

Participation of the second extracellular loop of claudin-5 in paracellular tightening against ions, small and large molecules

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Abstract Tight junctions control paracellular permeability. Here, we analyzed the impact of residues in the second extracellular loop (ECL2) of mouse claudin-5 on paracellular permeability. Stable expression of claudin-5_{wild type} in MDCK-II cells—but not that of mutants R145A, Y148A, Y158A or E159Q—increased transepithelial electrical resistance and decreased fluorescein permeation. Expression of claudin-5_{Y148A}, _{Y158A} or _{E159Q} enhanced permeability of FITC-dextran_{10 kDa}, which was unchanged in cells expressing claudin-5_{wild type} or claudin-5_{R145A}. In contrast, targeting to tight junctions, strand morphology and tight junction assembly were unchanged. It is concluded that R145 is unessential for *trans*-interaction of claudin-5, but necessary for tightening against small solutes and ions. The highly conserved residues Y148, Y158 and E159 in ECL2 of claudin-5 contribute to homo- and/or heterophilic *trans*-interaction between classic claudins and thereby tighten the paracellular space against ions, small and large molecules. These results provide novel insights into the molecular function of tight junctions.

Keywords Claudin · Tight junction · Paracellular permeability · Transmembrane protein · Protein–protein interaction

Abbreviations

Cld	Claudin
Da	Dalton
DMEM	Dulbecco's modified Eagle's medium
ECL	Extracellular loop
FITC	Fluorescein isothiocyanate
HBSS	Hank's buffered salt solution
HRP	Horseradish peroxidase
MDCK	Madin-Darby canine kidney
PBS	Phosphate buffered saline
P _{fluorescein}	Permeability coefficient of fluorescein
P _{FD10}	Permeability coefficient of 10 kDa FITC-dextran
SEM	Standard error of the mean
TEER	Transepithelial electrical resistance
TJ	Tight junctions

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Introduction

Tight junctions (TJ) are formed between endothelial or epithelial cells. They limit and regulate the paracellular permeation of small ions, solutes and proteins, thus maintaining the homeostasis of different tissue compartments. TJs are mostly located on the apical side of the lateral plasma membrane [1]. They are composed of transmembrane proteins of two opposing cells. Using transmission electron microscopy, TJs appear as fusions of the membranes of two neighboring cells. In freeze-fracture electron microscopic analyses, TJs appear as a continuous network of strands of transmembrane proteins [2].

Claudins, a protein family of about 24 members (subtypes), form the structural backbone of TJ strands. They are able to build up TJ strands under physiological conditions [3]. The various claudin subtypes differ in their permeability properties with respect to the size and charge of small ions or molecules and exhibit tissue-specific expression patterns [4]. For instance, claudin-5 is responsible for tightening the blood-brain barrier against small molecules up to 800 Da [5]. The combination of claudins expressed in a given tissue determines the characteristics of its paracellular tightness [6–8]. The influence of the first extracellular loop (ECL1) of claudins on the paracellular permeability has been studied [4]. For example, Asp65 in the ECL1 has been recently identified as mediating the charge selectivity of the paracellular cation pores that are formed by mouse claudin-2 [9]. For claudin-5, the involvement of the ECL1 in paracellular tightening was also investigated. The exogenous expression of claudin-5 in Madin-Darby canine kidney (MDCK)-II cells expressing other claudin subtypes but not claudin-5 increased the transepithelial electrical resistance (TEER). Alanine substitutions of either of the two conserved cysteine residues in the ECL1 abolished this increase [10].

The impact of the amino acid residues of the second extracellular loop (ECL2) on paracellular sealing has not yet been studied for Cld5 and hardly studied for any other classic claudin [4]. In previous experiments, we investigated the effect of single amino acid substitutions in the ECL2 of Cld5 on homophilic Cld5 interactions in TJ-free cells [11]. Some mutants (for example R145A) showed wild-type-like enrichment of Cld5 in the plasma membrane at contact sites between transfected cells indicating *trans*-interaction. Other mutants (for example, Y148A, Y158A or E159Q) did not show any enrichment at contact sites, but exhibited homogenous Cld5 distribution in the plasma membrane, indicating block of *trans*-interaction. The aim of this study was to investigate the impact of the amino acids R145, Y148, Y158 and E159 in the ECL2 of Cld5 on the TJ integrity after stable expression in MDCK-II cells. Only the expression of Cld5_{wt}—but not the expression of any of the Cld5 mutants—increased the paracellular tightness for ions and small solutes. In addition, some mutants disturbed the sealant against large molecules, too. The results demonstrate that the ECL2 is essential for the barrier function of Cld5.

Materials and methods

Antibodies and cell culture

Rabbit anti-Cld1, rabbit anti-occludin, mouse anti-Cld5, mouse anti-GAPDH, goat horseradish peroxidase (HRP)-conjugated anti-rabbit, goat HRP-conjugated anti-mouse,

goat Cy3-conjugated anti-mouse and goat 488-conjugated anti-rabbit (Invitrogen, Carlsbad, CA) were used for immunocytochemistry.

Madin-Darby canine kidney (MDCK)-II cells [12] were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U penicillin and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA).

