Distribution of the tight junction proteins ZO-1, occludin, and claudin-4, -8, and -12 in bladder epithelium

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Am J Physiol Renal Physiol 287: F305–F318, 2004. First published April 6, 2004; 10.1152/ajprenal.00341.2003. —In mammals, the bladder stores urine without permitting the passage of urine contents into the bloodstream, a function, in part, of the uroepithelial-associated tight junction complex. The protein constituents that make up this high-resistance barrier in the bladder are currently unknown, although the claudins, a multigene family, are thought to govern paracellular transport in other epithelia. Reverse transcriptase-polymerase chain reaction analysis was used to define that mRNA for claudin-2, -4, -8, -12, and -13 was expressed in mouse bladder tissue. The localization of these claudins, as well as the tight junction-associated proteins zonula occludens-1 (ZO-1) and occludin, within the bladder epithelium was determined by immunofluorescence microscopy. As expected, occludin and ZO-1 were localized to the tight junctions of rat, mouse, and rabbit umbrella cells. Intriguingly, ZO-1 in mouse epithelium, ZO-1 in the dome region of rabbit bladders and occludin in rat and mouse bladders were also expressed in the underlying intermediate and basal cell layers. Claudin-4, -8, and -12 were found in the umbrella cell tight junction; however, additional staining of claudin-4 was observed along the sites of cell-cell contact in the underlying cell layers of rat, mouse, and rabbit tissue. No claudin-2 staining was associated with tight junctions in the uroepithelium. Our results indicate that claudin-4, -8, and -12 are expressed in umbrella cells, where they may impart the high-resistance phenotype associated with this cell type, and that in some instances tight junction proteins are also associated at the sites of cell contact of the underlying cell layers, perhaps playing some role in cell-cell adhesion.

In mammal, the bladder must store urine for prolonged periods of time while limiting the passage of water, electrolytes, and highly permeable molecules such as ammonia into the bloodstream. The barrier function of the bladder lies in the uroepithelium, which is made up of three layers: the superficial umbrella cell layer, the underlying intermediate cell layer, and the basal cell layer (2, 17). The latter two serve as progenitors for the umbrella cell layer. The umbrella cell layer contains specialized apical membrane lipids as well as asymmetric unit membrane particles that form a barrier to unregulated movement of ions and solutes between adjacent cells (i.e., gate function) and prevent mixing of proteins and lipids in the outer leaflet of the apical and basolateral plasma membrane domains (i.e., fence function) and thus maintain the specialized plasma membrane domains crucial for epithelial function (15, 40, 41). The relatively modest levels of transcellular ion transport and high resistance to paracellular ion flow result in measured transepithelial resistances (TERs) across the uroepithelium as high as 75,000–135,000 Ω·cm² (24, 44).

Tight junctions are composed of a dense network of cytoplasmic proteins, cytoskeletal elements, as well as several transmembrane proteins (15). The cytoplasmic proteins, which include zonula occludens-1 (ZO-1), are thought to form a scaffold that links the tight junction-associated transmembrane proteins to the perijunctional actin cytoskeleton and may also be important in transducing regulatory signals that control the paracellular barrier (26). Transmembrane proteins associated with the tight junctions include junctional-associated membrane protein-1 (25), possibly the Coxackie and adenovirus-associated receptor (6, 43), the IgG antigen (30), and the tetraspan proteins including occludin (−65 kDa) and the claudins (−22 kDa) (10, 13). The latter two form continuous branching fibrils of transmembrane particles that completely encircle the apical aspect of the lateral surface of each cell, creating a complex barrier, which shows ion and size selectivity (40).

Occludin is the product of a single gene, is found in two splice variants, is expressed by most epithelial cells, and is localized to tight junctions (13, 15, 36). Expression of truncated versions of occludin in cultured epithelial cells disrupts gate and fence function (5); however, disruption of the gene encoding occludin does not affect tight junction formation, integrity, or function in embryonic stem cells (35). Knockout mice lacking expression of occludin have a complicated phenotype including inflamed and hyperplastic gastric epithelium, testicular atrophy, presence of calcifications in the brain, and thinning of compact bone tissue (36). The relationship between these abnormalities and tight junction formation or function is unknown.

