation of the resulting supernatant at 43,000g (4°C). Pellets were resuspended in TRIS-buffer, and the protein content was determined by employing BCA protein assay reagents (Pierce, Rockford, Ill., USA) and quantification with a plate reader (Tecan, Austria). Aliquots of 2.5 µg protein were mixed with SDS buffer (Laemmli), loaded onto a 12.5% SDS polyacrylamide gel, and electrophoresed. Proteins were detected by immunoblotting with antibodies raised against human occludin, claudin-1, -2, -3, -5, and FLAG peptide. Chemiluminescence was induced with a Lumi-Light^{PLUS} Western blotting kit (Roche, Mannheim, Germany), detected with an LAS-1000 imaging system (Fuji, Tokyo, Japan), and analyzed by quantification software (AIDA, Raytest, Straubenhardt, Germany). Testing of antibody specificity of rb-anti-claudin-5 was performed through competition with custom-synthesized immunization peptide provided by the company that produced the antibody (Zymed Laboratories, San Francisco, Calif., USA). Antibody (5 µg) was precipitated with 50× excess of immunization peptide prior to the standard immunoblotting procedure. Bands that disappeared were regarded as specific, whereas the remaining bands were regarded as the result of unspecific binding.

Immunofluorescence analysis Immunofluorescence studies analysis and photography were performed as reported by Florian et al. (2003). Briefly, cells were grown on coverslips (18×18 mm; Menzel, Braunschweig, Germany). Confluent monolayers were rinsed with phosphate-buffered saline (PBS), fixed with methanol, and permeabilized with PBS containing 0.5% Triton X-100. The concentrations of the primary antibody were 20 µg/ml. Fluorescence images were obtained with a confocal microscope (Zeiss LSM 510 META) at excitation wavelengths of 543 and 488 nm. Details concerning the microscopical set-up are available upon request.

Flux measurements Unidirectional tracer flux measurements were performed under short-circuit conditions with 25 kBq/ml [³H]-mannitol (Biotrend, Cologne, Germany) from the apical to the basolateral side as reported by Amasheh et al. (2002). The medium also contained non-

labeled mannitol (10 mM). Four 15-min flux periods were analyzed. A 100-µl sample was taken from the donor (apical) side upon initiation and completion, and 900 µl Ringer's solution and 4 ml Ultima Gold high flash-point liquid scintillation cocktail (Packard Bioscience, Groningen, The Netherlands) were added. Samples (1 ml) of the receiving (basolateral) side, replaced with fresh Ringer's, were mixed with 4 ml liquid scintillation cocktail. Sub-