Mammalian expression vectors, site-directed mutagenesis and transfection

Vectors for the expression of N-terminally FLAG-tagged mouse Cld5 were based on pEYFP-N1 (Clontech, Palo Alto, CA). FLAG-Cld5-YFP was generated by amplification of Cld5 using pGTCL-5 (provided by Dr. M. Furuse, Kobe) as template and primers, one encoding the N-terminal FLAG epitope. To generate FLAG-Cld5 without a YFP-tag, site-directed mutagenesis was performed as described in [11] with the introduction of a stop codon 3' behind the Cld5 open reading frame. The amino acid substitutions R145A, Y148A, Y158A and E159Q were again introduced by site-directed mutagenesis [11]. The amino acid sequence of Cld5-ECL2 wt and mutants is depicted in Fig. 1a. Transfections of MDCK-II cells with FLAG but not YFP tagged Cld5 were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) or Cell Line Nucleofection® Kit L (Amaxa, Cologne, Germany) according to the recommendations of the supplier. Stable lines were selected by adding 1 mg/ml G418 (Calbiochem, San Diego, CA). To exclude clonal variations, three independent clones with comparable Cld5 expression were initially analyzed, and two of them were used for quantifications for each Cld5 construct.

Immunoblotting

Cell lysis was performed in 50 mM Tris/HCL, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and complete protease inhibitor cocktail (Roche, Mannheim, Germany). SDS-PAGE and immunoblotting were performed as previously described [13].

Immunocytochemistry

For immunocytochemistry, cells were washed with phosphate-buffered saline (PBS), fixed with acetone (5 min, 4°C), incubated with ethanol and PBS (1 min, 4°C, each), soaked in blocking solution (BS: 1% bovine serum albumin and 0.05% Tween 20 in PBS) for 10 min and then incubated with the first primary antibody in BS for 1 h. After washing (5 × 2 min in BS), samples were incubated with

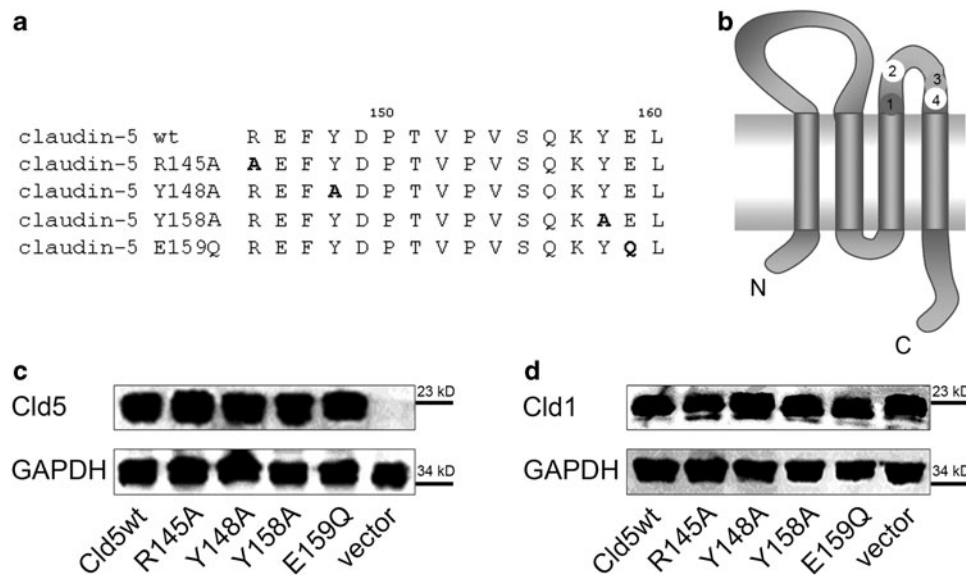


Fig. 1 Sequence and exogenous expression of claudin-5 (Cld5) constructs in stably transfected MDCK-II cells. **a** Predicted (swiss-prot) amino acid sequence of the second extracellular loop of mouse Cld5 wild type (wt) and its mutants. Amino acids substituted by site-directed mutagenesis are indicated in bold. **b** Schematic overview of Cld5 and the positions of the amino acid substitutions in the Cld5 mutants transfected. **c** Expression of the different Cld5 proteins in

stably transfected MDCK-II cell lines. Cld5 was not detected in cells transfected with the vector control. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as loading control. **d** The expression of the exogenous Cld5 proteins did not influence the expression of endogenous claudin-1 (Cld1). For C and D, Cld5 and Cld1 were detected by Western blot of cell lysates

the second primary antibody in BS for 1 h, washed (5×2 min in BS), incubated with secondary antibodies for 30 min in BS, and washed (5×2 min in BS) and mounted. Cells were examined using an LSM 510 confocal microscope (Zeiss, Jena, Germany) [11].

Measurement of transepithelial electrical resistance (TEER) and permeation coefficients

Stably transfected MDCK-II cells were seeded at a density of 1×10^5 into 24-well Millipore transwell filters (Schwalbach, Germany). TEER was determined using Endohm electrodes (Millipore, Eschborn, Germany). After stable TEER values had been reached, permeation studies were performed using 40 μ g/ml fluorescein (Sigma-Aldrich, Taufkirchen, Germany) or 25 μ g/ml 10 kDa fluorescein isothiocyanate dextran (FITC-dextran) in Hank's buffered salt solution (HBSS, Biochrom, Berlin, Germany) in the apical compartment and pure HBSS in the basal compartment. After 10 min incubation, 100- μ l aliquots were removed from the basal compartment, and the fluorescence intensity was measured using a fluorescence plate reader (Tecan, Crailsheim, Germany). Permeation coefficients were calculated as described [14]. Permeation studies with fluorescein have to be done with care since it could be transported by organic anion transporters. However, the

suitability of fluorescein as a paracellular tracer for MDCK-II cells has been demonstrated [15, 16]. In addition, the contribution of a potential fluorescein transport by monocarboxylate transporters is very unlikely for the measuring time used [17].