Claudins compromise a multigene family with 24 members identified to date (41). The expression pattern of claudins is tissue and segment specific (20, 33). In the kidney, for example, claudin-2 is expressed in the proximal tubule (9, 20), claudin-4 is expressed in the thin ascending limb of Henle and collecting duct (20), and claudin-16 is expressed in the thick
ascending limb of Henle and the distal convoluted tubule (37). Hereditary defects in expression of functional claudin-16 lead to a loss of paracellular recovery of Mg"^2+" resulting in severe hypomagnesaemia (37). Different claudins can exist within the same junction, where they interact in both a homotypic and heterotypic manner, and permutations of claudins expressed in each cell or tissue may define the unique paracellular properties of each epithelium (14, 40). In this regard, it has been shown that overexpression of claudin-1, -4, -8, or -15 in Madin-Darby canine kidney (MDCK) cells increases TER (7, 8, 27, 42, 45). In contrast, expression of claudin-2 decreases TER in high-resistance variants of this cell line that do not normally express this claudin isoform (1, 11). Electrophysiological studies confirm that claudin-2, -4, -8, and -12 act to regulate paracellular ion conductivity (1, 7, 8, 42). In claudin-4, this regulation is dependent on charged residues in the first extracellular loop (7). Similarly, substitutions of negative-charged amino acids with positive-charged ones in the first extracellular loop of claudin-15 convert the paracellular pathway from one selective for Na^+ over Cl^- to one that is selective for Cl^- over Na^+ (7).

At present, little is known about the specific tight junction proteins that form the high-resistance paracellular pathway that is associated with the bladder epithelium. ZO-1 and occludin have been localized to the umbrella cell layer (21, 22), and expression of claudin-4 has been described in the underlying intermediate and basal cell layers (29). However, it is unknown whether other claudins are expressed in the bladder or whether expression of tight junction proteins is limited to specific cell layers or regions of the bladder, or whether there is species variation in the expression and distribution of these proteins. We observed that mRNA for claudin-2, -4, -8, -12, and -13 was present in mouse bladder tissue. When immunolocalized, claudin-4 was expressed in the tight junctions of umbrella cells as well as along the sites of cell-cell contact in all three epithelial cell layers, and claudin-8 and -12 were localized to the tight junctions of the umbrella cell layer. Claudin-2 was not found associated with cellular junctions. ZO-1 and occludin were found in the tight junctions of the umbrella cells; however, in some instances they were also found at sites of cell-cell contact. Our results indicate that the high-resistance paracellular pathway observed in bladder epithelium may reflect the expression of claudin-4, -8, and -12. In addition, the localization of ZO-1, occludin, and claudin-4 to the sites of cell-cell contact provides evidence that tight junction proteins may also play some role in cell adhesion.

MATERIALS AND METHODS

Materials, antibodies, and other labeled reagents. Unless specified otherwise, all chemicals were obtained from Sigma (St. Louis, MO) and were of reagent grade or better. Rabbit antisera to claudin-2, -4, -8, or -12 were characterized previously (10, 11, 20, 32) and were of reagent grade or better. Rabbit antisera to claudin-2, -4, -8, -10, or -12 were characterized previously (10, 11, 20, 32) and were of reagent grade or better. Rabbit antisera were used at dilutions of 1:500–1:5,000. Purified rabbit polyclonal anti-claudin-2 or claudin-11 antibody (Zymed Laboratories, West Grove, PA) was used at a final concentration of 2.5 μg/ml. FITC-conjugated goat anti-rat (Jackson ImmunoResearch Laboratories, West Grove, PA) was used at a final concentration of 2.3 μg/ml; FITC-conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories) was used at a final concentration of 2.3 μg/ml; FITC-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories) was used at a final concentration of 6.8 μg/ml; rhodamine phalloidin (Molecular Probes, Portland, OR) was used at a dilution at a final concentration of 6 U/ml; and TO-PRO3 (Molecular Probes) was used at a dilution of 1:1,000.

Animals. Urinary bladders were obtained from female New Zealand White rabbits (3–4 kg), female Swiss-Webster or C57BL/6J mice (3–4 mo old), or female Sprague-Dawley rats weighing 275–350 g. All animals were fed a standard diet with free access to water before euthanization. Rabbits were euthanized with 300 mg of pentobarbital sodium (dissolved in 5 ml of water) injected into the ear vein. Mice and rats were euthanized by inhalation of 100% CO2. After euthanization and thorocotomy, the bladder was rapidly excised and processed for immunolocalization and/or RT-PCR. Bladders from two sets of three mice were placed in RNA Later solution (Qiagen, Valencia, CA) to prevent RNA degradation. Total RNA was extracted from the tissue using an RNeasy kit (Qiagen) following the manufacturer’s protocol for animal tissues. Homogenization was performed using a hand-held Polytron tissue homogenizer (Brinkmann, New Haven, CT). Purified total RNA was stored at −80°C. Total RNA for mouse brain and 17-day mouse embryos was purchased from Clontech. First-strand cDNA synthesis was accomplished using 1 μg of total RNA and a First-Strand Synthesis Kit (Roche Diagnostics, Pleasanton, CA) according to the manufacturer’s protocol. PCR using the first-strand reaction product was carried out using a Taq Core Master Mix PCR kit (Qiagen). Primers for each claudin subtype were designed using Primer3 software (www-genome.wi.mit.edu/cgi-bin/primer/primer3 WWW.cgi) and are shown in Table 1. The ideal annealing temperature for each claudin subtype was empirically determined by running the PCR reaction from 44 to 66°C in 2°C steps in a Gradient 96 Robocycler (Stratagene, La Jolla, CA). Cycling temperatures for the PCR reactions were as follows: 95°C for 10 min; then 35 cycles of 95°C for 1 min, 44–66°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min. Final products were run on a 2.0% agarose horizontal gel stained with ethidium bromide to visualize the PCR products. Products were compared with a 100-bp ladder (PGC Scientific, Frederick, MD) to estimate band size. Samples that showed a positive result in the gel were purified using a MiniElute Kit (Qiagen) for purification of PCR products and sent to the University of Pittsburgh’s DNA Sequencing Facility for verification of the sequence.

