Renal localization and function of the tight junction protein, claudin-19

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Submitted 19 February 2007; accepted in final form 17 March 2007

Angelow S, El-Husseini R, Kanzawa SA, Yu AS. Renal localization and function of the tight junction protein, claudin-19. Am J Physiol Renal Physiol 293:F166–F177, 2007. First published March 27, 2007; doi:10.1152/ajprenal.00087.2007.—Claudins form a family of transmembrane tight junction proteins that play a key role in control and selectivity of paracellular transport. Mutations in claudin-19, which is expressed in kidney, retina, and myelinated peripheral neurons, were identified in familial hypomagnesemia with hypercalciuria and nephrocalcinosis, a hereditary disease causing renal Mg²⁺ and Ca²⁺ wasting. Here, we studied the distribution and possible functional role of claudin-19 in the renal tubule. By immunofluorescence staining of mouse kidney, claudin-19 was found to be expressed at the tight junction of the thick ascending limb of Henle, the major site of paracellular Mg²⁺ reabsorption, where it colocalized with claudin-16, as well as in the thin ascending limb. The role of claudin-19 in paracellular transport was tested by stable transfection into Madin Darby canine kidney II TetOff cells to generate inducible cell lines. Claudin-19 increased the transepithelial electrical resistance and decreased permeability to monovalent and divalent cations, while anion and urea permeability were not affected. Our data suggest that claudin-19 acts as a selective cation barrier at the tight junction. This would be consistent with its physiological role to electrically seal myelinated peripheral neurons. The normal role of claudin-19 in renal tubule function remains to be determined.

POLORED EPITHELIAL CELLS form cellular barriers that control the exchange of molecules and ions between fluid compartments of different chemical composition by transcellular and paracellular transport. Diffusion through the paracellular route is limited by the tight junction, a heteromeric belt of protein complexes that encircles the apical end of the lateral membrane and forms cell-cell contacts by interaction of its integral membrane components within the paracellular space. Among the integral membrane proteins expressed at the tight junction, it is the claudins that are thought to determine size and charge selectivity of the paracellular diffusion barrier, properties that can vary markedly between different epithelia (see detailed review on claudins in Ref. 32). Claudins form a family (≥24 members in mammals) of tetraspan proteins that are usually expressed at the tight junction but can also be found in other cell compartments like the basolateral membrane. While some members, like claudin-1, are ubiquitously expressed in epithelia of various tissues, other isoforms show a very distinct expression, like claudin-16 (also known as paracellin-1) which is exclusively expressed in the thick ascending limb of Henle (TAL) (29). However, claudins constitute more than a simple diffusion barrier. As shown by Anderson, van Itallie, and co-workers (6, 7), claudins use their extracellular domains to control paracellular transport, supposedly by formation of selective pores that are oriented parallel to the lateral membrane. Differences in charge selectivity are based on charged amino acid residues of the first extracellular domain that line the paracellular pore and electrostatically interact with ions passing the paracellular route.

A common approach to study the specific roles of different claudins at the tight junction is overexpression in epithelial cell lines and analysis of the electrophysiological phenotype. The heterologous overexpression of most claudins, including claudin-1, -4, -5, -7, -8, and -14 (1, 22, 31, 36, 38), leads to an increase in the transepithelial electrical resistance (TER) and thus enhancement of the barrier, in most cases due to a selective decrease in cation permeability. In contrast, claudin-2 has been found to decrease the TER by increasing cation permeability, suggesting that it adds cation-permeable pores to the tight junction (3, 9).

Paracellular transport is considered to be a major pathway by which ions cross the renal epithelium during salt retention. In the late proximal tubule, approximately half of filtered NaCl is reabsorbed by paracellular transport (4). In the TAL, divalent cations like Mg²⁺ are reabsorbed via the paracellular route, a passive process that is driven by the transepithelial potential (reviewed in Ref. 27). The situation is different in the distal convoluted tubule (DCT) and collecting duct (CD) where high transepithelial gradients of salt concentration are established by hormone-regulated active transport and the role of the intercellular junction is to tightly seal the paracellular route to prevent back-leak of solutes. The heterogeneity of permeability characteristics of epithelia that line different nephron segments coincides with the expression of certain claudin isoforms or combination of claudins. Claudin-2, for instance, is expressed throughout the proximal tubule and in the early segment of the thin descending limb of Henle (8, 17). The cation pore-forming abilities of claudin-2 could be responsible for the high cation permeability of these segments. On the other hand, claudin-4 and claudin-8, which act as cation barriers when heterologously expressed in Madin Darby canine kidney (MDCK) cells (31, 38), are both located in the distal nephron, including the CD (17, 20), and could contribute to the low paracellular cation permeability of these segments.

As mentioned above, the expression of claudin-16 is restricted to the tight junction of the TAL. Mutations in claudin-16 cause a rare autosomal recessive disease, familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC) characterized by renal Mg²⁺ wasting, which results in severe hypomagnesemia, and renal Ca²⁺ wasting, resulting in renal parenchymal calcification and renal failure (29, 35).

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The expression of claudin-16 in epithelial cells induces a modest increase in Mg\(^{2+}\) and Ca\(^{2+}\) permeability across the cell monolayers. This effect is not observed when FHHNC mutants are expressed and can also be abolished by mutation of the COOH-terminal end of claudin-16 (11–13, 15). The data suggest that claudin-16 may play a role in forming paracellular pores for renal Mg\(^{2+}\) and Ca\(^{2+}\) reabsorption.