For the measurements of TEER and permeation of tracers, ≥ 9 filters of two different cell clones were analyzed for each construct.

Freeze-fracture electron microscopy

Transfected MDCK-II cells were grown until confluence, washed with PBS, fixed with 2.5% glutaraldehyde (electron microscopy grade; Sigma-Aldrich, Taufkirchen, Germany) in PBS for 2 h, washed and processed for freeze-fracture electron microscopy as reported [18].

Statistics

Data are mean $\pm$ standard error of the mean (SEM). The Kruskal-Wallis test was used to identify significant differences within experiments, and Dunn's multiple comparison post-test was used to assess the significance of differences between several experimental groups with $p < 0.05$ considered as significant. The computer package GraphPad Prism version 3.0 (San Diego, CA) was employed.

Results

The amino acid substitutions R145A, Y148A, Y158A and E159A in the second extracellular loop of claudin-5 do not alter its targeting to tight junctions

To investigate the impact of the amino acids, residues R145 (1), Y148 (2), Y158 (3) and E159 (4) of Cld5 on the paracellular permeability of epithelial cells expressing endogenous claudins, both Cld5_{wt} and the respective mutants of Cld5 (Fig. 1a and b), were stably transfected into low-resistance MDCK-II cells. All exogenous Cld5 proteins were clearly expressed in the cells, while no Cld5 was detected in MDCK-II cells transfected with the vector control (Figs. 1c, 2, 3). As it has already been demonstrated elsewhere that the expression of exogenous Cld5 does not influence the expression of other endogenous claudins [10, 19], the present analysis was restricted to demonstrating that the expression of endogenous Cld1 did not differ between MDCK-II cells transfected with exogenous Cld5_{wt} or Cld5 mutants (Figs. 1d, 3). Cld5 and mutants were expressed in >95% of the cells used (Figs. 2 and 3). To analyze whether the substitutions affect targeting of Cld5 to TJs, double immunostaining was performed against Cld5 and occludin (Fig. 2), as well as Cld5 and Cld1 (Fig. 3). Cld5_{wt} and all mutants colocalized with both TJ marker proteins, indicating that the substitutions did not change the proper TJ localization of Cld5. In addition, for all constructs similar amounts of Cld5 were detected at the cell-cell contacts (Figs. 2 and 3). These data allowed comparison between Cld5 and its mutants.

Amino acid substitutions in the second extracellular loop of claudin-5 affected tight-junction function

The expression of Cld5_{wt} in MDCK-II cells caused a significant, five-fold increase in TEER ($507 \pm 42 \Omega \text{ cm}^2$) relative to the vector control ($105 \pm 3.6 \Omega \text{ cm}^2$). In contrast, expression of any of the Cld5 mutants failed to alter the TEER significantly compared with vector control (Cld5_{R145A}: $152 \pm 10 \Omega \text{ cm}^2$, Cld5_{Y148A}: $107 \pm 5.2 \Omega \text{ cm}^2$, Cld5_{Y158A}: $80 \pm 7 \Omega \text{ cm}^2$, and Cld5_{E159Q}: $152 \pm 11.9 \Omega \text{ cm}^2$). These results indicated that all amino acid residues of the ECL2 of Cld5 analyzed are essential for the sealing of the paracellular cleft of MDCK-II cells against ions (Fig. 4a).

To study whether these amino acid residues of the ECL2 of Cld5 were also involved in the sealing of the paracellular cleft against small molecules, the permeability coefficient of fluorescein ($P_{\text{fluorescein}}$) was determined for the respective stably transfected MDCK-II cell lines. In cells expressing Cld5_{wt}, the $P_{\text{fluorescein}}$ ($1.5 \times 10^{-6} \pm 0.1 \times 10^{-6} \text{ cm/s}$) was significantly lower than the vector control by a factor of 2

