TWO SPLICE VARIANTS OF CLAUDIN-10 IN THE KIDNEY CREATE PARACELLULAR PORES WITH DIFFERENT ION SELECTIVITIES

Christina M. Van Itallie¹, Sarah Rogan², Alan Yu³, Lucia Seminario Vidal², Jennifer Holmes²

and James M. Anderson²

Departments of ¹Medicine and ²Cell and Molecular Physiology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7545 USA and ³Departments of Medicine and Physiology and Biophysics, University of Southern California Keck School of Medicine, Los Angeles, California 90033

Running title: Cldn-10a and 10b have different TJ charge selectivity

Please address correspondence to:

Christina M. Van Itallie
University of North Carolina at Chapel Hill
6314 MBRB CB#7545
103 Mason Farm Road
Chapel Hill, NC 27599-7545
Phone: 919-966-6412
Fax: 919-966-6413
Email: vitallie@med.unc.edu
Members of the large claudin family of tight junction proteins create the differences in paracellular conductance and charge selectivity observed among different epithelia. Previous studies demonstrated that ionic charge selectivity is influenced by acidic or basic amino acids on the first extracellular domain of claudins. We noted two alternatively spliced variants of claudin-10 in the database, 10a and 10b, which are predicted to encode two different first extracellular domains and asked if this might be a novel mechanism to generate two different permselectivities from a single gene. Using quantitative PCR, we found that claudin-10b is widely expressed among tissues including the kidney; however claudin-10a is unique to the kidney. Using a nondiscriminating antibody we found that claudin-10 (a plus b) is expressed in most segments of the nephron. In situ hybridization, however, showed mRNA for 10a is concentrated in the cortex and mRNA for 10b is more highly expressed in the medulla. Expression in MDCK II and LLC-PK1 cells reveals that both variants form low resistance pores, and that claudin-10b is more selective for cations than claudin-10a. Charge-reversing mutations of cationic residues on 10a reveal positions that contribute to its anion selectivity. We conclude that alternative splicing of claudin-10 generates unique permselectivities and might contribute to the variable paracellular transport observed along the nephron.

Key words: tight junction, claudin, kidney, permselectivity, MDCK, LLC-PK1
INTRODUCTION

Tight junctions regulate the movement of solutes and electrolytes through the paracellular pathway across epithelia (3; 11). Permeability characteristics of tight junctions vary widely among different tissues as characterized by electrical resistance, preference for cations or anions and the flux for noncharged solutes; these tight junction characteristics are collectively referred to as permselectivity (21). Current evidence suggests that these variable properties are based on differential expression of members of the claudin family of transmembrane proteins (reviewed in (29)).

Claudins are small proteins with four transmembrane segments and two extracellular domains. They assemble into rows of adhesive cell-cell contacts which appear as interconnected transmembrane strands in freeze-fracture electron micrographs (12). For many years the strand barriers have been modeled as rows of pores with size and charge selectivity (4; 10). There are at least 23 claudin genes in mammals and each epithelial cell type has a distinct expression profile (see for example, (18), (17), (14)), presumably resulting in its unique collective permselectivity.

When expressed in a cultured epithelial cell monolayers, different claudins cause different permselectivity phenotypes, that is, they behave like different pores (29). For example, claudin-2 forms electrically low resistance, cation-selective pores when expressed in high resistance MDCK I cells (1). In contrast, expression of claudin-8 (32) and -14 (2) results in increased transepithelial resistance and specifically decreased paracellular cation permeability. The result of charge reversing mutagenesis on two claudins reveals the ability of some charged residues on the first extracellular domain to influence ionic permeability, as if these residues lined the pore (8). We noted two alternatively spliced cDNAs for claudin-10 in mice and humans which encode two different first extracellular domains and asked if they represented a naturally
occurring mechanism to generate different permselectivities. Our findings are most relevant to
the physiology of the kidney which is the only organ were we observe detectable levels of both
splice variants (this paper).

Transcellular transport as well as paracellular permselectivity varies along the nephron as
the basis of water, solute, salt and pH homeostasis. In general, paracellular electrical resistance
increases along the nephron from the leaky proximal tubule (6-7 ohms x cm²) to tight collecting
ducts and bladder (300,000 ohms x cm²) (21). Most segments are more permeable for cations
than anions, although junctions of the superficial straight proximal tubule are anion selective
(27). While we still know little about the properties of most claudins, several groups have studied
their expression profiles along the nephron with the ultimate goal of explaining transport
difference among segments. Even a limited review of the published immunolocalization reveals
strong regional specialization: glomerulus expresses claudin-1 (6; 18), proximal tubule expresses
relatively high levels of claudin 2 (6; 18), the thick ascending limb of the loop of Henle
expresses claudin-16 (Paracellin-1) (25), the distal nephron expresses claudin-7 and -8 (19),
collecting tubule contains claudin-4 (6; 18), and claudin-5 (18) is localized to endothelial cells.
However, the detailed profiles are incomplete and there exists some disagreement about the
distribution of other claudins. Human mutations in claudin-16 (Paracellin-1) result in magnesium
wasting, which was rationalized as loss of paracellular cation pores in the thick ascending limb
of the loop of Henle, the only segment where appreciable Mg²⁺ resorption normally occurs (25).