Immunofluorescence labeling of bladder tissue. Bladders from rabbits, mice, or rats were fixed in 4% paraformaldehyde, 100 mM sodium cacodylate buffer, pH 7.4, for 2 h at room temperature and then either processed for immunofluorescence staining or cryosectioned. Rat bladders from 3–4 mo old, were either processed for immunofluorescence staining or cryosectioned. Rat bladders from 3–4 mo old, or female Sprague-Dawley rats weighing 275–350 g. All animals were fed a standard diet with free access to water before euthanization. Rabbits were euthanized with 300 mg of pentobarbital sodium (dissolved in 5 ml of water) injected into the ear vein. Mice and rats were euthanized by inhalation of 100% CO2. After euthanization and thorocotomy, the bladder was rapidly excised and processed for immunolocalization and/or RT-PCR. Bladders from two sets of three mice were placed in RNA Later solution (Qiagen, Valencia, CA) to prevent RNA degradation. Total RNA was extracted from the tissue using an RNeasy kit (Qiagen) following the manufacturer’s protocol for animal tissues. Homogenization was performed using a hand-held Polytron tissue homogenizer (Brinkmann, New Haven, CT). Purified total RNA was stored at −80°C. Total RNA for mouse brain and 17-day mouse embryos was purchased from Clontech. First-strand cDNA synthesis was accomplished using 1 μg of total RNA and a First-Strand Synthesis Kit (Roche Diagnostics, Pleasanton, CA) according to the manufacturer’s protocol. PCR using the first-strand reaction product was carried out using a Taq Core Master Mix PCR kit (Qiagen). Primers for each claudin subtype were designed using Primer3 software (www-genome.wi.mit.edu/cgi-bin/primer/primer3 WWW.cgi) and are shown in Table 1. The ideal annealing temperature for each claudin subtype was empirically determined by running the PCR reaction from 44 to 66°C in 2°C steps in a Gradient 96 Robocycler (Stratagene, La Jolla, CA). Cycling temperatures for the PCR reactions were as follows: 95°C for 10 min; then 35 cycles of 95°C for 1 min, 44–66°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min. Final products were run on a 2.0% agarose horizontal gel stained with ethidium bromide to visualize the PCR products. Products were compared with a 100-bp ladder (PGC Scientific, Frederick, MD) to estimate band size. Samples that showed a positive result in the gel were purified using a MiniElute Kit (Qiagen) for purification of PCR products and sent to the University of Pittsburgh’s DNA Sequencing Facility for verification of the sequence.

Immunofluorescence labeling of bladder tissue. Bladders from rabbits, mice, or rats were fixed in 4% paraformaldehyde, 100 mM sodium cacodylate buffer, pH 7.4, for 2 h at room temperature and then either processed for immunofluorescence analysis (whole mounts) or cryoprotected by incubation in PBS containing 30% (wt/wt) sucrose overnight at 4°C on a rotator (tissue sections). Cryoprotected tissue was then placed in OCT compound (Tissue-Tek, Torrance, CA) and frozen in molds placed on dry-ice blocks. Frozen tissue blocks were stored at −80°C. Sections were cut at a thickness of 10 μm using a CM1900 microtome (Leica, Bannockburn, IL), collected on Fisherbrand Superfrost/Plus slides (Fisher, Pittsburgh, PA), and then dehydrated by incubating the slides for 1 min at room temperature followed by three washes with PBS for 5 min. For both
whole mount tissue and cryosections, paraformaldehyde was quenched and the cells were permeabilized with PBS containing 20 mM glycine, pH 8.0, 75 mM ammonium chloride, and 0.1% (vol/vol) Triton X-100 for 10–15 min at room temperature. The cells were washed with block solution [PBS containing 0.7% (wt/vol) fish skin gelatin and 0.025% (wt/vol) saponin] and then incubated in block solution containing 10% (vol/vol) newborn calf serum for 15 min at ambient temperature. Tissue was subsequently washed in block solution three times over 15 min and then incubated for 60–120 min at ambient temperature with primary antibody diluted in block solution. After incubation with the primary antibody, the tissue was washed three times with block solution over a 15-min period of time. The tissue was then incubated with the secondary antibody for 60–120 min at ambient temperature and then washed three times with block solution over a 15-min time period. After three washes with PBS, the tissue was postfixed for 10–15 min in 4% (wt/vol) paraformaldehyde in 100 mM sodium cacodylate, pH 7.4. After a final wash with PBS, coverslips were placed over the stained cryosections using p-diaminobenzidine-containing mounting medium (3). In the case of whole mounts, it was necessary to place plastic spacers (50 μm high) underneath the coverslip to raise it above the tissue.