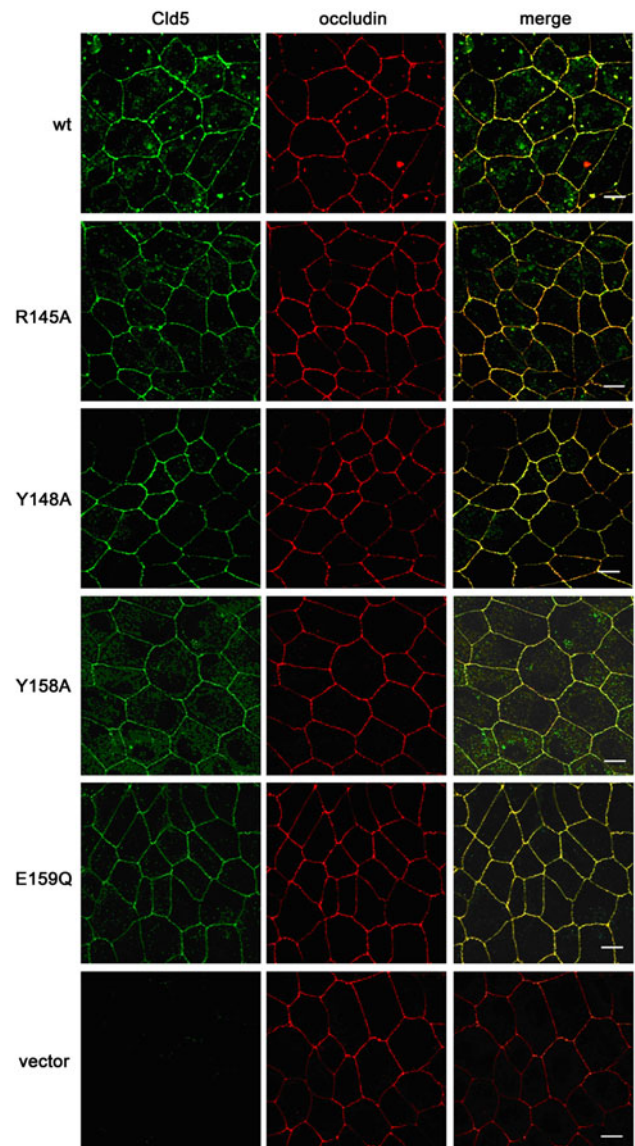


Fig. 2 Colocalization of exogenously expressed Cld5_{wt}, Cld5_{R145A}, Cld5_{Y148A}, Cld5_{Y158A} and Cld5_{E159Q} with the general tight junction marker occludin at cell-cell contacts. The subcellular localization of Cld5 (green) and occludin (red) in stably transfected MDCK-II cells was studied by immunostaining followed by confocal microscopy; bars 10 μm

($3.4 \times 10^{-6} \pm 0.2 \times 10^{-6} \text{ cm/s}$). In contrast, expression of Cld5_{R145A} ($9.3 \times 10^{-6} \pm 0.6 \times 10^{-6} \text{ cm/s}$), Cld5_{Y148A} ($6.8 \times 10^{-6} \pm 0.4 \times 10^{-6} \text{ cm/s}$), Cld5_{Y158A} ($6.3 \times 10^{-6} \pm 0.4 \times 10^{-6} \text{ cm/s}$) and Cld5_{E159Q} ($7.2 \times 10^{-6} \pm 0.4 \times 10^{-6} \text{ cm/s}$) resulted in two- to three-fold increases in the paracellular permeability of MDCK-II cells compared with the control (Fig. 4b). Taken together, the expression of Cld5_{wt} caused sealing of the paracellular cleft against small molecules, whereas the expression of all Cld5 mutants decreased the tightness against small molecules. Hence, the residues in the ECL2 of Cld5 analyzed are critically involved in this tightening.

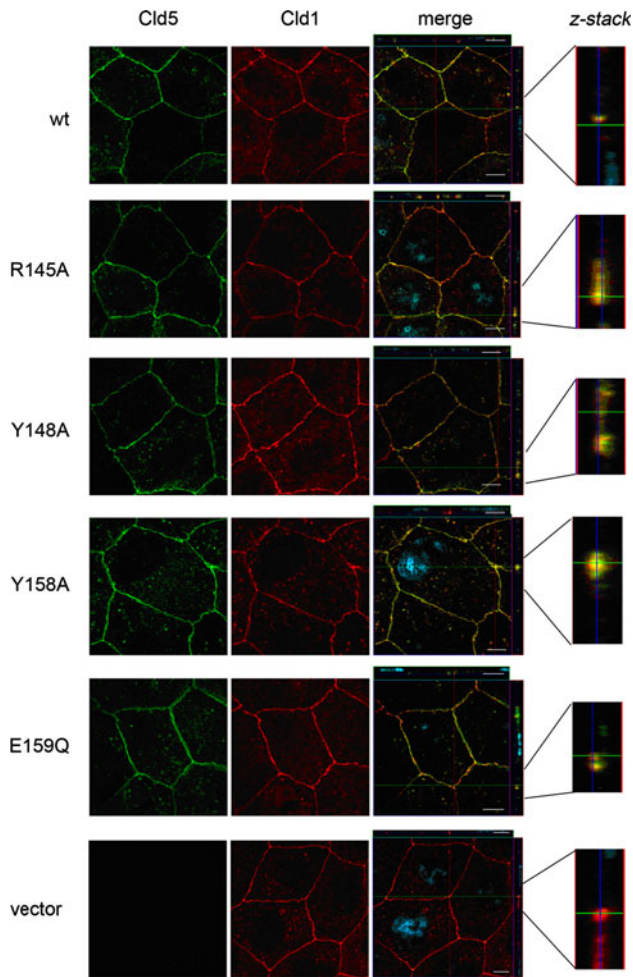


Fig. 3 Exogenously expressed Cld5_{wt}, Cld5_{R145A}, Cld5_{Y148A}, Cld5_{Y158A} and Cld5_{E159Q} showed strong colocalization with claudin-1 (Cld1) in the apical part of the lateral plasma membrane. The subcellular localization of Cld5 (green) and Cld1 (red) in stably transfected MDCK-II cells was studied by immunostaining followed by confocal microscopy. XY-Images are shown for Cld5 and Cld1 (large squares). In addition to the XY-images, for the merge, XZ-images are shown on top (flat rectangle), YZ-images on the right and a 5× magnification of the latter in the insert (z-stack). Blue, DAPI staining of the nuclei; bars 10 μm

In addition, it was studied whether the residues in the ECL2 of Cld5 are involved in sealing the paracellular cleft against larger molecules, too. The permeability coefficient of 10 kDa FITC-dextran (P_{FD10}) did not exhibit significant differences between cells expressing Cld5_{wt} ($2.6 \times 10^{-7} \pm 0.3 \times 10^{-7}$ cm/s), Cld5_{R145A} ($4.5 \times 10^{-7} \pm 0.3 \times 10^{-7}$ cm/s) or the vector control ($3.7 \times 10^{-7} \pm 0.2 \times 10^{-7}$ cm/s). However, expression of Cld5_{Y148A} ($7.3 \times 10^{-7} \pm 0.7 \times 10^{-7}$ cm/s), Cld5_{Y158A} ($9.0 \times 10^{-7} \pm 1.9 \times 10^{-7}$ cm/s), and Cld5_{E159Q} ($8.9 \times 10^{-7} \pm 1.1 \times 10^{-7}$ cm/s) increased the P_{FD10} significantly 2- to 2.4-fold (Fig. 4c). Thus, the expression of Cld5_{wt} and Cld5_{R145A} does not influence the paracellular permeability of MDCK-II cells

towards large molecules. In contrast, expression of Cld5_{Y148A}, Cld5_{Y158A} and Cld5_{E159Q} caused an increase in the paracellular permeation of large molecules compared with vector control.