There remains much to learn about how claudins make selective pores. Insights about the
basis for charge selectivity are inferred from a single study of experimental mutagenesis (8). To
address this limitation we investigated the consequences of expressing in cultured epithelial cells
two naturally occurring isoforms of claudin-10 which differ only in the first transmembrane and
extracellular domains and share the remaining sequence. Inspection of the charged residues of the first extracellular domain suggested to us that the claudin-10b isoform was likely to be a cation pore, while claudin-10a was likely to create a pore with less selectivity for cations. Claudin-10 had been previously reported to be expressed in proximal tubule and thick ascending limb of the loop of Henle by Tsukita and colleagues (18) and in proximal and distal tubule, thick ascending limb of Henle and vasa recta by Inai et al. (16). However, neither group addressed the issue that claudin-10 is expressed as two different isoforms, and in each case the detecting antibody did not discriminate between these isoforms. Because the tissue distribution of these two isoforms in the body has not been previously described, we first characterized their distribution by quantitative PCR and the distribution of claudin-10 by immunoblotting and immunofluorescence microscopy. Each isoform was then expressed separately in the cation-selective background of MDCK II cells and the anion-selective background of LLC-PK1 cells and the physiologic properties assessed. The results presented here suggest that the two naturally occurring isoforms of claudin-10 differ in their localization in kidney and in their physiologic properties.

**MATERIALS AND METHODS**

**Plasmid constructs and cell lines.** Mouse claudin-10a (clone ID 5356256) and 10b (clone ID 5040100) cDNAs were obtained from Open Biosystems (Huntsville, AL), cloned into the pTRE vector (Clontech Laboratories, Palo Alto, CA) and verified by sequencing in both directions. Claudin-10a as obtained from Open Biosystems contains a deletion within the carboxyl terminal half, which we corrected by replacement with sequence encoding the shared
carboxyl terminal domain from claudin-10b. Mutants of claudin-10a were created with the PCR-mediated Quik-change mutagenesis kit (Stratagene, La Jolla, CA) using appropriate oligonucleotide primers to generate the charge-reversing mutations m1 (R32D), m2 (R61D) and m3 (K68D) and the resulting mutations were verified by sequencing. Clonal cell lines of both MDCK II and LLC-PK1 cells were generated as described previously (30); 3-5 separate stable clones were derived for each construct. Cells were maintained in the presence of 50 ng/ml doxycycline to repress transgene expression and plated for experiments onto removable filters (Snapwell, Corning Life Sciences, Acton, MA) in the presence (not induced) or absence (induced) of doxycycline as described previously (30). Physiological studies, immunoblots and immunofluorescence microscopy of the cultured cells were performed as described previously (8; 30). All antibodies except the anti-ZO-1 rat monoclonal (26) were from Zymed Laboratories (Invitrogen R&D, Carlsbad, CA).

Quantitative Real-Time PCR, tissue immunoblotting and immunofluorescence microscopy. Tissue for RNA isolation and for immunofluorescence microscopy (except for kidney) was obtained from 3 or 4 adult C57 Black6 mice and preserved by submersion in RNALater RNA stabilization solution (Ambion, Inc., Austin, TX). Tissues for immunofluorescence were flash frozen in liquid nitrogen in O.C.T embedding compound (Tissue-Tek #4583, Sakura Finetek U.S.A., Inc., Torrance, CA). RNA was isolated using an RNeasy kit (Qiagen, Inc., Valencia, CA) according to kit protocols. 5 ug of RNA for each tissue was treated with TURBO DNase (TURBO DNA-free kit, Ambion, Inc., Austin, TX) per instructions to remove genomic DNA contamination. cDNA was synthesized from 2.5 ug of treated RNA using Superscript III reverse transcriptase (Invitrogen Corporation, Carlsbad, CA) with an equal amount of RNA included in a no-RT control for each separate RNA sample.
Quantitative PCR was performed with 1:50 dilutions of both the cDNA (in triplicate) and no-RT control for each sample as well as no-template reaction controls. Reaction conditions, threshold cycle determinations and quality control measures have been previously described (14). The cycle at which each sample crossed a fluorescence threshold, C_t (at 0.1-0.2 fluorescence units), was determined, and the triplicate values for each cDNA were averaged. RNA for Eef1a1, eukaryotic translation elongation factor 1 alpha 1, served as a housekeeping gene for normalization between samples and was included in each cycling run (13); (14)). Gene expression was normalized to Eef1a1 expression by calculating a \( \Delta C_t \) (C_t of claudin-10a or 10b minus the C_t of Eef1a1). Relative expression values were calculated as 2^{(-\Delta C_t)}), setting the expression value of Eef1a1 to 1.0. Experimental error was estimated for each gene in each tissue by the CV (%) of the average \( \Delta C_t \) value of that gene between animals, error = \((2^{\%CV})/100)\)* relative expression value. If a sample signal had not risen above the threshold value within 37 cycles, it was considered not detected (nd). Primers for quantitative PCR are shown in Table 1.