Konrad et al. (18) recently characterized nine families that do not carry any mutations in claudin-16 but show a renal phenotype indistinguishable from that of FHHNC patients. Affected individuals of these families additionally exhibit severe visual impairment. Gene mapping revealed an association of this form of renal Mg\(^{2+}\) wasting with missense mutations in claudin-19 that could potentially affect the correct trafficking, dimerization, and folding of the protein. Thus claudin-19 could potentially play a role in renal reabsorption of divalent cations, just like claudin-16. According to Konrad et al. (18), immunostaining of mouse kidney showed the expression of claudin-19 in the TAL, colocalizing with claudin-16, and in the DCT. In addition, Lee et al. (19) previously reported high levels of claudin-19 expression in the kidney, among the organs that were screened by Northern blot. Claudin-19 has also been detected in the retinal pigment epithelium of the zebrafish (18), linking dysfunction of claudin-19 to the phenotype of visual impairment, and is also expressed in the tight junction of Schwann cells where it could participate in electrical insulation of axons. Claudin-19-deficient mice exhibited behavioral abnormalities that could be due to peripheral nervous system deficits (23). However, the specific function of claudin-19 at the tight junction and its impact on size and charge selectivity of the epithelium is still unknown and has not been tested in overexpression studies so far.

In the present study, we performed a detailed localization of claudin-19 in the mouse kidney using segment-specific markers of renal tubules and detected claudin-19 at the tight junction of TAL, colocalizing with claudin-16, and in the thin ascending limb. To test the role of claudin-19 in paracellular transport, we generated stable cell lines of MDCK II that were induced to express claudin-19. Electrophysiological and flux studies showed that claudin-19 decreases monovalent and divalent cation permeability, indicating that the role of claudin-19 in renal Mg\(^{2+}\) reabsorption is complex and cannot be explained by simple formation of Mg\(^{2+}\)-permeable pores by claudin-19.

METHODS

Generation of MDCK II TetOff Claudin-19 Cell Lines

The claudin-19 coding sequence was amplified from mouse kidney by PCR, confirmed to be identical to the Refseq sequence (Genbank accession no: NM 153105) by DNA sequencing, then ligated into our modified retroviral Tet response vector, pRevTREP. Retrovirus encoding mouse claudin-19 was generated, transduced into MDCK II Tet-Off cells, and stable clonal cell lines were selected in hygromycin modified retroviral Tet response vector, pRevTREP. Retrovirus encoding mouse claudin-19 was generated, transduced into MDCK II Tet-Off cells, and stable clonal cell lines were selected in hygromycin.

Immunoblotting and Immunofluorescence

Antibodies. Polyclonal claudin-19 antiserum was generated (Zymed Custom Antibody Service, San Francisco, CA) by immunizing a rabbit against a synthetic 23-residue peptide (CERANSIPQYPYRS-GPSTAAREYV) corresponding to the unique COOH terminus of mouse claudin-19 (of which 18/22 residues are identical to the predicted orthologous canine peptide sequence), then purified over a Protein A column, and used at a dilution of 1:100 for immunofluorescence, and 1:1,000 for immunoblotting. This peptide sequence is unique among claudin isoforms and a BLAST search revealed no other close matches in the National Center for Biotechnology Information nonredundant protein database. Rabbit anti-claudin antibodies, mouse anti-ZO-1 antibody, and rabbit anti-occludin antibody were purchased from Zymed, rabbit anti-CIC-K from Alomone Labs, and rabbit anti-NKCC2 was a gift of Dr. Steven Hebert. Secondary antibodies used were goat anti-rabbit or -mouse IgG conjugated to Alexa Fluor 488 or 555.

Immunofluorescence staining of cryosections. Immunofluorescence staining was performed using cryosections cut from kidneys of adult male CD1 mice that had been perfusion-fixed with 4% paraformaldehyde. This was performed essentially as described previously (8) except that instead of using SDS, we now routinely use microwave pretreatment (15 min on 10% power in 1 mM EDTA, pH 8), which we find to be more effective at antigen retrieval for claudins. For double-staining experiments in which both primary antibodies were of rabbit origin, the first primary antibody was applied at a very low concentration and amplified with the TSA kit using Alexa Fluor 488 tyramide (Molecular Probes, Eugene, OR). The second primary antibody was subsequently added and detected using goat anti-rabbit Alexa Fluor 555. Images were acquired at the USC Center for Liver Diseases using a Nikon PCM confocal microscopy system with argon and helium-neon lasers. For double-labeled slides with a large disparity in fluorescence brightness between the two channels, images were acquired sequentially for each fluorophore in single-label mode to minimize spectral overlap between channels.

Immunohistochemistry of cultured cells. Heterologous expression of claudin-19 and endogenous expression of tight junction proteins in MDCK II cells were studied by indirect immunofluorescence staining as described previously (38) using paraformaldehyde-fixed cells grown on Transwell filters (0.4-µm-pore size, Costar). Primary antibodies were diluted 1:100–1:200 in PBS containing 1% BSA, 5% goat serum, and 0.3% Triton X-100. Secondary antibodies (Molecular Probes), coupled to either Alexa Fluor 488 (green) or 555 (red), were used at 1:1,000 dilution. Filters were mounted on glass slides using Vectashield antifade mounting medium (Vector Laboratories) and analyzed either with a conventional epifluorescence microscope or a Nikon PCM confocal microscope.