Amino acid substitutions in the second extracellular loop of claudin-5 do not alter the tight-junction strand architecture

To study whether the increase in paracellular permeability with the expression of the Cld5 mutants was due to changes in TJ strand morphology, freeze-fracture electron microscopy was performed. The MDCK-II cells transfected with vector control showed the typical morphology and protoplasmic fracture face (P-face) association of the strands (Fig. 5). After transfection of Cld5_{wt}, Cld5_{R145A} or Cld5_{Y148A}, no differences were detectable in the morphology of the TJ strands formed by the different cells. The strand networks were still associated with the P-face and exhibited similar density and branching (Fig. 5). Nevertheless, morphology of individual samples within one experimental group may vary. These results indicate that the residues R145 and Y148 in the ECL2 of Cld5 are not required for maintaining the morphology of heteropolymeric TJ strands.

The amino acid substitution Y148A in the second extracellular loop of claudin-5 does not alter disassembly or reassembly of tight junctions

Then, we investigated whether the amino acid substitution Y148A in the ECL2 of Cld5 alters the disassembly or reassembly of TJs. The subcellular localization of Cld5 and Cld1 (serving as an endogenous TJ marker) was therefore studied with MDCK-II cells stably expressing Cld5_{wt} or Cld5_{Y148A}, using a calcium-switch assay. After Ca^{2+} removal in Cld5_{wt} as well as in Cld5_{Y148A} expressing cells, Cld1 and Cld5 were internalized with comparable kinetics and to a similar extent after Ca^{2+} removal (Supplementary Figure S1). Likewise, after Ca^{2+} addition, both in Cld5_{wt}- and Cld5_{Y148A}-expressing cells, Cld5 and Cld1 reappeared in the plasma membrane in a comparable manner and to a similar extent during TJ reassembly upon Ca^{2+} reintroduction (Supplementary Figure S1). These data indicate that Y148A does not affect the assembly of tight junctions.

Discussion

To obtain novel insights into the molecular mechanism of paracellular tightening by claudins, we have previously demonstrated that defined amino acid residues within the ECL2 of the transmembrane TJ protein Cld5 are essential

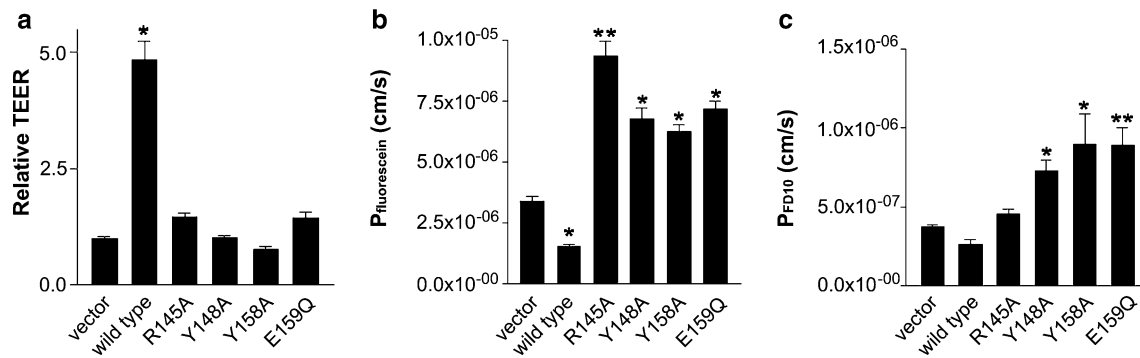
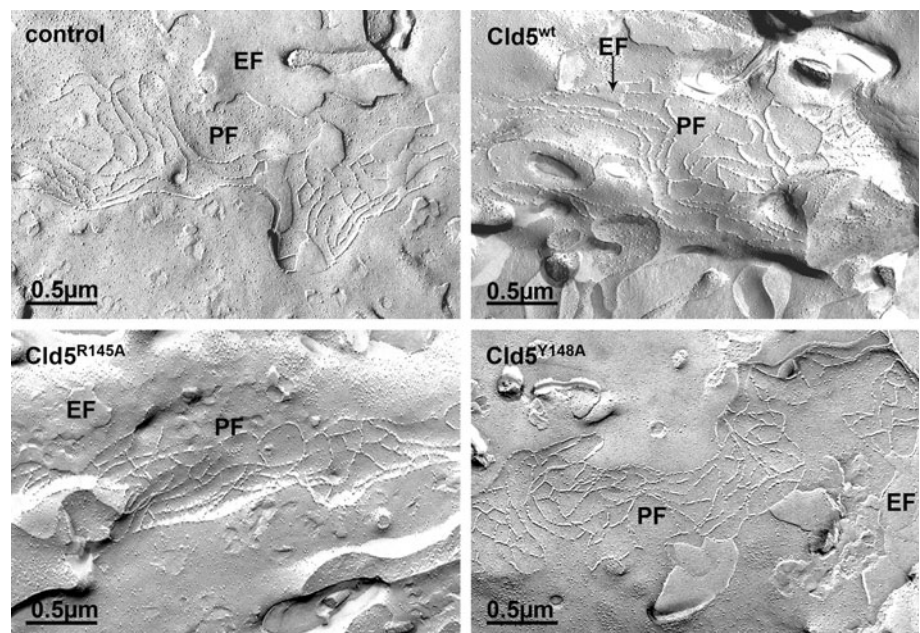