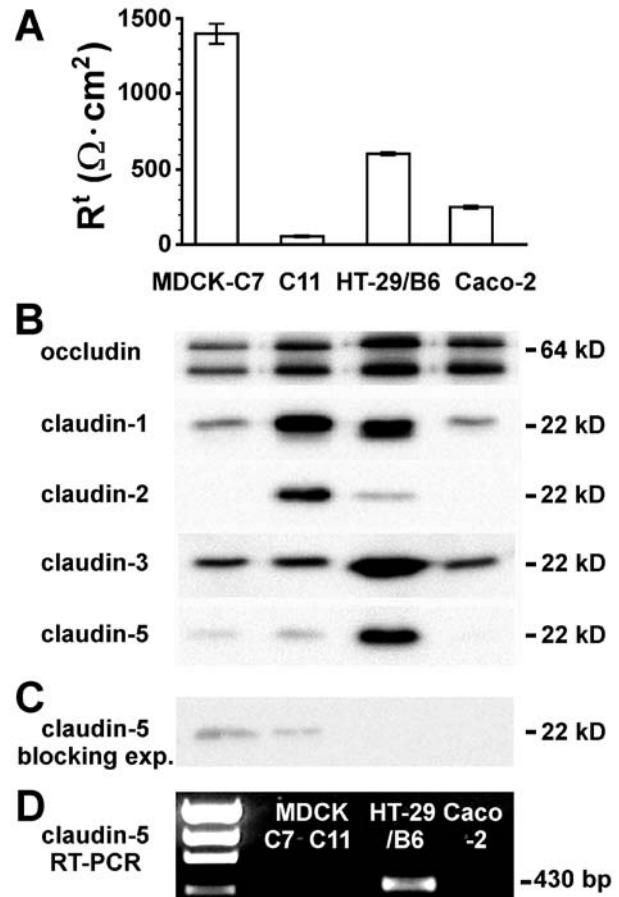


Fig. 1 Transepithelial resistance measurements and detection of tight junction proteins. **a** R^t of MDCK-C7, -C11, HT-29/B6, and Caco-2 cells revealed the highest values for MDCK-C7, representing a “tight” tight junction, and low values for MDCK-C11 and Caco-2 cells, representing relatively permeable tight junctions. **b** Membrane fractions of MDCK-C7, -C11, HT-29/B6, and Caco-2 revealed differential tight-junctional protein synthesis. Occludin and claudin-1 were detected in all cell types, whereas the pore-forming tight-junctional protein claudin-2 was not detectable in MDCK-C7 and Caco-2 cells. Claudin-5 showed no signals in Caco-2 cells and only marginal signals in MDCK-C7 cells. **c** Demonstration of antibody specificity through immuno-neutralization with the immunization peptide. After the blocking of a fraction of claudin-5 antibody with 50× excess of claudin-5 peptide supplied by the producer, the respective band of HT-29/B6 cells was not detectable, whereas MDCK signals remained, demonstrating unspecific binding. **d** Results of a PCR employing primers corresponding to both human and canine cDNA sequences, and the cDNA preparations of MDCK, HT-29/B6, and Caco-2 cells. The expected 430-bp fragment of the claudin-5 sequence could be detected in HT-29/B6 preparations, whereas no specific signals were detected in cDNA obtained from MDCK and Caco-2 cells (marker: low-DNA-mass ladder; Invitrogen, Carlsbad, Calif., USA)

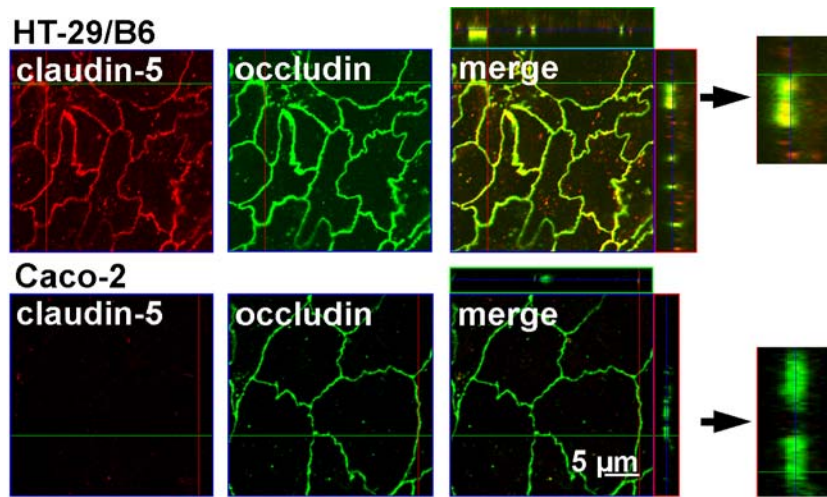


Fig. 2 Immunofluorescent staining of HT-29/B6 and Caco-2 cells. Confluent monolayers grown on coverslips were stained with a monoclonal antibody against occludin (green) and a polyclonal antibody against claudin-5 (red). Claudin-5 was not specifically detectable in tight junctions of Caco-2, whereas in HT-29/B6, a colocalization with occludin was detected. We computed an *xz*-projection at the

sequently, all 5-ml samples were analyzed in a Tri-Carb 2100TR Liquid Scintillation counter (Packard, Meriden, Conn., USA).