Immunoblotting. For protein detection by immunoblotting, confluent cells, grown on tissue culture dishes, were homogenized in buffer (pH 7) containing 0.25 M sucrose, 30 mM histidine, 1 mM EDTA and protease inhibitors (Complete Mini, Roche Diagnostics). For further fractionation, the homogenate was centrifuged briefly to spin down cell debris followed by centrifugation at 100,000 g, separating membrane-associated proteins (pellet) from the cytosolic fraction (supernatant). The 100,000 g membrane pellet was resuspended in sucrose-histidine buffer and boiled in reducing loading buffer containing 2% SDS. Samples were loaded on a 10% denaturing polyacrylamide gel in aliquots of 20 µg protein and separated by electrophoresis. Proteins were then transferred onto a polyvinylidene difluoride membrane (BioRad) and visualized using antibodies at 1:1,000 dilution and the ECL chemiluminescence detection kit (Amersham).

TER Monitoring

The time course of TER of cells, plated on Transwell filters at confluent density (~2 × 10⁶ cells/cm²), was monitored using a Millicell-ERS voltmeter (Millipore). Chopstick-style Ag/AgCl-covered electrodes were immersed into the culture medium of the
apical and basolateral compartment and a 20-μA AC square-wave current was applied across the cell monolayer. TER of blank filters was subtracted from the resistance measured for cell-covered filters.

**Diffusion Potential Measurements**

The permeability of MDCK II monolayers to Na\(^+\) and Cl\(^-\) was determined by dilution potential measurements using Ussing chambers as described before (38). Cells grown on Snapwell filters (Costar) were mounted in Ussing chambers, filled with Ringer solution containing 3 M KCl. The electrodes were connected via head stage amplifiers to a microcomputer-controlled voltage/current clamp (VCC-MC6, Physiologic Instruments, San Diego, CA). Before measurements, asymmetry voltage was compensated by adjustment of the offset potentiometer. The spontaneous transepithelial potential was negligible, since all studies were performed in K\(^+\)-free solutions to inactivate the Na-K-ATPase. The monolayer conductance G (TER\(^-1\)) was calculated from the voltage evoked by a 90-μA current pulse. The solution in the basolateral chamber was then replaced by a modified Ringer solution containing 75 mM NaCl (osmolarity adjusted with mannitol) and the 2:1 NaCl dilution potential was read immediately under open-circuit conditions (values indicated refer to the apical side). The basolateral solution was then sequentially replaced by Ringer solution in which NaCl was substituted by 150 mM LiCl, KCl, RbCl, CsCl, and arginine chloride, respectively, and the bi-ionic potential was immediately read under open-circuit conditions. G, NaCl dilution potential, and bi-ionic potentials of cell monolayers were corrected by subtracting the values determined for blank filters.

The relative permeability ratios P_{Na}/P_{Cl} and P_{X}/P_{Na}, with X being another alkali metal cation, were calculated from the NaCl dilution potential and the bi-ionic potentials, respectively, using the Goldman-Hodgkin-Katz voltage equation. In all calculations, activities rather than concentrations were used, assuming that the activity coefficient of each XCl salt is the same as that of NaCl. The absolute permeabilities P_{Na} and P_{Cl} were derived by the method of Kimizuka and Koketsu (16) using the equation

\[
P_{Na} = \frac{RT}{F} \frac{G_M}{\alpha(1 + \beta)}
\]

where G\(_M\) is monolayer’s conductance per unit surface area, \(\alpha\) is the ratio of NaCl activity in the apical and the basolateral compartment, \(\beta\) is the ratio of the permeability of Cl\(^-\) and Na\(^+\) (P_{Cl}/P_{Na}).

**Solute Flux Measurements**

Transepithelial flux studies were performed in 12-well plates of Transwell filters in an incubator at 37°C. Substrates were added to the apical (cis) compartment and after different time intervals samples were taken from the basolateral (trans) compartment and replaced with an equal volume of fresh medium. During the experiments, the filter plates were frequently shaken to minimize formation of unstirred layers underneath the filter inserts. The initial rate of flux was used to determine the permeability of filters with cell monolayers (PC\(_M\)) and whole cell lysates of human embryonic kidney (HEK) cells transiently transfected with mouse claudin-19 (Cldn) or vector control (V).

For assays of net Mg\(^2+\) flux, Ringer solution containing 10 mM MgCl\(_2\) was added to the cis compartment and the osmolarity of the trans side was adjusted with mannitol. Samples from the trans compartment were diluted 1:33-fold in H\(_2\)O containing 0.16% HCl and 0.1% La\(_2\)O\(_3\), and analyzed by atomic absorption spectrophotometry (Spectra AA-220, Varian, Walnut Creek, CA). Ca\(^2+\) permeability was determined in unidirectional tracer flux experiments using culture medium that already contained 1.8 mM Ca\(^2+\); 3 μM 45Ca\(^{2+}\) was added to the cis side and samples from the trans side were analyzed by liquid scintillation counting. Urea permeability was determined using culture medium that was supplemented with 1 mM nonlabeled urea on both sides and 1 μCi 14C-urea in the cis compartment. To determine dextran flux, 5 mg/ml of 4-kDa FITC-dextran was added to culture medium without phenol red in the cis compartment. Samples were analyzed with a Hitachi F-2000 fluorometer at excitation and emission wavelengths of 485 and 520 nm, respectively.