**Immunodepletion of claudin-specific antisera.** cDNAs encoding fusion proteins of glutathione-S-transferase (GST) fused to the COOH terminus of claudin-2, -4, -8, or -12 were kindly provided by Dr. M. Furuse.

_Escherichia coli_, transfected with GST-claudin cDNA, was induced with 100 μg/ml IPTG for 2 h to produce GST-claudin fusion protein, lysed by sonication, and GST-claudin proteins were purified on glutathione agarose (Amersham Biosciences, Piscataway, NJ) using the manufacturer’s protocol (Amersham Biosciences). After elution and dialysis, the GST-claudin fusion proteins were stored at −80°C. For immunodepletion, 250–500 μg of GST alone or GST-claudin fusion protein were bound to 50 μl of glutathione-agarose beads in PBS for 2 h at ambient temperature and then washed three times with PBS to remove unbound protein. The washed beads were then incubated overnight at 4°C on a rotator with claudin-specific antisera diluted in 0.5 ml of block solution. The beads were recovered by centrifugation, and the remaining block solution was then used during the primary antibody incubation step described in the previous section.

**Scanning laser confocal analysis of fluorescently labeled cells.** Imaging was performed on a TCS-SL confocal microscope equipped with argon and green and red helium-neon lasers (Leica, Dearfield, IL). Images were acquired by sequential scanning using a ×40 (1.25 numerical aperture) or a ×100 (1.4 numerical aperture) plan-apochromat oil objective and the appropriate filter combination. Settings were as follows: photomultipliers set to 600–800 V, 1 Airy disk, and Kalman filter (n = 4). For cryosections, serial (z) sections were captured with a 0.5-μm step size. For whole mounts, z sections were captured with a 0.25-μm step size. The images (512 × 512 pixels) were saved as TIFF files. For the cryosections, the OpenLab program (Improvision, Lexington, MA) was used to project the serial sections into one image. For whole mounts, the Velocity program (Improvision) was used to generate x-z sections and the three-dimensional, tilted projections of the images. The contrast level of the final images was adjusted in the Photoshop program (Adobe, Mountain View, CA), and the contrast-corrected images were imported into FreeHand (Macromedia, San Francisco, CA).

**RESULTS**

**RT-PCR reveals expression of claudin-2, -4, -8, -12, and -13 in mouse bladders.** Our initial goal was to define which claudins were expressed in the mammalian bladder. Mice were chosen for this analysis because many of the claudin isoforms have been identified and cloned in this species, thus facilitating primer design for the PCR analysis (see Table 1). Unambiguous mouse sequences or no sequences were available for claudin-17 and -20–24. PCR products of the expected size were obtained for claudin-2, -4, -8, -12, and -13 (Fig. 1A). The product for claudin-2 was not abundant. The PCR fragments

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<th>Claudin Subtype</th>
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<tr>
<td>1</td>
<td>AGCTCCGCGCGACATTAGTGG</td>
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<tr>
<td>2</td>
<td>GTGCTTGTAGTGTTGAGGT</td>
<td>178</td>
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<tr>
<td>3</td>
<td>CCAAAGAAAACACGGCTGAG</td>
<td>247</td>
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<tr>
<td>4</td>
<td>ATGGCCTCTATGGGACTACA</td>
<td>633</td>
</tr>
<tr>
<td>5</td>
<td>CCTCTCGGAGCACAAGACAGT</td>
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</tr>
<tr>
<td>6</td>
<td>GTCTCGTGCTGCTGACAGCA</td>
<td>660</td>
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<tr>
<td>7</td>
<td>TCTCTGTGAGGTCGTGCTG</td>
<td>158</td>
</tr>
<tr>
<td>8</td>
<td>CCGGAACCGAGCAGAAGACGAC</td>
<td>208</td>
</tr>
<tr>
<td>9</td>
<td>AGAGGAGACTGGGCTTGG</td>
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<tr>
<td>10</td>
<td>AACCAGAGGAATTGGAAGTAG</td>
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</tr>
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<td>12</td>
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<tr>
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<tr>
<td>14</td>
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<td>17</td>
<td>AGATCGCCGAGTCTCTGT</td>
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<tr>
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Primer design for the PCR analysis (see Table 1). Unambiguous mouse sequences or no sequences were available for claudin-17 and -20–24. PCR products of the expected size were obtained for claudin-2, -4, -8, -12, and -13 (Fig. 1A). The product for claudin-2 was not abundant. The PCR fragments...
were purified, and DNA sequencing confirmed that the products corresponded to the known DNA sequence of the appropriate claudin isoform.