**Mouse kidney immunofluorescence.** Mouse kidneys were perfusion fixed, immunofluorescently stained and micrographic images acquired as described previously (19). Secondary antibodies used were goat anti-rabbit, -mouse, or -guinea pig IgG conjugated to Alexa Fluor 488 or 555. Identification of tubule segments was made based on histological criteria and immunolocalization with the segment-specific marker proteins, Na-Ca exchanger/NCX1 (in the connecting tubules and cortical collecting duct principal cells) and vacuolar H-ATPase (apical membrane of \( \alpha \)-intercalated cells, \( \alpha \)-IC, and brush border of the proximal convoluted tubule) (19).

**In situ hybridization.** In situ hybridization was performed on fresh frozen kidney sections. PCR primers containing T7 and Sp6 promoter sites were used to generate DNA templates
specific for mouse claudin-10a or 10b. Digoxin-labeled RNA probes were used for hybridization and developed with HNPP (2-hydroxy-3-naphtoic acid-2’-phenylalanilide phosphate) fluorescent substrate by Dr. Yongqin Wu in the UNC Neuroscience Center Expression Localization Core.

RESULTS

Alternative splicing generates two variants of claudin-10. Our previous work showed that the different charge selectivities of claudins are influenced by the number and position of charged amino acid residues in the first extracellular domains (7; 8; 15). Thus, it was of interest to find two splice variants of claudin-10 in the cDNA sequence database, which differ in their first exon, and encode proteins which differ from the N-terminus to the last seven residues of the first extracellular domain, Figure 1. Inspection of the mouse claudin-10 gene (Mouse Genome Informatics Number 1913101, chromosome 14) reveals exon 1a precedes 1b and their splicing to shared exons 2 through 5 generates transcript variant-1 (claudin-10a, NM_023878) and variant-2 (claudin-10b, NM_021386), respectively. This led us to ask if the two forms of claudin-10 have different tissue distributions and charge selectivities and thus represent a novel mechanism to generate physiologically diversity from a single claudin gene. Claudin-18 has already been reported to undergo an analogous splicing of the first extracellular domain (20) although physiologic implications were not investigated.

Quantitative Real-Time PCR reveals that claudin-10b is ubiquitously expressed, while claudin-10a is unique to the kidney. Since none of the available antibodies discriminate between claudin-10a and 10b, we used quantitative PCR to determine whether the relative expression of the two transcripts differs among mouse tissues. Expression was measured relative to a control housekeeping transcript Eef1a1. Claudin-10b was found to be expressed in all tissues tested,
although it is most highly expressed in brain (cortex), kidney and lung, Figure 2A, with lower levels in other tissues including esophagus, jejunum, cecum, colon, heart and spleen. Claudin-10a, on the other hand, was only detected in kidney, where its level was similar to that of claudin-10b, Figure 2A.

Although the available antibodies do not distinguish between the isoforms, we attempted to verify expression of claudin-10 protein by immunoblotting in those tissues with the highest RNA levels, Figure 2B. Consistent with the qRT-PCR results, claudin-10 protein levels were highest in brain and kidney, but are also detectable in both lung and cecum. In liver, claudin-10 levels were undetectable by qRT-PCR or by immunoblot, although immunofluorescence microscopy revealed weak claudin-10 immunoreactivity in the tight junctions of hepatocytes (data not shown). For comparison, claudin-5 expression is ubiquitous and especially high in lung, Figure 2B.

Localization of claudin-10 in kidney and other tissues. We previously described the distribution of claudin-10 protein along the gastrointestinal tract where it localizes to both endothelial and epithelial tight junctions (14). In retrospect, this represented only the claudin-10b isoform. In the current study we performed additional immunolocalization of claudin-10 starting with tissues where the signal, based on mRNA analysis, represents exclusively the 10b isoform. Some atypical localizations were observed. In cecum, claudin-10, Figure 3B&D, colocalizes with its cytoplasmic binding partner ZO-1, Figure 3A&C, at epithelial tight junctions, but is also located on the lateral membranes in a subset of cells along the crypt-surface axis, Figure 3B. It is not obvious based on histological grounds what this unusual claudin distribution reveals and how these cells are unique. In brain, claudin-10 is colocalized with ZO-1 in endothelial cells, Figure 3E&F (arrows), as well as in as yet unidentified small fibrillar structures. In lung, claudin-10,
Figure 3H, is strongly colocalized with ZO-1, Figure 3G, in large airway epithelial cells and alveoli. In heart, claudin-10 is found with ZO-1 in endothelial cells, but not in the ZO-1 positive intercalated disks (data not shown).

In the kidney claudin-10 is widely expressed along the nephron; this is in contrast to other claudins which show restricted patterns, such as claudin-2 in the proximal convoluted tubule (PCT) (18) and claudin-16 in the TALH (Paracellin-1) (25). Claudin-10 colocalized with ZO-1 in numerous tubule types, including weak expression in both PCT and distal convoluted tubule, Figure 4A-C, high expression in the macula densa (Figure 4E), very high expression in both cortical and medullary thick ascending limbs of the loop of Henle (cTAL and mTAL), Figures 4D, 4G and both cortical (CCD) and inner medullary collecting ducts (IMCD). In the inner medulla, claudin-10 is strongly coexpressed with ZO-1 in the thin ascending limbs (Fig. 4H and at higher magnification in 4I) where the tight junctions appear tortuous due to the extensive lateral interdigitations between adjacent cells (24). Only the various cell types of the glomerulus lack detectable expression of claudin-10.