**Statistical Analysis**

The data are presented as means ± SE. Statistical significance was determined using the unpaired two-tailed Student’s t-test, where appropriate.

**RESULTS**

**Nephron Segment Localization of Claudin-19 in Mouse Kidney**

To determine the tubule localization of claudin-19, we generated a custom polyclonal antibody against a unique COOH-terminal peptide. By Western blot, this antibody recognized a band of the expected size (22.2 kDa) in mouse claudin-19-transfected HEK cells, as well as in native mouse kidney membranes (Fig. 1). By immunofluorescence, claudin-19 was detected in tubules throughout the cortex, where it completely colocalized with the apical Na-K-2Cl cotransporter, NKCC2 (Fig. 2), indicating that these are cortical thick ascending limbs (CTAL). Within CTAL cells, claudin-19 was expressed at the tight junction, where it colocalized with the tight junction marker, ZO-1 (Fig. 3, A and C). Interestingly, claudin-19 exhibited a subsegmental distribution: in any transverse section of the CTAL, it was found to be expressed in a

![Fig. 1. Validation of the rabbit claudin-19 antibody by Western blot. The lanes show (from left to right) mouse kidney cortex 47,000 g membrane fraction (MK) and whole cell lysates of human embryonic kidney (HEK) cells transiently transfected with mouse claudin-19 (Cldn) or vector control (V).](http://ajprenal.physiology.org/...)}
tight junctions between some cells of the CTAL but not others, whereas ZO1 was ubiquitous [Fig. 3C and Supplemental Fig. 1 (the online version of this article contains supplemental data)]. Claudin-19 was absent from the macula densa (Fig. 3B). In the outer medulla, claudin-19 was similarly expressed in a subsegmental distribution at the tight junctions of medullary thick ascending limbs (MTAL), although the levels of expression appeared to be lower than in CTAL (Supplemental Fig. 2). Because claudin-16 (paracellin-1) has also been found in the TAL, we examined whether it was colocalized with claudin-19. Indeed, we found that there was complete colocalization of claudins 16 and 19 in the CTAL (Fig. 4 and Supplemental Fig. 1) and MTAL (Supplemental Fig. 2).

In the inner medulla, claudin-19 was expressed in the thin ascending limbs, which were identified with an antibody to the CIC-K chloride channel isoforms (Fig. 5). There was no colocalization of claudin-19 with tubules that expressed AQP1, a marker for the thin descending limbs, or AQP2, a marker for the inner medullary collecting duct (data not shown). In the thin ascending limb, claudin-19 and ZO1 colocalized and exhibited a tortuous, serpentine pattern that presumably reflects the course of tight junction strands as they follow the known extensive infoldings of the lateral membranes in this segment (28). Like in the TAL, claudin-19 in the thin ascending limbs exhibited a segmental distribution and was found in a minority of the cells in these tubules (Fig. 5), where it also colocalized with claudin-16 (Supplemental Fig. 2). In contrast to an earlier report (19), we found no evidence for claudin-19 expression in proximal tubule, distal convoluted, or connecting tubule, or any part of the collecting duct.

**Generation of MDCK II TetOff Cells with Inducible Expression of Claudin-19**

To study the functional role of claudin-19 in paracellular transport, we overexpressed it by stable transfection into MDCK II TetOff cells, which have no detectable endogenous claudin-19 (by Western blot analysis). We identified two clones with high levels of inducible claudin-19 expression that showed similar phenotypic characteristics (the data from one representative clone is presented). In Western blots of cell lysates claudin-19 appeared as a strong band at ~22 kDa (Fig. 6A). High levels of claudin-19 expression were detected 4 days after seeding and culturing the cells in the absence of Dox (Dox−). The expression of exogenous claudin-19 in induced cells remained high for at least 8 days in culture and was suppressed by supplementing the culture medium with Dox (Dox+). Separation of the cell membranes and the cytosolic fraction by 100,000 g centrifugation of the cell lysate showed that claudin-19 was entirely membrane associated (Fig. 6B).

Furthermore, we analyzed the distribution of claudin-19 by double-immunofluorescence staining of claudin-19 and ZO-1. Confocal images obtained by laser-scanning microscopy revealed that claudin-19 largely colocalizes with ZO-1 at the tight junction of stably transfected MDCK II cells (Fig. 6C). In contrast to ZO-1, the staining of claudin-19 is less homogenous and appears to be weaker at some cell borders and brighter at others. The apical membrane was also stained, in a punctate pattern. Vertical sections of confocal images confirmed localization at the tight junction and additional staining of the apical membrane and, to a lesser extent, the lateral membrane.

Fig. 2. Overview of nephron segment distribution of claudin-19 in mouse kidney. Low-magnification views show complete colocalization of claudin-19 (green) and NKCC2 (red) to cortical thick ascending limb segments in the cortex. In the medulla, claudin-19 is expressed at very low levels in NKCC2-positive outer medullary (OM) thick ascending limbs and strongly stains a subset of tubules in the inner medulla (IM).
Previous studies showed that the heterologous expression of claudins in host cell lines can affect the composition of the tight junction. As previously discussed, changes in endogenous tight junction proteins make the interpretation of the electrophysiological phenotype more complex. Thus we tested whether the expression of claudin-19 has any effect on occludin and claudins endogenous to the host cell line. By testing various anti-claudin antibodies, we previously found that MDCK II cells endogenously express claudin-1, -2, -3, -4 and -7. Western blot analysis showed that the induction of claudin-19 has no effect on the expression of these endogenous claudins and occludin (Fig. 7A).