Positive control reactions for claudin-1–16 were also performed (Fig. 1B). Products of the expected sizes were observed for claudin-1–8, -10, and -12–16, confirming that primers for these particular isoforms were able to amplify claudin isoform-specific products from tissues known to express these proteins. However, we were unable to obtain positive controls for claudin-9, -11, -18, or -19. Claudin-9 and -18 were recently localized to the epithelia associated with the inner ear (19); however, obtaining RNA from the inner ear is difficult and was not attempted. Two different strains of mice (Swiss-Webster or C57BL/6J) and two different sets of PCR primers were used to identify message for claudin-11, a protein known to be expressed in the nephron of the kidney (20); however, all attempts were unsuccessful. No expression was observed when bladder tissue was stained with a commercially available claudin-11-specific antibody (data not shown). There are no published reports of claudin-19 tissue distribution.

Distribution of ZO-1 and occludin in rabbit, mouse, and rat uroepithelium. Before characterizing the specific claudin proteins expressed by the bladder epithelium, we first examined the expression and distribution of well-characterized tight junction proteins, ZO-1 and occludin, in rabbit, mouse, and rat bladder tissue. Bladders from multiple species were examined to define whether there were any interspecies differences in expression of these proteins. Furthermore, rabbit bladders were subdivided into three regions, i.e., neck, equatorial, and dome, to determine whether there were any regional differences in the expression of these proteins. The neck region was defined as the third of the bladder, just above the urethra, that included the sites of ureter insertion. The equatorial region included the middle third of the bladder and the dome region of the last third of the organ. Finally, to define the regions of cell–cell contact in the different cell layers and to identify the apicolateral junction in the umbrella cell layer, the tissue was stained with rhodamine-phalloidin. This phalloidin labels the filamentous actin-rich cortical cytoskeleton of the cells and pronouncedly labels the actin-rich junctional ring at the apicolateral border of the cells. ToPro-3 was used to label cell nuclei.

A three-dimensional reconstruction of the umbrella cell layer from the equatorial region of whole mounted rabbit bladder tissue is shown in Fig. 2A. ZO-1 was localized at the periphery of the large polyhedral umbrella cells where it colocalized with actin at the apicolateral junction of the cells. Colocalization was apparent when the tissue was viewed in tilted reconstructions (Fig. 2A, right) or in x-z cross section (arrows in Fig. 2B). Similar results were observed for the other two regions of the rabbit bladder and for mouse and rat bladders. In preliminary experiments, we observed that some of the antibodies used in our analysis penetrated poorly into the underlying cell layers of whole mounted tissue. To obviate this problem, we also examined the localization of these proteins in tissue cryosections. The three cell layers of the cryosectioned rabbit uroepithelium are annotated in Fig. 2, C–E, middle: umbrella cells (UC) are marked by long thin arrows, intermediate cells by filled circles, and basal cells by filled triangles. In the neck and equatorial regions of the rabbit bladder, ZO-1 was localized to the tight junctions of the umbrella cell layer (Fig. 2, C and D). ZO-1 staining was also observed in the capillary endothelial cells that underlie the basal cell layer (marked with * in Fig. 2, C and D). In the dome region of rabbit bladder, ZO-1 was localized to both the apicolateral tight junctions (marked with arrows in Fig. 2E, right and left) as well as to the...
sites of cell-cell contact in the intermediate and basal cell layers (Fig. 2E).

Compared with rabbit bladder, the mouse epithelium was relatively thin and comprised only a few cell layers (which like Fig. 2 are annotated in Fig. 3A). In this tissue, ZO-1 was localized to punctate structures at the apicolateral borders of adjacent umbrella cells (see arrows in Fig. 3A, right and left), consistent with localization to tight junction. However, significant ZO-1 staining was also found associated with the underlying cell layers in what appeared to be a cytoplasmic distribution. The rat uroepithelium was also relatively thin, and ZO-1 was observed in the superficial umbrella cell layer at the
apicolateral junction (see arrows in Fig. 3B, right and left) and to a lesser extent along the basolateral surface of this cell layer (Fig. 3B).