Because the claudin-10 antibody does not distinguish between 10a and 10b isoforms, we used in situ hybridization to determine if their transcripts were expressed in different regions of the kidney, Figure 5. The in situ probes include only sequences from either exon 1a or 1b and have no shared sequence. As one control for their specificity we performed in situ hybridization on sections of mouse brain (data not shown). We detected a signal for only the claudin-10b transcript, consistent with RT-PCR results which detect only the claudin-10b transcript in brain Figure 2A. Relative expression for claudin-10a mRNA is higher in tubules of the cortex than the medulla. In contrast, claudin-10b mRNA shows the opposite relative expression, higher in the medulla than cortex. Although claudin-1 is found in endothelia in other parts of the body, we
found no protein or transcript in the vasa recta. Based on the available data we conclude claudin-10 is widely expressed along the nephron and that 10a and 10b transcripts are more highly expressed in cortex and medulla, respectively.

Claudin-10a and 10b have different charge selectivities, which are conferred by charged residues in the first extracellular domain. Claudin-10a and 10b differ in the charge characteristics of their first extracellular domains. Most claudins share a set of membrane proximal charged residues in this domain which in claudin-10a correspond to positions E26, K28, and R75, Figure 6A. The corresponding charges on claudin-10b are at positions D29, K32 and R81, Figure 6A. When consideration of these shared positions is excluded, claudin-10a is found to contain more positively charged residues (R32, R59 and K66, 3 positive residues) and claudin-10b more negatively charged residues (D37, K52, D57, K65, D66 and D74, 4 negative and 3 positive residues). This led us to predict that claudin-10a might form paracellular pores that are more permeable to anions than those formed by claudin-10b. To test this hypothesis, we expressed both isoforms of claudin-10 separately in cultured epithelial models which are either cation-selective (MDCK II) or anion selective (LLC-PK1) and looked for transgene-induced changes from cell background selectivities. In addition, to test the idea that the positively charged residues in claudin-10a might enhance permeability of anions relative to cations, we mutated the three positive residues, indicated in Figure 6A, to negative aspartic acid residues and tested the physiologic effect of their expression in MDCK II cells. These mutations correspond to m1 (R32D), m2 (R59D) and m3 (K66D). Several (3-5) stable, independent clonal cell lines expressing claudin-10a, 10b and the mutants of 10a were generated for each construct, using a tetracycline-repressible promoter system and the Tet-off MDCK II cells previously described (28). This system allows maintenance of the transfected cells in the absence of transgene
expression and permits comparison of each clonal line with and without induction of the claudin. Claudin-10 was not detectable in the parental MDCK II cells nor in the transfected lines when expression was repressed, Figure 6B top panel, “-” lanes. Claudin-10a, 10b and the three mutants of 10a could all be induced by removal of doxycycline, Figure 6B top panel, “+” lanes. Induction of any form of claudin-10 had little consistent effect on the expression of claudin-2, claudin-4 (data not shown) or occludin; β-tubulin was used as a control to verify approximately equal loading of cell proteinCl. Claudin-10a, 10b, Figure 6C, and the three mutants, Figure 7, colocalized with ZO-1 at cell contacts as well as in intracellular vesicles, similar to the pattern seen for several other claudins (30).

MDCK II cells normally form a paracellular barrier with a strong cation preference. Using our protocol, dilution potentials for NaCl are in the range of +8 to +12 mV, consistent with a \( P_{Na}/P_{Cl} \approx 3.5 \). As predicted, expression of claudin-10a in this background resulted in a sharp decrease in dilution potential, from +10 mV down to +3 mV, consistent with a relative decrease in the ratio \( Na^+ \) to \( Cl^- \) permeability, Figure 8A and Table 2. Mutation of either of the first two positively charged amino acid residues to aspartic acid (m1-R32D and m2-R59D) eliminates their ability to decrease \( P_{Na}/P_{Cl} \), while the third mutant (m3-K66D) retain the ability of wild-type claudin-10a to decrease \( P_{Na}/P_{Cl} \). We conclude the first two but not the third position can influence permeability of ions moving through the tight junction. Expression of claudin-10b did not significantly change the dilution potential from that of uninduced MDCK II cells. We conclude claudin-10b was likely to confer high \( P_{Na}/P_{Cl} \) similar to and thus indistinguishable from the background properties of MDCK II cells.