Furthermore, no changes in the expression and distribution of the investigated proteins were detected by immunofluorescence staining (Fig. 7B).

Effect of Claudin-19 on TER and Monovalent Ion Permeability

In the next set of experiments, we investigated the influence of claudin-19 expression on the electrical tightness of the cell monolayers by measurements of the TER. The TER of leaky epithelial monolayers, like those formed by MDCK II cells, is predominantly determined by the paracellular component of transepithelial ion permeability. The TER of MDCK II TetOff claudin-19 cells, cultured in the presence of Dox, peaked 2 days after seeding the cells on permeable filters, followed by a decline to steady-state levels (Fig. 8A). When cells were grown under Dox− conditions, no statistically significant differences in the TER were detected compared with Dox+-treated cells, within the first 3 days after seeding. Note that, according to Western blot studies, during the first days in culture induced cells express only minor amounts of claudin-19 (Fig. 6A). The strong induction of claudin-19 expression that has been observed on day 4 correlated with a further increase in TER which peaked on days 4 and 5 and reached values that were ~30% higher than peak values measured for noninduced cells. Subsequently, the TER of claudin-19-expressing cells declined to steady-state levels, which were still higher than those of Dox+ cells 2 wk after seeding.

The role of claudin-19 in the reassembly of the tight junction of a confluent monolayer was studied in a Ca2+-switch experiment (Fig. 8B). Exposure of MDCK II TetOff claudin-19 cells to Ca2+-free medium led to a breakdown of the tight junction, marked by a drop in TER, for both Dox+- and Dox−-treated cells. In the phase of tight junction reassembly induced by adding back Ca2+, the rate of increase in TER was much greater for claudin-19-expressing cells (Dox−). Steady-state TERs were reached ~1.5 h after readdition of Ca2+ and were about twice as high for Dox−-treated cells compared with Dox+-treated cells. The findings suggest that exogenous expression of claudin-19 enhances paracellular tightness to charged ions during the formation of the barrier as well as in established, mature tight junctions.
We determined the NaCl dilution potential to identify the ion(s) that mainly carry the transepithelial electrical current and to investigate the effect of claudin-19. MDCK II TetOff claudin-19 cells, grown on Snapwell filters, were mounted in Ussing chambers, and the transepithelial conductance was determined under current clamp conditions. A transepithelial concentration gradient of NaCl was imposed to determine the dilution potential, from which the permeability ratio $P_{Na}/P_{Cl}$ and absolute $P_{Na}$ and $P_{Cl}$ were calculated (Fig. 9A and Table 1). The results demonstrate that the increase in TER observed in

![Image](https://via.placeholder.com/150)

Fig. 4. Colocalization of claudins-16 and -19. Low (top)- and high (bottom)-magnification fields of the kidney cortex show complete colocalization of claudin-16 and -19 at the tight junctions of CTALs. Scale bar represents 10 μm.

Fig. 5. Localization of claudin-19 in the inner medulla. A: low- and high-magnification fields of merged 2-color immunofluorescence. Claudin-19 (green) is expressed in a subset of cells (arrowheads) within thin ascending limbs, which are identified by apical and basolateral membrane expression of ClC-K1 (red). B: double staining of the same thin ascending limb with antibodies to claudin-19 (green) and ZO-1 (red). Note the tortuous, serpentine appearance of the tight junction of this nephron segment, both in cells that express claudin-19 (arrowhead) and those that do not (arrow). L, tubule lumen. Scale bar represents 10 μm.
cells induced to express claudin-19 is due to a selective decrease in $P_{\text{Na}}$ by 52% while $P_{\text{Cl}}$ is not affected, suggesting that claudin-19 selectively enhances the paracellular barrier to cations.

As ions pass through paracellular pores, it is thought that they undergo electrostatic interaction with charged sites within the pore, in a manner dependent on the diameter, and hence charge density and energy of hydration, of the permeating ion. To assess whether claudin-19 changes the physicochemical nature of the paracellular cation pores, we determined the order of permeabilities of various alkali metal ions by bi-ionic potential measurements (Fig. 9B). Calculation of absolute permeabilities (Table 1) showed that the expression of claudin-19 significantly decreases the monolayer permeability to all alkali metal cations to roughly the same extent as observed for Na$^+$, so that the selectivity sequence was essentially unchanged. This indicates that claudin-19 does not alter the properties of the paracellular cation pores.

The permeability of the monovalent organic cation, arginine (mean diameter 6.96 Å), was also tested by the bi-ionic potential method, and was also found to be decreased, although to a lesser extent than for alkali metal ions (Table 1).

**Effect of Claudin-19 on the Permeability of Divalent Cations and Uncharged Molecules**

Within the kidney, claudin-19 was found to be mainly expressed in the TAL, a segment of high paracellular Ca$^{2+}$ and Mg$^{2+}$ permeability, and claudin-19 mutations have been associated with renal Ca$^{2+}$ and Mg$^{2+}$ wasting. These findings suggest that claudin-19 could participate in the paracellular transport of divergent cations. To test this hypothesis, permeability studies of divalent cations were performed. These showed that the induction of claudin-19 decreased permeability to Mg$^{2+}$ and Ca$^{2+}$ by 24% and 45%, respectively (Table 1), suggesting that claudin-19 also enhances the paracellular barrier to divalent cations.