Fig. 4 Amino acid residues in the second extracellular loop of claudin-5 (Cld5) are necessary for tightening the paracellular cleft. **a** Cld5_{wt} increased the transepithelial electrical resistance (TEER) after stable transfection into claudin-5-free MDCK-II cells, whereas mutants with the amino acid substitutions R145A, Y148A, Y158A and E159Q in the second extracellular loop of Cld5 did not. **b** Cld5_{wt} reduced the permeability coefficient of fluorescein (P_{fluorescein}) after

stable transfection in MDCK-II cells, whereas mutants with the amino acid substitutions in the second extracellular loop of Cld5 exhibited higher P_{fluorescein} compared to the vector control. **c** The expression of Cld5_{Y148A}, Cld5_{Y158A} and Cld5_{E159Q} increased the permeability coefficient for 10 kDa FITC-dextran (P_{FD10}), whereas that of Cld5_{wt} and Cld5_{R145A} did not. Data are mean ± SEM, $n \geq 9$; * $p < 0.05$, ** $p < 0.01$ vs. vector control

Fig. 5 Amino acid substitutions in the second extracellular loop of claudin-5 (Cld5) did not alter the morphology of tight junction strands. MDCK-II cells, stably transfected with control vector, Cld5_{wt}, Cld5_{R145A} or Cld5_{Y148A} were fixed and processed for freeze-fracture electron microscopy. In all cases similar network-like intramembranous strands, associated with protoplasmic face (PF) but not with the exoplasmic face (EF), were found



for its homophilic *trans*-interaction between two opposing cells [11, 36]. However, it was not clear if these residues contribute to the paracellular tightening mediated by epi-/endothelial tight junctions consisting of several transmembrane and associated proteins. To address this issue in this study, we stably expressed mutants of claudin-5 in MDCK-II cells that contain endogenous claudins and form functional TJs [20, 21]. We demonstrated that residues within the ECL2 of claudin-5 are essential for paracellular tightening.

The effect of amino acid substitutions (human pathogenic as well as designed mutations) in claudins on paracellular permeability has been analyzed for the ECL1 of Cld2 [9], Cld4 and Cld15 [21], Cld5 [10], Cld7 [22] and

Cld16 [23]. These studies indicate that the ECL1 is mainly responsible for paracellular tightening, pore formation and charge selectivity. However, the contribution of the ECL2 has been neglected so far.

To obtain better mechanistic insights into the role of the ECL2 of Cld5 in paracellular barrier formation, we have previously analyzed the effect of systematic single amino acid substitutions in the ECL2 on plasma membrane targeting, homophilic *cis*- and *trans*-interaction as well as formation of tight junction strands [11]. By expressing mutants of claudin-5 in TJ- and claudin-free HEK cells, we identified residues in the ECL2 that are specifically involved in folding or in strand formation and in *trans*- but not *cis*-interaction. The single amino acid substitutions

F147A, Y148A, Q156A, Y158A and E159Q strongly inhibited *trans*-interaction and strand formation [11]. Due to the lack of polarity and additional TJ machinery, claudin-transfected HEK cells could not be used for the measurement of paracellular permeability. In contrast, MDCK-II cells are a well-established model to study the latter [10]. For this reason, stably transfected MDCK-II lines expressing Cld5_{wt}, Cld5_{R145A}, Cld5_{Y148A}, Cld5_{Y158A} and Cld5_{E159Q} were generated to analyze the impact of the ECL2 on paracellular sealing.

Exogenous claudin-5 improves the paracellular tightness of MDCK-II cells

The expression of Cld5_{wt} in MDCK-II cells caused a fivefold increase in TEER and halved the permeability coefficient for fluorescein (376 Da) compared to the control. In contrast, only a slight but not significant reduction was found for the permeability coefficient of 10 kDa-FITC-dextran. These results are consistent with earlier reports obtained in MDCK-II cells [10], Caco-2 cells [19], bEND3 brain endothelial cells [24] and knock-out mice [5]. This verified that Cld5 tightens the junctions against small ions and small molecules, but does not significantly enhance the barrier against large molecules that is formed by other endogenous TJ proteins [4].

The residues R145, Y148, Y158 and E159 in the ECL2 are not necessary for incorporation of claudin-5 into preexisting tight junctions and the polymerization of heteropolymeric claudin strands

In addition to MDCK-II lines that express Cld5_{wt}, lines that express the previously characterized ECL2 mutants Cld5_{R145A}, Cld5_{Y148A}, Cld5_{Y158A} and Cld5_{E159Q} [11] have been generated. In all cell clones analyzed, the Cld5 constructs were clearly expressed, as judged by Western blot and immunostaining. A comparable staining indicates similar amounts of Cld5 at TJs for all constructs. After expression of Cld5_{wt} or Cld5 mutants, no change in the expression of endogenous Cld1 was detected. This is consistent with earlier reports showing no change in expression of endogenous Clds, including Cld2, after Cld5 transfection of MDCK-II [10], MDCK-C7 or Caco-2 cells [19]. Similarly, overexpression of claudin-7 also does not affect expression and localization of endogenous Cld1, -3, -4 and -7 in LLC-PK1 cells [25]. On the other hand, replacement of endogenous claudin-2 in MDCK-II is possible by exogenous claudin-8 [26]. Hence, it is not a general phenomenon for all claudins that overexpression does not change expression of endogenous claudins.