 Transepithelial electrical resistance was not significantly changed by expression of claudin-10a, 10b or the mutants, Figure 8B. The ability of claudin-10a to reduce the dilution
potential without changing TER is the result of an opposing decrease in Na⁺ permeability and increase in Cl⁻ permeability, Table 2. This is the first time we have seen an increase in Cl⁻ permeability in MDCK II cells and the result is a leaky pore with half the normal cation selectivity that characterizes the MDCK II background. This pattern of opposing affects on ionic permeabilities is distinct from those observed following expression of claudin-4 (28), claudin-11 (30) or claudin-14 (2) in this same cell line. These claudins decrease Na⁺ permeability, with no effect on Cl⁻ permeability, with the result that total permeability and conductance (1/TER) are decreased.

The inability of claudin-10b to alter the dilution potential in MDCK II cells could either be attributed to intrinsic cation selectivity or might mean that the protein was not functional. To determine whether expression of 10b in a different cell background could reveal a functional cation-selectivity, we expressed it and 10a in the anion-selective LLC-PK₁ cell line. Both claudin-10 isoforms were inducible in the tet-off LLC-PK₁ cells, Figure 9A, and localize to both cell borders and in some intracellular vesicles, Figure 9B, as was seen in the MDCK II cells. The normal dilution potential for untransfected LLC-PK₁ cells under conditions described here (30) is -2 to -3 mV, Figure 9C, expression of claudin-10a in this cell background resulted in a slightly but not significantly more negative dilution potential. In contrast, expression of claudin-10b resulted in a significant increase in the dilution potential from approximately -2 to +1 mV.

Expression of both claudin-10a and 10b decreased TER, Figure 9D, however, in the case of claudin-10a, this was attributable to a significant increase in Cl⁻ permeability while in the case of claudin-10b, this was due mainly to a significant increase in Na⁺ permeability, Table 3. We conclude that claudin-10a creates leaky pores relatively more Cl⁻ permeable than Na⁺ and claudin-10b creates pores more permeable to Na⁺ than Cl⁻.
DISCUSSION

In this study, we investigated whether claudin-10a and 10b, two naturally occurring splice variants confer different effects on tight junction permselectivity. The two variants use alternative first exons resulting in different first extracellular domains, which could be significant because previous studies on other claudins implicate the first extracellular domains in charge selectivity (7). We find that claudin-10b is widely expressed in many tissues, but consistent with the possibility of distinct physiologic functions, claudin-10a is unique to the kidney. While we are unable to assign expression of claudin-10a and 10b to specific nephron segments, their mRNAs are preferentially expressed in either the medulla or cortex respectively. Directly expressing the two isoforms in cultured epithelial monolayers reveals that claudin-10a creates pores preferentially permeable to anions and 10b to cations. Site-specific mutagenesis reveals some but not all positively charged residues in the first extracellular domain of claudin-10a are required for its anionic selectivity. We conclude that alternative splicing of the first extracellular domain of claudin-10 represents a novel mechanism to generate diversity in paracellular permselectivity. This may also be true of claudin-18 (20) which shows a similar splicing pattern although its physiology has not been studied.

The finding that claudin-10a appears unique to kidney and the fact that we lack antibodies to distinguish it from claudin-10b raise the question of whether it is a real splice variant and protein. We tried several different biochemical approaches to distinguish between the isoforms. Isoelectric focusing followed by immunoblot did not resolve the two isoforms, but this technique is generally difficult for small, basic membrane proteins. We were unable to identify unique chemical or enzymatic cleavage sites which would generate different sized products from
10a and 10b on immunoblots. Attempts at proteomic discrimination of the variants in immunoprecipitated material was also unsuccessful; both proteins are of low abundance in native kidney. Nevertheless, we think the circumstantial evidence is overwhelming that claudin-10a is a real protein and not, for example, a pseudogene. First, 10a and 10b splice forms can be found in the database for humans, mice, dogs and cows. Second, the fact that highly homologous cDNAs have been sequenced for multiple species strongly supports proper RNA transcription and splicing from active genes. Protein encoded by human exon 1a is 97% identical to the same regions of both the mouse and dog sequences. In addition, when protein is expressed from the cldn-10a cDNA in either MDCK or LLC-PK1 cells it localizes properly to the junction and has unique affects on tight junction ion selectivity. We thus conclude 10a is a real splice variant and protein from the claudin-10 gene.

Claudin-10 has previously been reported in both endothelial (14) and epithelial (14; 16; 17) cells, raising the question of whether the splice variants segregate between these cell types. This is not the case since in tissues like cecum which express only claudin-10b, the protein is detected by immunofluorescence microscopy in both epithelial and endothelial cells. Also, in kidney, transcripts for both variants are detected in tubular epithelial cells; in some segments they appear to be expressed in the same cells. Beyond its expression in epithelial and endothelial cells, claudin-10b in the brain is located in small fibrillar structures which could represent glial or neural cell process. Identification of these structures awaits further study.