In most simple epithelia, occludin is concentrated in the tight junctional ring at the apex of the cell. This expected distribution was observed in all three layers of rabbit bladder epithelium (Fig. 4, A-C). Unlike ZO-1, significant occludin was not observed in the capillary bed. In rat and mouse bladder, occludin was also found in an apicolateral distribution (marked with arrows in Fig. 4, D and E). However, significant occludin was also expressed along the basolateral surface of the umbrella cell layer and on the plasma membranes of the underlying cell layers (Fig. 4, D and E).

Localization of claudin-4, -8, and -12 to bladder epithelium. Because whole mouse bladders were used in the RT-PCR analysis, immunofluorescence was used to determine which claudins were expressed in the uroepithelium. The distribution of claudin-2, -4, -8, or -12 was examined in mouse and rat tissue using purified rabbit polyclonal antibodies or rabbit polyclonal antibodies found in serum, whereas claudin-4 expression was examined in rabbit tissue using a commercially available mouse monoclonal antibody. No antibodies are currently available for assessing the distribution of claudin-13.

When mouse or rat bladder tissue was stained with claudin-2-specific antiserum, expression was limited to the uroepithelium. The distribution of claudin-2, -4, -8, or -12 was examined in mouse and rat tissue using purified rabbit polyclonal antibodies or rabbit polyclonal antibodies found in serum, whereas claudin-4 expression was examined in rabbit tissue using a commercially available mouse monoclonal antibody. No antibodies are currently available for assessing the distribution of claudin-13.

When mouse or rat bladder tissue was stained with claudin-2-specific antiserum, expression was limited to the uroepithelium. The staining pattern was not as expected for a tight junction-associated protein. In both mouse and rat bladder, claudin-2 was found under the apical membrane of the surface umbrella cells, as well as to a lesser extent in what appeared to be the cytoplasm of the underlying intermediate and basal cell layers (our unpublished observations). No accumulation of claudin-2 was observed in the expected location of the tight junctions. Staining was not significantly diminished when GST-claudin-2 protein was used to immunodeplete serum of claudin-2-specific antibodies, indicating that the staining was likely to be nonspecific. Use of other tissue preparation methods, such as freezing tissue, followed by fixation of cryosections with methanol, also resulted in no specific staining (our unpublished observations). In addition, no staining was observed when a commercially available purified polyclonal antibody to claudin-2 was used, and expression of claudin-2 protein could not be detected when rat or mouse bladder lysates were screened by Western blot analysis (our unpublished observations).

Claudin-4 was previously localized to the intermediate and basal cell layers of mouse uroepithelium, but not the umbrella cell layer (29). Consistent with this previous observation, we found that claudin-4 was distributed at regions of cell-cell contact in the intermediate and basal cell layers of all three regions of rabbit bladder uroepithelium (Fig. 5, A-C) and of the mouse (Fig. 6A) and rat (Fig. 6B) uroepithelium. Little staining was observed when GST-claudin-4 protein (but not GST alone) was used to immunodeplete serum of claudin-4-specific antibodies. In contrast to previous reports, we also observed claudin-4 association with the lateral surface of adjacent umbrella cells. This was readily apparent when the distribution of ZO-1 and claudin-4 was examined in the umbrella cell layer of whole mounted rabbit (Fig. 5, D-F) or mouse tissue (Fig. 6C).
Fig. 4. Distribution of occludin in bladder epithelium. Cryosections of bladder tissue from rabbit neck region (A), rabbit equatorial region (B), rabbit dome region (C), mouse (D), or rat (E) were labeled with antibodies to label occludin (left) or rhodamine-phalloidin and TO-PRO3 (middle). Right: merged images. E: mucosal fold. Images were collected as a z-series with a confocal microscope and then summed and displayed as a single composite projection. Arrows show location of tight junctions. Bar = 25 μm.
Fig. 5. Distribution of claudin-4 in rabbit bladder epithelium. A–C: cryosections of bladder tissue from the rabbit neck region (A), rabbit equatorial region (B), or rabbit dome region (C) were labeled with antibodies to claudin-4 (left) or rhodamine-phalloidin and TO-PRO3 (middle). Right: merged images. Images were collected as a z-series with a confocal microscope and then summed and displayed as a single composite projection. Left and right: arrows show location of tight junctions. Bar = 25 μm. D–I: whole mounted epithelium from the neck region (D and G), equatorial region (E and H), or dome region (F and I) was labeled with antibodies to ZO-1 (red) and claudin-4 (green). A merged image is shown in each of the panels. D–F: images are 3-dimensional reconstructions of z-series collected with a confocal microscope. The images were tilted 65° about the x-axis. The grid is a 3-dimensional scale marker, with each side of the square equivalent to ~16.8 μm (D), ~18.3 μm (E), or ~17.9 μm (F). G–I: x-z confocal sections of whole mounted bladder epithelium. The arrows mark the location of tight junctions.
or when the distribution of actin and claudin-4 was examined in whole mounted rat tissue (Fig. 6D).