Chemicals All chemicals, unless otherwise noted, were purchased from Sigma Aldrich. Antibodies raised against claudin-1, -2, -3, -5, and occludin were purchased from Zymed Laboratories (San Francisco, Calif., USA). Secondary antibodies, viz., Alexa Fluor 488 goat anti-mouse and Alexa Fluor 594 goat anti-rabbit (both used at concentrations of 2 µg/ml), were purchased from Molecular Probes (MoBiTec, Göttingen, Germany).

Statistical analysis Data are expressed as means \pm standard error of the mean. Statistical analysis was performed by using Student's *t* test. $P < 0.05$ was considered significant.

Results

Presence of claudin-5 in epithelial cells with different genuine R^t Confluent monolayers of the epithelial cell line (MDCK) subtypes C7 and C11 and the human colon carcinoma cell lines Caco-2 and HT29/B6 revealed general differences of transepithelial resistance (R^t). Among the cells studied, MDCK-C7 showed the highest R^t ($1,396 \pm 67 \Omega \text{ cm}^2$, $n=12$), and MDCK-C11 revealed the lowest R^t value ($58 \pm 1.3 \Omega \text{ cm}^2$, $n=12$). Transepithelial resistances of HT-29/B6 and Caco-2 cells were 603 ± 6 and $251 \pm 3 \Omega \text{ cm}^2$, respectively ($n=15$ each, Fig. 1a).

To correlate functional and structural parameters, the presence of tight junction proteins occludin, and claudin-1, -2, -3, and -5 was analyzed as shown in Fig. 1b. Occludin, claudin-1, and claudin-3 were detectable in all cells, whereas the pore-forming protein claudin-2 was not detectable in MDCK-C7 and Caco-2 cells. Claudin-5 was not specifically detected in Caco-2. An antigen-antibody competi-

tion experiment employing 50 \times excess of blocking peptide is shown in Fig. 1c; the strong signal of HT-29/B6 was not detectable after blockage of the antibody with immunization peptide and thus was specific. In contrast, signals of MDCK-C7 and -C11, albeit weak, were still detectable after immuno-neutralization of the antibody, demonstrating unspecificity. In addition, a 430-bp fragment of the claudin-5 sequence could be detected by RT-PCR from HT-29/B6 preparations, whereas no specific signals were detected in MDCK and Caco-2 preparations (Fig. 1d).

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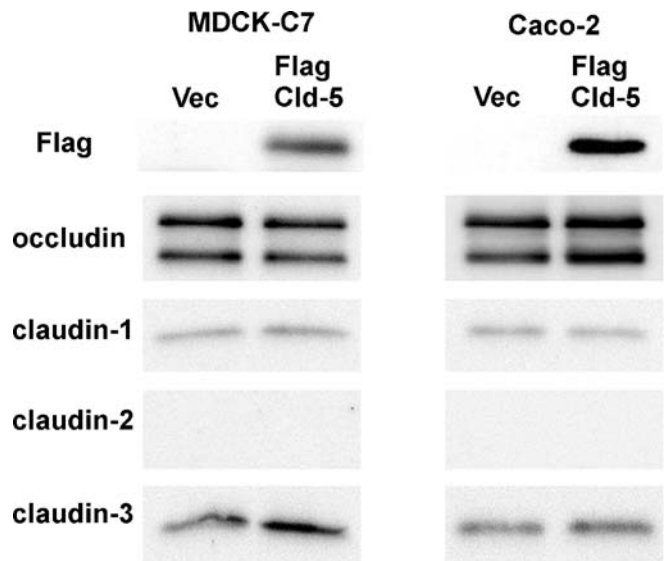


Fig. 3 Western blots of transfected cells after stable transfection; the synthesis of FLAG-claudin-5 (*FLAG Cld5*) was detected by Western blotting in both MDCK-C7 and Caco-2 cells. Clones with the strongest *FLAG Cld5* signals were selected for further comparative analyses (C7-FLAG-Cld5 and Caco-2-FLAG-Cld5, respectively). Controls were transfected with vector cDNA alone (*Vec*). No change of endogenous occludin, claudin-1, -2, and -3 was detectable

The localization of endogenous claudin-5 was determined in immunofluorescence studies in Caco-2 and HT-29/B6 cells by employing laser scanning confocal microscopy (Fig. 2). Z-scans revealed tight-junctional presence in colocalization with occludin in HT-29/B6, whereas immunostainings revealed no specific claudin-5 signals in the tight junction of Caco-2 cells.