Ion permeability analysis of the two isoforms of claudin-10 revealed some distinct differences from other claudins. In the cation-selective MDCK II cell background, expression of a number of claudins were shown to decrease paracellular conduction by specifically decreasing cation permeability and without changing anion permeability. These include claudin-4 (28),
claudin-5 (31), claudin-8 (32), claudin-11 (30), and claudin-14 (2). In contrast, expressing claudin-10a simultaneously decreased $P_{Na^+}$ while increasing $P_{Cl^-}$, creating a unique type of pore with less overall ionic discrimination. The result of these counterbalancing changes is that overall resistance does not change. This increase in $P_{Cl^-}$ was also observed when this claudin was expressed in the LLC-PK$_1$ cell line. In contrast to claudin-10a, claudin-10b behaved in a fashion similar to that previously observed for claudin-2 (1; 30), in that it makes highly conductive and cation-selective pores in both MDCK and LLC-PK$_1$ cells. Consistent with our previous work on other claudins, some but not all, charged residues on the first extracellular domain of claudin-10a influence ionic permeability. In claudin-10a, R32 and R61 limit Na$^+$ permeability and presumably line the junctional pores. To summarize our observations on six different claudins, charge selectivity can be influenced by residues along the entire first extracellular loop, but not all charged residue positions exert equal influence. Further rationalization of selectivity will require more structural information on the pore. The unique permselectivity of each tissue likely results from the profile of claudins expressed, with each claudin having a unique influence on anion and cation permeability and resistance.

Some claudins have very restricted expression patterns along the nephron where they are proposed to confer segment-specific paracellular resistance and ion selectivity (6; 18; 22; 25). In contrast, we find that claudin-10 is expressed in all segments except the glomerulus. The most obvious finding from the in situ hybridization studies was that claudin-10b was very heavily expressed in the TAL, where by immunofluorescence the protein is also highly expressed and localized not only to tight junctions but also found on the lateral cell membrane. In contrast, claudin-10a mRNA was more abundant in the kidney cortex than in medulla, but there was considerable overlap between 10a and 10b mRNA expression. Immunofluorescent analysis
revealed that claudin-10 protein was expressed in almost every tubule segment in the kidney, usually but not always colocalized with ZO-1. This finding is different from results reported by Kiuchi-Saishin et al. (18), who reported the presence of claudin-10 only in proximal tubule and the thick ascending limb of the loop of Henle, but more similar to findings of Inai et al. (16) and Chabardes-Garonne et al. (6). This latter group used microarray analysis in freshly dissected kidney tubules to determine the overall gene expression profile and found claudin-10 expression in all tubule segments except glomerulus and collecting duct, although the heaviest expression was in the thick ascending limb of Henle. Our immunofluorescence results suggest that collecting duct does express claudin-10, although at relatively low levels, while glomerulus appears to be negative. Unlike what we have seen in other tissues, claudin-10 immunofluorescent labeling of blood vessels was either faint or undetectable, although others have reported vasa recta staining with the same claudin-10 antibody (16). The relatively heavy staining for claudin-10 in macula densa is intriguing but the physiological relevance is unclear.

It is tempting to speculate that the general distribution of the claudin-10 isoforms as identified by in situ hybridization in the kidney could be rationalized with current knowledge about the functions of different parts of the tubule. Claudin-10b creates leaky, cation-permeable pores. It is more highly expressed in kidney medulla than is claudin-10a, apparently in both the thin and thick ascending limbs of the loop of Henle. If this is true, it is consistent with the previously described Na\(^+\) and Cl\(^-\) transport in these segments, since in these tubule segments paracellular Na\(^+\) resorption is driven by lumen positive voltage, but Cl\(^-\) resorption is mediated mostly by transcellular transporters. Claudin-10a, which forms leaky anion permeable pores, is more highly expressed in cortex than medulla. By immunofluorescence, claudin-10 is more prominent in proximal tubules than in distal tubules. Claudin-2, which is a leaky cation pore, is
highly expressed in the proximal tubules, but if claudin-10a is also a component of this barrier, it would help explain the high paracellular Cl⁻ permeability that characterizes this tubule segment, particularly the distal proximal tubule. The expression of claudin-10 (if present as 10a) in collecting duct would be consistent with the reported paracellular Cl⁻ permeability in this segment, but the resolution of the in situ hybridization was insufficient to indicate the exact localization of claudin-10a. In any case, the exact molecular constituents of the tight junctions of these tubule segments, their organization and relative abundances are still unknown, and it is premature to infer much about the physiologic properties of the junction based on the reported presence of even several claudins. The role of claudin-10 in the kidney requires further study although our observations clearly show that alternative splicing is an additional mechanism for generating diversity in permselectivity of tight junctions.
Disclosures: none

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Figure Legends

Figure 1. Alternative splicing generates two isoforms of claudin-10, which differ in their first extracellular domains. A: Schematic organization of the exons within the mouse claudin-10 gene (Mouse Genome Informatics Number 1913101) on chromosome 14. Alternative slicing of exons 1a and 1b to shared exons 2 through 5 generates transcript variant-1 (claudin-10a, NM_023878) and variant-2, (claudin-10b, NM_021386). Start and stop codons are noted. B: Amino acid sequence alignments of claudin-10a and 10b. Identical sequences encode by exons 2-5 are underlined and shaded. The four transmembrane segments are boxed. The sequences of claudin-10a and 10b differ up to exon 2, which begins with the last 7 residues of the first extracellular domain, GYIQACR. Sequences encoded by exons 1a and 1b are 53% homologous and their identical residues are shaded.