Furthermore, we observed that claudin-4, in a region- and species-specific manner, was concentrated at the apicolateral junction of the umbrella cell layer, consistent with localization to tight junctions. In the dome region of the rabbit bladder, there was significant localization of claudin-4 to the tight junction (examples of these regions are marked with arrows in

Fig. 6. Distribution of claudin-4 in rat and mouse bladder epithelium. A and B: cryosections of mouse (A) or rat (B) bladder epithelium were labeled with antibodies to claudin-4 (left) or rhodamine-phalloidin and TO-PRO3 (middle). Right: merged images. Images were collected as a z-series with a confocal microscope and then summed and displayed as a single composite projection. Left and right: arrows show location of tight junctions. Bar = 25 μm. C-F: whole mounted epithelium from the mouse (C and E) was labeled with antibodies to ZO-1 (red) and claudin-4 (green), whereas rat tissue (D and F) was labeled with antibodies to claudin-4 (green) and rhodamine-phalloidin (red). A merged image is shown in each of the panels. C and D: images are 3-dimensional reconstructions of z-series collected with a confocal microscope. The images were tilted 65° about the x-axis. The grid is a 3-dimensional scale marker, with each side of the square equivalent to ~18.8 μm (C) or ~12.5 μm (D). E and F: x-z confocal sections of whole mounted bladder epithelium. The arrows mark the location of tight junctions.
Fig. 7. Localization of claudin-8 in bladder epithelium. A and B: whole mounted epithelium from rat bladder was labeled with antibodies to claudin-8 (green) and rhodamine-phalloidin (red). A merged image is shown. A: image is a 3-dimensional reconstruction of a z-series collected with a confocal microscope. The image was tilted 65° about the x-axis. The grid is a 3-dimensional scale marker, with each side of the square equivalent to ~12.3 μm. B: x-z confocal section of the tissue. The arrows mark the location of tight junctions. C and D: cryosections of bladder tissue from mouse (C) or rat (D) were labeled with antibodies to label claudin-8 (left) or rhodamine-phalloidin and TO-PRO3 (middle). Right: merged images. Images were collected as a z-series with a confocal microscope and then summed and displayed as a single composite projection. Arrows show location of tight junctions. Bar = 25 μm.
Fig. 8. Distribution of claudin-12 in bladder epithelium. A-D: whole mounted epithelium from the mouse (A and C) was labeled with antibodies to ZO-1 (red) and claudin-12 (green), whereas rat tissue (B and D) was labeled with antibodies to claudin-12 (green) and rhodamine-phalloidin (red). A merged image is shown in each of the panels. A and B: images are 3-dimensional reconstructions of z-series collected with a confocal microscope. The images were tilted 65° about the z-axis. The grid is a 3-dimensional scale marker, with each side of the square equivalent to ~18.8 μm (A) or ~12.5 μm (D). C and D: x-z confocal sections of whole mounted bladder epithelium. The arrows mark the location of tight junctions. E-H: cryosections of bladder tissue from mouse (E) or rat (F) were labeled with antibodies to label claudin-12 (left) or rhodamine-phalloidin and TO-PRO3 (middle). Right: merged images. Left and right panels: arrows show location of tight junctions. G and H: cryosections were labeled with TO-PRO3 and antisera preincubated with glutathione-S-transferase (GST; G) or GST-claudin-12 (H). Staining for claudin-12 is in green and staining for TO-PRO3 is in red. G: short arrows indicate localization of claudin-12 to tight junctions. When antiserum was preabsorbed with GST-claudin-12, no staining at tight junctions was observed. E-H: all images were collected as a z-series with a confocal microscope and then summed and displayed as a single composite projection. Bar = 25 μm.
Fig. 5, F and I); however, in the neck and equatorial regions claudin-4 association with the tight junctions was less marked (Fig. 5, D and G, and E and H). In mouse umbrella cells, there were only occasional regions where claudin-4 localized to tight junctions (see arrows in Fig. 6C and left arrow in Fig. 6E), but significant localization of claudin-4 was observed at the apicolateral junctions of rat umbrella cells (Fig. 6, D and F).