The effect on cation permeability was relatively specific because induction of claudin-19 had no effect on urea permeability. By contrast, the permeability of a macromolecular marker, 4-kDa FITC-dextran, was decreased when cells were induced to express claudin-19, perhaps due to changes in tight junction strand dynamics (Table 1).

**DISCUSSION**

**Tubule Localization of Claudin-19**

We found claudin-19 expression at the tight junction in a subset of cells in the thick and thin ascending limbs of the mouse kidney. Lee et al. (19) initially reported expression of claudin-19 predominantly in the distal tubule and collecting duct, with less expression in the proximal tubule and MTAL. The reason for the differences between their findings and ours is not completely clear, but they did not confirm the specificity of their antibody (which we did by Western blot of claudin-19-transfected cells, Fig. 1). They may also have incorrectly misidentified tubules that lacked aquaporin-1 and aquaporin-2, which they broadly classified as “distal tubules” but obviously includes many other segments, including the thick and thin ascending limbs. In a subsequent paper, Konrad et al. (18) used the same antibody and found claudin-19 in the TAL and distal tubule. However, no markers were used to confirm the identity of the distal tubule, and the main corroborative evidence appears to be analysis of mRNA in microdissected nephron segments by reverse transcription-PCR, which can potentially yield falsely positive results.

Our observation that claudin-19 is expressed in TAL and colocalizes with claudin-16 is consistent with the findings of Konrad et al. (18). The TAL is known to have a very low transepithelial resistance (11–34 Ω·cm$^2$) and a high paracellular permeability to Na$^+$, Ca$^{2+}$, and Mg$^{2+}$, but not to water (5). This is functionally important because transcellular, active NaCl reabsorption via apical Na-K-2Cl cotransport and recycling of K$^+$ back into the lumen through ROMK channels generates a lumen-positive voltage that provides the driving force for passive paracellular reabsorption of divergent cations, as well as driving further Na$^+$ to be reabsorbed via the paracellular route. The observation that mutations in either claudin-16 (29) or claudin-19 (18) lead to familial hypomagnesemia with hypercalciuria and nephrocalcinosis strongly suggests that claudin-16 and -19 might both be involved in...
paracellular divalent cation reabsorption in the TAL and might act together in the same pathway.

We also found high levels of claudin-19 expression in thin ascending limbs. This segment is known to have shallow tight junctions and lateral membranes that interdigitate extensively between neighboring cells (28), suggesting that it may function to mediate high rates of paracellular transport. Thin ascending limbs have a high passive permeability to Cl\(^-\)/H\(^+\), which is known to be primarily transcellular (21), and to Na\(^+\), which is probably paracellular, along with a very low water permeability (14). NaCl is concentrated in the luminal fluid of the thin descending limb and then delivered to the thin ascending limb, whose unique permeability properties appear to be important for passive reabsorption of NaCl, thus generating the high inner medullary interstitial NaCl gradient necessary for urinary concentration. It is interesting to speculate that claudin-19 might play a role in paracellular Na\(^+\) permeability in this segment.

One of our most remarkable findings is the striking heterogeneity of claudin-19 expression in both the thick and thin ascending limbs. It is in fact now well established that the TAL is heterogeneous. Morphologically, it consists of two populations of cells: R cells are rough-surfaced with prominent microvilli and extensive lateral processes, and are more abundant in the cortex, while S cells are smooth-surfaced with a paucity of microvilli, rare lateral processes, and are more abundant in the medulla (2). Immunofluorescence staining of transport proteins has shown that expression of both ROMK (37) and the shortened COOH terminal splice variant of the apical Na-K-2Cl cotransporter NKCC2 (25) in the TAL is heterogeneous. Finally, electrophysiological studies showed two distinct cell populations in the TAL that have either high

Fig. 7. Effect of claudin-19 induction in MDCK II TetOff claudin-19 cells on tight junction composition. Western blots of lysed cells (A) and immunofluorescence staining of confluent monolayers (B) show similar expression levels and distribution of occludin and endogenous claudins in noninduced and induced cells (bar = 10 µm).
or low basolateral K⁺ conductance (30). How these differently classified cell populations correspond to each other and to the claudin-19-expressing cells in the TAL remains to be determined. In the thin limb, alternating short segments with morphological and immunohistochemical properties of descending and ascending thin limbs have been observed (26). However, heterogeneity from cell to cell, as we observed for claudin-19, has to our knowledge never been reported before in the thin ascending limb and its significance is unknown.