Figure 2. Relative expression of mRNA for claudin-10a and claudin-10b and for total claudin-10 protein in various mouse tissues. A: Expression of claudin-10a and 10b transcripts determined by qRT-PCR in brain cortex, whole kidney, lung and cecum expressed relative to Ef1a1. Claudin-10b is widely expressed but at different levels, while 10a is detected only in the kidney. In most cases the variation (see methods) is too small to be visible on the graph. B: Relative protein expression of claudin-10 in brain cortex, whole kidney, lung, cecum and liver. Antibodies used do not discriminate claudin-10a from 10b. Expression profile for claudin-5 is shown for comparison. 5 ug of protein was loaded in each lane. Mr markers in kDa.
**Figure 3.** Immunofluorescent protein colocalization of ZO-1 (left panels) with claudin-10 (right panels). In cecum, claudin-10 (B and D) colocalizes ZO-1 (A and C) at epithelial tight junctions from the crypt base to the surface. Claudin-10 is also found on the lateral membranes of a subset of unidentified epithelial cells (arrow). In cerebellar cortex (E and F), claudin 10 colocalizes with ZO-1 in endothelial cells (arrows) and is also found in fine fibrillar structure with lack ZO-1 staining (arrowhead). In the lung, ZO-1 (G) and claudin-10 colocalize to tight junctions in alveoli (AL) and large airway surface cells (AW). Bar, 20µm.

**Figure 4.** Immunolocalization of claudin-10 in mouse kidney cryosections. Claudin-10 is stained green in every panel. **A-C.** Double staining for claudin-10 (A), ZO-1 (red, B) and merged image (C). Claudin-10 is weakly expressed in proximal convoluted tubule (PCT) and distal convoluted tubule (DCT). Claudin-10 overlaps with ZO-1 at the tight junction; note though that ZO-1 extends further along the lateral membrane, presumably into the adherens junction, where claudin-10 is absent (arrowheads in C). **D.** Claudin-10 is strongly expressed in the cortical thick ascending limb (cTAL) at the basolateral membrane (note deep infoldings of the basal membrane) and at the tight junction (arrowheads). **E.** Claudin-10 expression in a section of the cTAL (asterisk denotes the lumen) at the level of the juxtaglomerular apparatus. Note strong claudin-10 staining along the basolateral membrane of macula densa cells (arrow, note the simplified basal membrane), which abut the vascular pole of the glomerulus (G), as well as in more typical cTAL cells on the opposite side of the tubule (with typical deep infoldings of the basal membrane). The section is counterstained with antibody to claudin-2 which happens to highlight the glomerular basement membrane and PCT. **F.** Claudin-10 is expressed in tight junctions of the cortical collecting duct (arrows, CCD) and connecting tubule (CNT). Tubules
are identified by staining with antibody against the Na-Ca exchanger, NCX1 (red, CNT and CCD principal cells) and the vacuolar H-ATPase (blue, apical membrane of α-intercalated cells, α-IC, and brush border of the PCT). G. Claudin-10 is also strongly expressed at the basolateral membrane and tight junction (arrowheads) of the medullary thick ascending limb (mTAL) and the tight junction of the outer medullary collecting duct (OMCD). Here the tight junctions are highlighted with anti-ZO-1 (red). H. In the inner medulla, claudin-10 is strongly expressed in thin ascending limbs (arrows) and weakly expressed at the tight junctions of thin descending limbs (asterisks) and inner medullary collecting ducts (IMCD). I. Higher magnification view of two thin ascending limbs shows claudin-10 colocalization with ZO-1 (red) at their tight junctions, which appear serpentine and tortuous due to the extensive lateral interdigitations between neighboring epithelial cells. Scale bars represent 25 µm.

**Figure 5.** In situ hybridization for claudin-10a and 10b transcripts in mouse kidney. Collages from cortex to inner medulla were assembled digitally from sequential immunofluorescence microscopic images all taken at a single exposure setting. The relative expression of 10a is higher in cortex and 10b in medulla. C (cortex), OM (outer medulla) and IM (inner medulla).

**Figure 6.** Expression of claudin-10a, 10b and three charge-reversing mutations of claudin-10a in MDCK II cells monolayers. A: Sequences of the first extracellular domains of claudin-10a, 10b and 10a mutants m1(R32D), m2(R52D) and m3 (K66D). The final seven residues of the first extracellular domains are encoded by exon 2 and are underlined. Identical residues in 10a and 10b are shaded. Positive residues are indicated in blue, negative residues are indicated in red. Position numbers of the first and last residues in the extracellular domains are indicated. B:
Immunoblot showing inducible expression of all proteins in representative cell lines. Repressed in the presence of doxycycline (-) and induced in its absence (+). Induction has no reproducible effect on the levels of endogenous claudin-2 and occludin. Beta-tubulin was used as a loading control. C: Immunofluorescent co-localization of ZO-1 (left) with claudin-10a and 10b before (-) and after (+) induction. The induced proteins colocalize with ZO-1 at tight junctions and additional pools are observed in the cytoplasm.