Stable transfection of MDCK-C7 and Caco-2 with FLAG-claudin-5 cDNA In order to determine the contribution of claudin-5 to paracellular permeability, stable transfection of MDCK-C7 and Caco-2 with claudin-5 cDNA was performed. For specific determination of elevated claudin-5 synthesis, the 5' terminal region of the claudin-5 open reading frame was linked to a DNA sequence encoding the FLAG epitope, resulting in a protein with a N-terminal FLAG peptide sequence.

Clones were screened for successful transfection by Western blotting with the anti-FLAG antibody. FLAG-specific signals were detected in stably transfected clones, whereas controls did not show FLAG-specific signals. Clones of MDCK-C7 and Caco-2 that showed the highest levels of FLAG-claudin-5 synthesis were selected for further characterization (C7-FLAG-Cld5 and Caco-2-FLAG-Cld5). In addition, levels of the endogenous claudins were

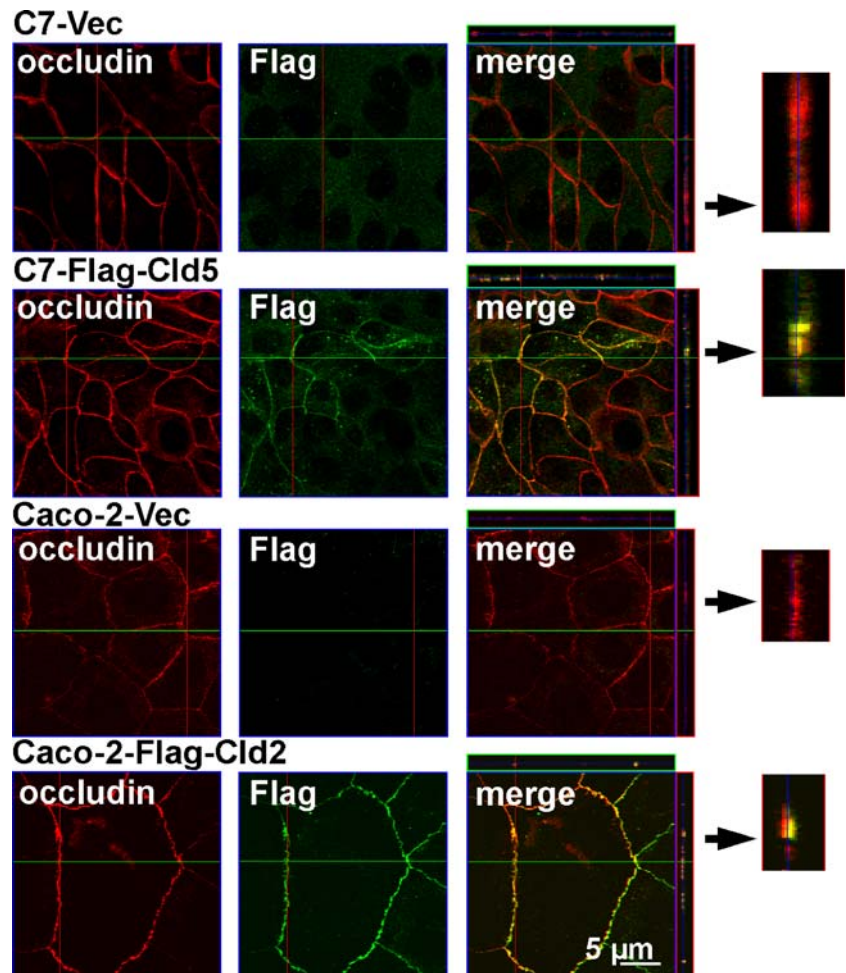
analyzed after transfection. No change of the presence of claudin-1, -2, -3, and occludin was detectable in stably transfected cells (Fig. 3).