In TJ-free HEK cells, Cld5_{wt} and Cld5_{R145A} but not Cld5_{Y148A}, Cld5_{Y158A} and Cld5_{E159Q} showed enrichment at

cell-cell contacts demonstrating a lack of *trans*-interaction for the latter [11]. In contrast, in our MDCK cells Cld5_{wt} and all mutants were strongly enriched at cell-cell contacts where they co-localized with occludin and claudin-1 in the apical part of the lateral plasma membrane as known for the TJ proteins in polarized epithelial cells. These data indicate that the residues R145, Y148, Y158 and E159 in the ECL2 of Cld5 are not involved in protein interactions that are necessary for incorporation of exogenous claudin-5 into endogenous tight junctions. This has been expected since the substitutions in Cld5-ECL2 may not affect binding to ZO-1 [27] and *cis*-interaction with endogenous claudins [11]. Furthermore, Ca²⁺ switch assays [13] indicated that none of the substitutions affected TJ dis- or reassembly. Finally, the expression of either Cld5_{wt}, Cld5_{R145A} or Cld5_{Y148A} (the latter as an example of those substitutions that inhibit *trans*-interaction, Y148A, Y158A and E159Q) did not change the morphology of the intramembranous TJ strands. Taken together, these data demonstrate that the analyzed substitutions in the ECL2 of Cld5 do not affect the formation of heteropolymeric strands consisting of endogenous claudins and exogenous Cld5.

Amino acid substitutions in the second extracellular loop affect the barrier properties of claudin-5

The effect of mutations in claudins on paracellular permeability has been analyzed in several studies [22, 28]. For example, it is well known that Cld2 forms paracellular cation pores and that the residue Asp65 (mouse Cld2) in the ECL1 is involved in pore formation and charge selectivity [9]. However, the molecular structure of this pore is not known. Compared to pore formation, even less is known about the mechanism that leads to the formation of tight paracellular barriers, e.g., by Cld1, Cld3, Cld4 and Cld5 [4]. It has been shown that C54, C64, L50 and Trp51 in the ECL1 of CLD5 are involved in paracellular barrier formation against small molecules [10]. However, distinct molecular interactions have not been addressed, and the ECL2 has been largely neglected. In our approach, we performed permeability measurements with MDCK-II cells transfected with claudin-5-ECL2 mutants of which the interaction properties had been characterized before by cellular reconstitution of TJ strands in TJ-free cells [11, 12]. Interestingly, each substitution in the ECL2 analyzed (R145A, Y148A, Y158A, E159Q) impeded the Cld5-induced increase in TEER. In addition, they showed a dominant negative effect on the permeability of small molecules, since $P_{\text{fluorescein}}$ was not reduced, but greatly increased, compared to the control. There is no evidence that transcellular transport of fluorescein contributes to the observed changes in $P_{\text{fluorescein}}$. In addition to other reports [15, 16], it is particularly unlikely that the point mutants

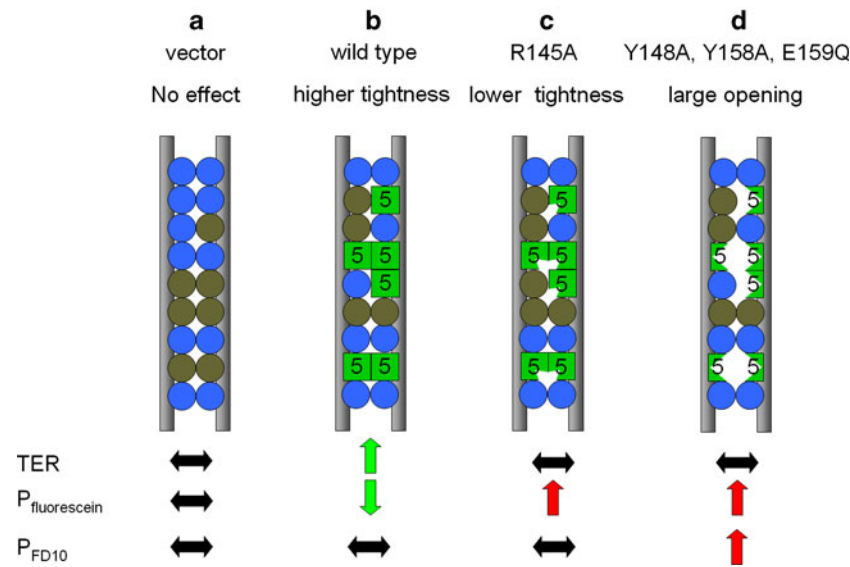


Fig. 6 Scheme of the claudin-5-based tightening of the paracellular cleft between two plasma membranes (gray). The additional expression of Cld5_{wild type} (green square) in MDCK-II cells that contain endogenous claudins (blue and olive circles) caused an increase in the transepithelial electrical resistance (TER) and a decrease in paracellular permeation of small molecules ($P_{\text{fluorescein}}$), without influencing the paracellular permeation of large molecules (P_{FD10}). Removal of the basic side chain R145 of Cld5 (indicated by the slot) does not block *trans*-interaction but diminishes the tightening against small ions (no increase in TER) and elevated the paracellular permeation of

small molecules (increase in $P_{\text{fluorescein}}$). However, Cld5_{R145A} had no effect on the paracellular permeation of large molecules (no change in P_{FD10}). This demonstrates that R145 is essential for the tightening function of Cld5 against small ions and small but not large molecules. Blocking the *trans*-interaction of Cld5 by the substitutions Y148A, Y158A or E159Q (indicated by the gaps) does not block the formation of heteropolymeric strands but results in clefts that reduce the tightening against small ions (increase in TER), small and even large molecules (increase in $P_{\text{fluorescein}}$ and P_{FD10})

with very specific effects on *trans*-interaction [11] should influence transcellular transport of fluorescein in the opposite way than Cld5_{wt}.