**Figure 7.** Immunofluorescent co-localization of claudin-10a mutants (left) with ZO-1 (right) in MDCK cell monolayers induced to express the transgenes. All the induced mutant proteins (m1, m2 and m3) colocalize with ZO-1 at tight junctions and additional pools are observed in the cytoplasm.

**Figure 8.** Electrophysiologic consequences of expressing claudin-10a, 10b and three charge-reversing mutations of claudin-10a in MDCK II cell monolayers. A: Dilution potential (mV) across MDCK monolayers following a 120 to 60mM apical NaCl dilution for uninduced (open bars) and their induced matched clones (filled bars). The positive dilution potential reveals the cation selectivity of the parental MDCK monolayers. Expression of claudin-10a reduces cation selectivity while expression of claudin-10b confers no change. M1 and m2 eliminate the effect of expressing claudin-10a while m3 shows the selectivity of wild-type 10a. B. Despite different affects on charge selectivity there was no statistically significant effect on transepithelial electrical resistance (TER). Average of 3-5 clones and duplicate measurements on each; means ± SE. * P < 0.05. Ion permeabilities are reported in Table 1.
Figure 9. Consequences of expressing claudin-10a and 10b in anion-selective LLC-PK₁ cell monolayers. **A:** Immunoblot showing doxycycline-regulated induction of claudin-10a and 10b in representative LLC-PK₁ clones. (-) promoter suppressed in the presence of doxycycline; (+) activated in its absence. Five clones were studied for each claudin. Immunoblotting for β-tubulin on the same blot confirms similar protein loading. Mr markers in kDa. **B:** Immunofluorescent microscopic colocalization of ZO-1 with induced claudin-10a (top panels) and 10b (bottom panels). **C:** Dilution potentials (mV) across LLC-PK₁ monolayers following a 120mM to 60 mM NaCl dilution for untransfected cells (LLC-PK₁), claudin-10a and 10b. The negative dilution potential reveals the anion selective background of LLC-PK₁ monolayers. Expression of claudin-10a does not alter the anion selectivity while expression of claudin-10b produces a cation-selective monolayer. **D:** Despite different affects on charge selectivity both 10a and 10b cause a statistically significant reduction in transepithelial electrical resistance. Average of 3-5 clones and duplicate measurements on each, means ± SE. * P < 0.05.
Tables and table legends

**Table 1. Quantitative PCR Primers**

All primers were designed using Primer3 software (23).

**Table 2. Permselectivity of MDCK II monolayers before and after expressing claudin-10a, claudin-10b and charge-reversing mutants of claudin-10a.**

\(^a\)Calculated using the constant field equation (5; 9); \(^b\)calculated using the Kimiuzuka-Kokerzu equation (9); \(^c\)P<0.05, ANOVA followed by Tukey’s test.

**Table 3. Permselectivity properties of LLC-PK1 monolayers before and after expressing Cldn-10a or Cldn-10b.**

\(^a\)Calculated using the constant field equation (5); \(^b\)calculated using the Kimiuzuka-Kokerzu equation (9); \(^c\)P<0.05, ANOVA followed by Tukey’s test.
### Table 1. Quantitative PCR Primers

<table>
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<tr>
<th>Gene</th>
<th>Accession</th>
<th>5' primer</th>
<th>3' primer</th>
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All primers were designed using Primer3 software (23).
Table 2. Permselectivity of MDCK II monolayers before and after expressing claudin-10a, claudin-10b and charge-reversing mutants of claudin-10a.

<table>
<thead>
<tr>
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<th>P_{02}/P_{H2}</th>
<th>P_{36} (10^{-5} cm/s)</th>
<th>P_{CE} (10^{-6} cm/s)</th>
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<td>Induced</td>
<td>Uninduced</td>
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<td>Clhn-10b</td>
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<td>0.182±0.009</td>
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*Calculated using the constant field equation (5, 9); †calculated using the Kimura-Kochka equation (9); ‡P<0.05, ANOVA followed by Tukey’s test.
Table 3. Permeability properties of LLC-PK1 monolayers before and after expressing Cldn-10a or Cldn-10b.

<table>
<thead>
<tr>
<th></th>
<th>$P_{cl}/P_m$</th>
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<th>$P_{oc}$ ($10^{-6}$ cm/s)</th>
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$^a$Calculated using the constant field equation (5); $^b$calculated using the Kimizu-Kohzu equation (2); $^c$P<0.05, ANOVA followed by Tukey's test.
25 + + + 75
10A NE-WKVTRASSVITATWVYQGLWMNCAGNALGSFHCRPHTIFPVAGYIQACR
M1 R32D NE-WKVTRASSVITATWVYQGLWMNCAGNALGSFHCRPHTIFPVAGYIQACR
M2 R59D NE-WKVTRASSVITATWVYQGLWMNCAGNALGSFHCDFPVAGYIQACR
M3 K66D NE-WKVTRASSVITATWVYQGLWMNCAGNALGSFHCDFPVAGYIQACR

10B TDYWVKVSTIDGTIVITTATYWANLWKACVTDSTGVSNCKDFPSMLALDGYIQACR
28 - + + + - 81