Immunolocalization of FLAG-Cld5 Confocal microscopy revealed the strongly focused presence of FLAG-claudin-5 in the tight junctions of the majority of both C7-FLAG-Cld5 and Caco-2-FLAG-Cld5 cells grown as monolayers. FLAG-claudin-5 was colocalized with occludin and remained stable during the first passages (Fig. 4).

Electrophysiology and flux measurements Functional analysis of the clones was performed by measurements of transepithelial resistance (R^t) and [^3H]-mannitol flux analyses. No change of R^t was detected in transfected MDCK cells ($1,426.7 \pm 90.1 \Omega \text{ cm}^2$ for C7-Vec; $1,208.1 \pm 95.4 \Omega \text{ cm}^2$ for C7-FLAG-Cld5; $n=15$ and 14 measurements, respectively), whereas a marked increase of resistance was detectable in transfected Caco-2 cells ($242.1 \pm 8.4 \Omega \text{ cm}^2$ for Caco-2-Vec; $457.7 \pm 18.1 \Omega \text{ cm}^2$ for Caco-2-FLAG-Cld5); $n=18$ measurements each, $P < 0.001$; Fig. 5a).

Flux measurements employing [^3H]-mannitol (182.2 Da) did not differ in MDCK C7 clones with or without FLAG-Cld5 ($29.3 \pm 1.8 \text{ nmol h}^{-1} \text{ cm}^{-2}$ for C7-Vec vs. $28.0 \pm 1.8 \text{ nmol h}^{-1} \text{ cm}^{-2}$ for C7-FLAG-Cld5; $n=9$, respectively),

Fig. 4 Immunofluorescent staining with anti-occludin and anti-FLAG antibodies. Immunofluorescent staining with polyclonal or monoclonal antibodies against the *occludin* (red) and the *FLAG* epitope of FLAG-Cld5 (green) were detected in the tight junction region of both MDCK-C7-FLAG-Cld5 (C7-FLAG-Cld5) and Caco-2-FLAG-Cld5 (Caco-2-FLAG-Cld2) cells, whereas in mock-transfected cells (C7-Vec, Caco-2-Vec), only occludin was detectable. *Extreme right* Higher magnification of single strand regions



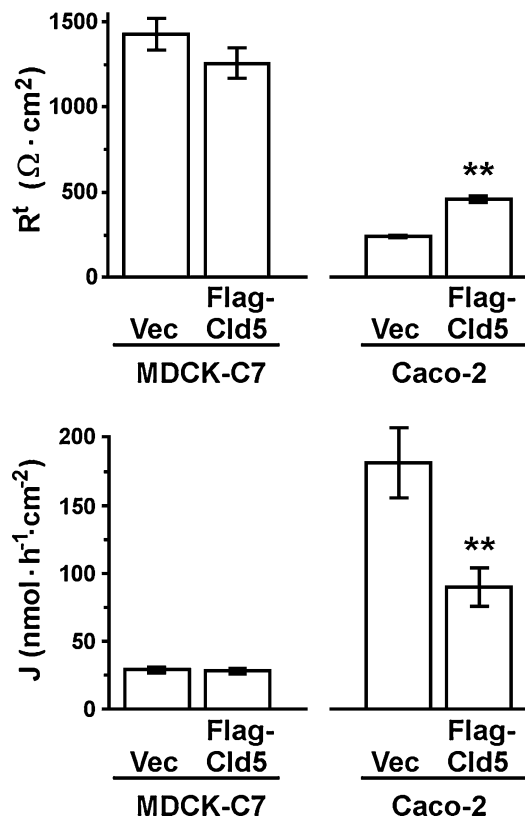


Fig. 5 a R^t and b $[^3\text{H}]$ -mannitol flux measurements of transfected cells. Transfection of the tight epithelial cell clone MDCK-C7 revealed no significant alterations of transepithelial resistance or $[^3\text{H}]$ -mannitol fluxes. In contrast, Caco-2 cells exhibited a marked increase of R^t and a decrease of mannitol fluxes after stable transfection with claudin-2-cDNA (Caco-FLAG-Cld5)