In contrast to the substitution R145A that does not strongly affect *trans*-interaction [11], the substitutions Y148A, Y158A and E159Q that inhibit *trans*-interaction also showed a dominant negative effect on the permeability of large molecules, since $P_{10 \text{ kDa FITC-dextran}}$ has been strongly increased compared to the control. These findings demonstrate that, in the ECL2 of Cld5, residues Y148, Y158 and E159 are necessary for tightening against ions as well as against small and large molecules. In contrast, R145 is involved in the tightening against ions and small molecules, only.

We previously demonstrated that the substitutions R145A, Y148A and E159Q, in contrast to others (e.g., K157A, V152A), do not influence, and Y158A only slightly influences, the plasma membrane targeting of Cld5 [11]. This indicates that these substitutions do not change the conformation of Cld5. In addition, R145A, Y148A and E159A did not affect the homophilic *cis*-interaction of Cld5. In contrast, Y148, Y158 and E159, but not R145, are involved in homophilic *trans*-interaction and thereby in the formation of homopolymeric Cld5 strands. Molecular modeling indicated that Y148 and Y158 together with F147A from two opposing Cld5 molecules form an

aromatic binding core triggering the *trans*-interaction [11]. According to this model, the residues R145, E159 and the earlier described Q156 point to additional interaction mechanism(s). However, the respective binding residues are not identified. Hence, they could interact with any loop in the neighbourhood, which could be an ECL2 or an ECL1 of another claudin molecule or the ECL1 of the same claudin-5 molecule. It is likely, but not proven, that both loops cooperate in tightening the paracellular space.

Interestingly, the residues, necessary for *trans*-interaction, are highly conserved within the classic claudins [4] and are likely to be involved in heterophilic *trans*-interaction, too. Heterophilic interaction has been reported for different claudin pairs, including Cld5-Cld3, Cld1-Cld3, Cld2-Cld3, Cld3-Cld4 [6, 29, 30] and Cld16-Cld19 [31, 32]. Hence, it is likely that in MDCK-II cells endogenously expressed claudins (claudin-1, -2, -3, -4, -7; [20]) and exogenous Cld5 form homophilic as well as heterophilic claudin-claudin interactions. However, the precise pattern of heterophilic *cis*- and *trans*-interactions still has to be identified.

Model of claudin-based barrier formation

Taken together, we propose the following model of claudin-based barrier formation (Fig. 6):

In non-transfected MDCK-II cells, endogenous claudins (e.g., Cld1, -2, -3, -4, -7) copolymerize in heteropolymeric intramembranous TJ strands [6] that form an effective barrier against large and, to a lesser extent, against small molecules (Fig. 6a). Small ions are able to permeate—due to the pore formation mediated by claudin-2 [33, 34]. Due to heterophilic *cis*- and/or *trans*-interactions, exogenously expressed Cld5 is incorporated in the heteropolymeric strands. This tightens the barrier against small ions and small molecules (Fig. 6b). This might be due to a higher packing density or an altered molecular organization of the claudins in the TJ. The side chain of R145 seems to be directly involved in this tightening, and if it is missing, then the seal is slightly loosened (Fig. 6c). The substitutions Y148A, Y158A and E159Q do not inhibit the homo- and heterophilic *cis*-interactions of Cld5, but specifically inhibit its homo- and heterophilic *trans*-interactions. In contrast, *trans*-interactions between endogenous claudins are not affected (Fig. 6d). The resulting partial inhibition of *trans*-interaction is not sufficient to block heteropolymerization of the claudins, but causes larger clefts at the position within the polymer where Cld5 molecules are located. According to the model, these clefts are responsible for the increase in paracellular permeability of small and large molecules. All substitutions reduce TER relative to Cld5_{wt}, but not below the level of the vector control. This is due to the leakiness for ions caused by ion pores that are formed by endogenous Cld2 in MDCK-II.

We used epithelial MDCK-II cells as an established TJ-containing but Cld5-free model system [10] to analyze mechanistic aspects of TJ formation. Cld5 is mainly expressed in endothelial cells. In the blood-brain barrier, it is likely to be coexpressed with Cld3, Cld12 and maybe Cld1 as well as other potential claudins [4]. We propose that the mechanism of homophilic and heterophilic Cld5 *trans*-interactions (e.g., Cld5 and Cld3) is similar for epithelial and endothelial cells. In addition, we showed that the substitution Y148A of Cld5, in zebrafish Cld5a, blocked its function in a cerebral barrier consisting of epithelial cells during the development of the zebrafish embryo [35]. Hence, the identified molecular determinants are of relevance *in vivo*, too.

In conclusion, we have identified molecular mechanisms by which the ECL2 of claudin-5 essentially contributes to the paracellular tightening against ions, small and large molecules. These sealing properties of the ECL2 are assumed to be largely shared by the classic group of claudin proteins, as most of the ECL residues discussed in this paper are highly conserved [4].

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