Claudins can act predominantly either as barriers or pores. They can increase TER and selectively limit ion permeability, like claudin-4 for example (31), or decrease TER and selectively increase ion permeation, as observed for claudin-2 (9). The heterogeneous expression of claudin-19 is more consistent with a pore function because in that case expression of claudin-19 in even a minority of cells in the tubule segment would be sufficient to allow a paracellular leak. If claudin-19 acts as

Table 1. Summary of permeability data for MDCK II TetOff claudin-19 cells determined by diffusion potential measurements and tracer flux studies

<table>
<thead>
<tr>
<th>Permeability (×10⁻⁶ cm/s)</th>
<th>Dox+</th>
<th>Dox⁻</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monovalent cations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Li⁺</td>
<td>36.0±0.2</td>
<td>17.8±0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Na⁺</td>
<td>36.2±0.7</td>
<td>17.3±0.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>K⁺</td>
<td>41.3±0.2</td>
<td>19.3±0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Rb⁺</td>
<td>38.7±0.3</td>
<td>17.8±0.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cs⁺</td>
<td>30.9±0.4</td>
<td>14.7±0.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Arginine</td>
<td>6.7±0.3</td>
<td>5.7±0.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Divalent cations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>5.2±0.4</td>
<td>4.0±0.1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>23.1±0.4</td>
<td>12.6±2.9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Anions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl⁻</td>
<td>2.7±0.3</td>
<td>2.2±0.3</td>
<td>0.28</td>
</tr>
<tr>
<td>Neutral solutes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea</td>
<td>2.0±0.1</td>
<td>2.2±0.1</td>
<td>0.19</td>
</tr>
<tr>
<td>Dextran</td>
<td>0.77±0.07</td>
<td>0.48±0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE.

**Fig. 8.** Effect of claudin-19 expression in MDCK II TetOff claudin-19 cells on TER. A: cells, seeded on filters in confluent density, were cultured under Dox⁺ and Dox⁻ conditions and the time course of the TER was monitored. Cells induced to express claudin-19 developed a higher TER at the peak of the initial overshoot that remained above values of noninduced cells throughout the experiment. B: Ca switch experiment: confluent cell monolayers were deprived of Ca²⁺ for 1 h to break down the tight junction barrier. Upon reintroduction of Ca²⁺ to stimulate reassembly of the tight junction, the rate of recovery of TER and the subsequent steady-state TER were greater in the presence of claudin-19. Data points represent means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001 (Dox⁻ vs. Dox⁺).

**Fig. 9.** Permeability of MDCK II TetOff claudin-19 cells to small inorganic ions. A: Na⁺ and Cl⁻ permeability of confluent monolayers determined from transepithelial NaCl dilution potential measurements in Ussing chambers. The data show that claudin-19 selectively decreases Na⁺ permeability while Cl⁻ permeability is not affected. B: bi-ionic potential measurements to determine permeability of alkali metal cations, expressed as relative values compared with Na⁺, show minimal change in the Eisenman sequence with induction of claudin-19. Data points represent means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001 (Dox⁻ vs. Dox⁺).
a barrier, such heterogeneous expression would make it poorly effective on its own because neighboring cells that lack claudin-19 would act as a shunt pathway. In that case, one would predict that another claudin isoform must act as the barrier between the claudin-19-negative cells.

Functional Expression of Claudin-19 Leads to Selective Decrease in Cation Permeability

To test the function of claudin-19 at the tight junction, we used stably transfected cell lines of MDCK II that could be induced to express claudin-19. Besides its predominant expression at the tight junction, where it colocalized with ZO-1, claudin-19 was also found at the apical and lateral membrane (Fig. 6C). In contrast, in the native mouse kidney, claudin-19 is expressed only at the tight junction of the TAL and thin ascending limbs. It is not clear at this point whether the presence of claudin-19 in MDCK II cells in membrane domains other than the tight junction is an artifact of overexpression or has a functional role. The induction of claudin-19 did not affect the expression of other tight junction membrane proteins that are, to our knowledge, expressed endogenously in MDCK II cells (Fig. 7). This is important since our previous investigations have shown that a heterologously expressed claudin (claudin-8) can replace an endogenous claudin (claudin-2) at the tight junction of the host cell line (38). If replacement occurs, changes in the phenotype represent not only the contributions of the newly inserted exogenous claudin but also reflect in part the loss of the endogenous claudin that is replaced and the loss of its contribution to the barrier’s properties. Because of a lack of availability of specific antibodies to all claudin isoforms, we cannot exclude the possibility that additional claudins are endogenously expressed by MDCK II cells and affected by exogenously expressed claudin-19.

In the present study, we observed that claudin-19 augments the paracellular cation barrier in MDCK II cells (Fig. 8). The TER of the induced cells was significantly increased, compared with noninduced cells, and this correlated with the induction of high levels of claudin-19 protein (Fig. 6A). In general, claudins that improve the barrier under steady-state conditions also augment the rate of reassembly of the barrier, for example after Ca switch, an effect which was also observed for claudin-19. Diffusion potential measurements showed that the increase in TER is due to a specific decrease in the permeability to monovalent and divalent cations (Table 1), while the permeability of anions and small neutral molecules does not seem to be affected. In an elegant series of experiments, Anderson, van Itallie, and co-workers (6, 7, 33) found that the pore or barrier properties of claudins depend on charged amino acid residues located in the first extracellular domain of claudins. Positively charged amino acids enable a claudin to act as a barrier to cations while claudins with negatively charged amino acids are predicted to act as cation pores. Mutation of a single lysine at position 65 in the first extracellular loop of claudin-4 to aspartate abrogates its ability to discriminate against cations. It is interesting to note that claudin-19 also expresses a lysine at that same position, raising the possibility that this might play a role in its cation barrier function.