whereas Caco-2 cells stably transfected with claudin-5 cDNA showed a decreased permeability for mannitol ($181.0 \pm 25.8 \text{ nmol h}^{-1} \text{ cm}^{-2}$ for Caco-2-Vec; $89.8 \pm 14.4 \text{ nmol h}^{-1} \text{ cm}^{-2}$ for Caco-2-FLAG-Cld5; $n=9$ and 7 , respectively $P < 0.001$; Fig. 5b).

In an additional set of experiments, MDCK-C11 cells exhibiting low genuine resistance, because of the predominant presence of claudin-2, were stably transfected with claudin-5 cDNA. This led to no alterations of transepithelial resistance (not shown).

Discussion

A number of hereditary diseases have been identified as being linked to mutations of the genomic claudin-5 sequence. Expression of the claudin-5 gene is changed in glioblastoma multiforme (Liebner et al. 2000) and deletions within the genomic claudin-5 sequence have been associated with two related autosomal dominant genetic disorders, viz., velo-cardio-facial syndrome (VCFS) and DiGeorge syndrome (DGS). VCFS is characterized by craniofacial anomalies, learning disabilities, and conotruncal cardiac abnormalities (Shprintzen et al. 1978, 1981; Lipson et al. 1991; Wilson et al. 1992). The penetrance and

expressivity of the phenotypes are highly variable, and more than 40 additional phenotypes have been associated with the syndrome, including skeletal muscle hypotonia, inguinal hernia, thrombocytopenia, mental retardation, psychiatric illness, and hypospadias (Goldberg et al. 1997). These findings demonstrate the physiological importance of claudin-5.

Tight-junctional proteins in epithelial cell lines of renal and intestinal origin

We have studied the presence of claudins in the human intestinal cell lines Caco-2 and HT-29/B6 and in MDCK subclones that bear properties of collecting duct cells. The MDCK-C7 clone has “tight” tight junctions, whereas the MDCK-C11 clone exhibits “leaky” tight junctions (Gitter et al. 1997). Occludin has been used as a reference for the location of tight junction strands, with the immunostaining of the claudins being compared with occludin detection.

Like occludin, claudin-1 and claudin-3 have been detected in all the cell types examined here. These claudins appear to belong to the basic pattern of epithelial tight junction proteins, as both are found in combination in many organs, such as liver, lung, kidney, colon (Morita et al. 1999a,b), and skin (Tebbe et al. 2002). Whereas claudin-1 is known to be crucial for the “sealing” of the barrier (Furuse et al. 2002), claudin-3 appears not to have a direct effect on tight junction barrier properties (Furuse et al. 2001) or may be easily replaced.

Claudin-2 forms a cation-selective paracellular pore that is not present in the tight barrier of MDCK-C7 cells (Amasheh et al. 2002). As this study demonstrates, it is, however, also absent in Caco-2 cells, which means that the lack of claudin-2 does not necessarily lead to the tight sealing of the tight junction; an adequate presence of “tightening” proteins is thus necessary to obtain a definitive seal.

Presence of claudin-5 in epithelial cells

Morita et al. (1999a,b) have detected claudin-5 in endothelial cells in some segments of blood vessels, but not in epithelial cells of the native mouse intestine. By contrast, Rahner et al. (2001) have found claudin-5 in the epithelial tight junctions of stomach (surface and gastric glands) and throughout the intestine without gradations along the crypt-to-villus surface axis. Caco-2 cells, although of colonic origin, represent a model for the functions of small intestinal epithelium (Hidalgo et al. 1989). However, claudin-5 is absent in Caco-2 cells, whereas it is present in the native intestine (Rahner et al. 2001). We have nevertheless demonstrated that claudin-5 is present in HT-29/B6 cells, which originate from human colon (Kreusel et al. 1991) and exhibit features of differentiated epithelial crypt cells (Gitter et al. 2000).

Claudin-5 has not been detected in native epithelial cells of the kidney, despite its presence in kidney endothelia

(Kiuchi-Saishin et al. 2002; Morita et al. 1999a,b; Reyes et al. 2002). In our study, endogenous claudin-5 signals in MDCK cells seem not to be specific. Although synthesis in low-resistance MDCK cells leads to an increase of barrier properties (Wen et al. 2004), this effect is not universal. We have found, in our experiments, that claudin-5 synthesis in moderately high resistance Caco-2 cells (which lack claudin-2) increases their resistance, but that claudin-5 does not induce further “sealing” of the paracellular barrier in high resistance MDCK cells (subclone C7).

As tight junction strands can be regarded as a heteropolymer of various tight-junctional proteins that contribute different properties to the characteristics of the tight junction, the displacement of a critical number of stronger “tightening” tight junction proteins might lead to a decrease in the effectiveness of the paracellular barrier; the insertion of a “tightening” or “sealing” tight-junctional protein may reverse this effect. Whether these effects can be achieved in MDCK-C7 cells by higher levels of claudin-5 synthesis remains to be elucidated.

Thus, Caco-2 cells seem to be a suitable model for the characterization of claudin-5 by the transfection approach because of the lack of both claudin-2 and -5 and its low genuine transepithelial resistance.

Role of claudin-5 in epithelial tight junctions

This study has shown that claudin-5 is able to tighten the barrier to a certain extent. In MDCK-C7 cells, which contain genuine “tight” tight junctions, however, no contribution of claudin-5 to barrier properties has been detected.

Furthermore, if claudins are able to interact in a heterophilic manner in tight junction strands between cells, certain binding partners may be able to compensate for the elevation of a single molecule. This principle has been described recently for the combination of claudin-2 and claudin-8 (Yu et al. 2003), supporting a model whereby tissue-specific binding partners may play an important role for the final determination of paracellular permeability. Thus, the stable transfection of cell lines may lead to different, sometimes divergent, results depending on the genuine presence of potential binding partners. In our study, the presence of several other tight-junctional proteins is unchanged after stable transfection. Additional effects on any of the remaining tight-junctional proteins, however, cannot completely be ruled out.

The “sealing” capacity of claudin-5 has also been suggested by Nitta et al. (2003) in studies focusing on blood-brain barrier endothelia. In their study, the knockout of claudin-5 leads to a dramatic size-selective degradation of the blood-brain barrier function; the remaining barrier is permeable to molecules smaller than 800 Da. Moreover, claudin-5-deficient mice die within 10 h after birth. In accordance with these findings, our study shows that the permeability to mannitol is reduced by the synthesis of claudin-5 in Caco-2 cells. We have focused on permeability measurements of mannitol, as potential changes in

ionic permeability may indicate the displacement of other claudins; no such changes have however been observed.

In the knockout experiments, lethal effects resulting from the perturbation of the epithelial barrier have not been ruled out (Nitta et al. 2003; Matter and Balda 2003). These results raise the question of whether claudin-5 directly exhibits barrier properties in epithelia. Our study demonstrates that claudin-5 is present in cells derived from colon epithelia. In addition, in the tight junction of Caco-2 cells, exogenous claudin-5 can be classified as a “sealing” tight junction protein as the presence of this protein induces a “tightening” of the paracellular barrier, whereas the synthesis of claudin-5 in cells with high endogenous transepithelial resistance does not add to barrier properties.

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