The observation that claudin-19 acts as a cation barrier is surprising from a renal physiology viewpoint, for three reasons. First, we have found that claudin-19 is expressed in the TAL, which is an important segment for paracellular cation reabsorption. Second, mutations in claudin-19 cause a form of FHHNC (18), also suggesting that claudin-19 likely acts as a pore to divalent cations. Third, claudin-19 and claudin-16 colocalize in the kidney, and mutations in both genes cause essentially the same disease, suggesting that these two claudins function in a common pathway. The functional role of claudin-16 is somewhat controversial. Its observed effect on TER and Na+ permeability differ markedly between different studies (11, 13, 15), perhaps because different renal epithelial cell lines were used for expression (MDCK II, MDCK-C7, and LLC-PK1). However, all investigators have found that wild-type claudin-16 increases Mg2+ permeability to some extent [Hou et al. (11) observe effects only in LLC-PK1 cells but not in MDCK II cells], supporting a possible role for claudin-16 in the formation of Mg2+-permeable pores for renal Mg2+ reabsorption. Thus the question arises as to why claudin-16 seems to mediate Mg2+ permeation while claudin-19 appears to inhibit it.

One explanation might be that, in the renal tubule, claudin-19 is designed to act as a nonspecific cation barrier to prevent wholesale paracellular leakage of cations like K+ and organic cations. In this case, there must be other claudins, at least in the CTAL, that are presumably inserted in parallel with claudin-19 and act as specific pores for individual cations such as Na+, Mg2+, and Ca2+ that need to be reabsorbed paracellularly. Besides claudin-16, which is a likely candidate for paracellular transport of divalent cations, claudin-3, -10, and -11 have been reported to be expressed in the TAL (17). While the electrophysiological properties of claudin-3 are unknown, claudin-10b, one of the two splice variants of claudin-10 that are expressed in the kidney, acts as a cation pore when expressed in epithelial cells (34).

The notion that claudin-19 acts as a cation barrier in vivo is supported by studies of its role in neuronal function. Miyamoto et al. (23) found claudin-19 in tight junction-like structures in Schwann cells of myelinated neurons of the peripheral nervous system. Schwann cells of claudin-19-deficient mice lack tight junctions, and the mice show behavioral abnormalities and changes in conduction velocity of myelinated fibers. Interestingly, claudin-19 is completely absent in oligodendrocytes of the central nervous system, and another member of the claudin family, claudin-11, also known as oligodendrocyte-specific protein, takes over the role of tight junction formation within their myelin sheaths (10, 24). Saltatory conduction in myelinated axons depends on electrical sealing of the myelin sheaths in the internodal region that act as a permeability barrier to Na+ and K+, thus allowing action potentials to hop between nodes of Ranvier. Claudin-11 and -19 seem to have a similar physiological function to maintain saltatory conduction. Like claudin-19, claudin-11 increases the TER when overexpressed in MDCK II cells and decreases the cation selectivity of MDCK II monolayers (33). Thus claudin-11 and -19 probably act as the cation barriers for myelin sheaths in central and peripheral nervous systems, respectively.

Pathogenesis of FHHNC Due to Claudin-19 Mutations

If claudin-19 reduces cation permeability, it is difficult to understand how mutations in claudin-19 lead to the phenotypic
features of FHHNC, such as renal Mg²⁺ wasting. Our data indicate that the simplest explanation, that it is due to loss of function mutations in Mg²⁺-permeable pores, cannot be correct. Indirect mechanisms, like changes in transepithelial electrochemical potentials, could be involved. It is also possible that claudin-19 behaves differently in vivo than in our cell culture model. A limitation of the method of overexpression of a claudin in a cell line to determine its electrophysiological characteristics is that the host cell line might lack some of the proteins that are coexpressed and regulate or interact with this claudin in vivo. Although MDCK II cells are derived from renal tubules, the claudins that this cell line expresses (claudins-1, -2, -3, -4 and -7) differ from those present in the tubule segments where claudin-19 is expressed (claudins-3, -10, -11 and -16 in TAL). Thus our study could miss potential interactions between claudin-19 and other claudins from the TAL that might be functionally important in vivo. Given that claudin-19 colocalizes precisely with claudin-16 along the nephron, it is possible that these isoforms interact with each other at the tight junction, for example by forming heteromultimers with permeability properties that differ from those observed when they are overexpressed individually in cell lines. Such an interaction between claudin-19 and claudin-16 could provide a plausible explanation for the observation that FHHNC can arise from mutations in either gene. Although the overexpression of claudins in cell lines provides important information, further investigations, such as characterization of the renal phenotype of claudin-19 knock-out mice, are necessary to determine the in vivo functions of claudin-19.

In summary, we showed that in the kidney, claudin-19 is expressed in the CTAL and thin ascending limb of Henle. Functional expression of claudin-19 in inducible MDCK II cells enhances the paracellular barrier to cations. The function of claudin-19 as a cation barrier is consistent with its physiological role in myelinated peripheral neurons, but its normal in vivo functions of claudin-19.

ACKNOWLEDGMENTS

We thank R. Rude and L. Wei for generously sharing equipment and helping to perform the magnesium flux measurements by atomic absorption spectrophotometry, and J. Peti-Peterdi for help with preliminary confocal imaging studies.

GRANTS

This work was supported by National Institutes of Health Grants RO1-DK-062283 (to A. Yu) and DK-48522 (to the USC Center for Liver Diseases, for the Microscopy Subcore).

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