Finally, we examined the distribution of claudin-8 and -12 in mouse and rat bladders. In whole mounted rat tissue, claudin-8 was localized to the apicolateral junction between umbrella cells (Fig. 7, A and B). Because of problems with uneven antibody penetration, we were unable to obtain similar images for mouse bladder. Nonetheless, claudin-8 localization to tight junctions was observed in the uroepithelium of cryosectioned mouse and rat tissue (Fig. 7, C and D). No localization of claudin-8 to the underlying layers of the uroepithelium was noted. Some staining was occasionally observed in blood vessels similar to that seen with ZO-1 and again likely represents staining of tight junction complexes in the vasculature. Staining for claudin-8 was blocked by GST-claudin-8 immunodepletion. A similar localization was obtained using a claudin-12 antibody. Claudin-12 was found concentrated in the apicolateral junction of adjacent mouse and rat umbrella cells in both whole mounts and in tissue cryosections (Fig. 8, A-F). The claudin-12 antisera also contained “nonspecific” antibodies that recognized an unidentified antigen that labeled the nuclear membrane (Fig. 8, A, B, E, F). The tight junction-specific labeling was confirmed by immunodepletion with GST-claudin-12, which blocked the tight junction-associated signal, but not the nuclear staining (Fig. 8, G and H).

DISCUSSION

An essential feature of all multicellular organisms is the ability to create and maintain specific tissue barriers, often times generating compositionally distinct fluid compartments on either side of the barrier. This is especially important in the bladder, which has to store urine for prolonged periods of time without permitting unregulated flow of toxins, ions, and water across the epithelium and into the blood supply. The tightness of the epithelium is likely defined by the combination and mixing ratios of the different claudin species within the individual tight junctions (41). Through homotypic and heterotypic interactions across the lateral cell-cell contact regions of the tight junction, specific “pores” are likely to be created that impart unique paracellular properties including ion selectivity and permeability characteristics (1, 7, 8, 41, 42, 45). The combinatorial possibilities offered by all 24 claudins could easily account for the extensive physiological variability observed among different epithelial tissues. Using RT-PCR, we found that claudin-2, -4, -8, -12, and -13 are expressed in the bladder, and we confirmed by immunofluorescence that claudin-4, -8, and -12 are expressed in the uroepithelium. Antibodies to claudin-13 are not yet available. Positive controls were not obtained for claudin-9, -11, -18, or -19, and sequence information was not available for claudin-17 or -20-24. As such, it remains unknown whether these claudin species are expressed in the uroepithelium.

Claudins-4, -8, and -12 are associated with high-resistance tight junctions (20, 23, 32, 39, 42, 45), and expression of these claudins in the tight junctions of umbrella cells is consistent with the high-resistance, low-permeability barrier associated with this cell type (23). Claudin-4 is found in other tight epithelial cells such as those that line the collecting duct of the kidney (20). Overexpression of claudin-4 in MDCK cells increases TER by approximately two-to-threefold and reduces transmonolayer conductance by decreasing paracellular Na+ permeability without a significant effect on Cl− permeability (42). In addition, when high-resistance type I MDCK cells are incubated with a claudin-4-binding peptide, claudin-4 is removed from TJ strands, resulting in a significant increase in TJ permeability (39). Similar to claudin-4, claudin-8 is expressed in “tight” segments of the nephron including the distal tubule, in the collecting duct, and in the thin descending limb of Henle (20, 24a). When claudin-8 is expressed in MDCK cells, it is localized to tight junction strands and reduces paracellular permeability to monovalent inorganic and organic cations and to divalent cations but not to anions or neutral solutes (45). Interestingly, claudin-8 expression is associated with a decrease in expression of endogenous claudin-2, which is thought to form a low-resistance cation-selective pore (45). Finally, claudin-12 was recently localized to the blood-brain barrier, which is composed of high-resistance endothelial cells (32). Because of a lack of reagents, little is presently known about the function or distribution of claudin-13.

Our ability to detect message for claudin-2 by PCR, but our inability to detect claudin-2 by Western blot analysis or to immunolocalize the protein to tight junctions, indicates that there might be low levels of claudin-2 mRNA translation into protein, rapid protein turnover, or low amounts of claudin-2 protein in bladder tissue. The apparent lack of expression of claudin-2 in the bladder epithelium is not surprising, as this claudin is associated with “leaky” epithelia such as those found in the proximal tubule of the kidney where the glomerular filtrate is almost fully reabsorbed (20, 45). Furthermore, when expressed in high-resistance MDCK cell lines, claudin-2 forms a cation-selective pore that increases paracellular permeability (1). However, it is worthwhile noting that claudin-2 overexpression results in some increase in TER in low-resistance strains of MDCK cells (7).

An additional finding of our analysis is that many of the “tight junction” proteins we studied were localized not only in tight junctions but in a species- and region-dependent manner were also found along the sites of cell-cell contact in the umbrella, intermediate, and basal cell layers. One example is claudin-4, which was previously localized to the periphery of the basal and intermediate cell layers (29). Our analysis indicates that claudin-4 is also found along the basolateral margins of the umbrella cell layer. Localization of claudins to sites of cell-cell contact has been noted previously in other tissues and cell lines (21). Claudin-1 is localized along the entire basolateral surface of the epithelium lining the epididymis (16), and this claudin also circumscribes the plasma membrane of all of the cellular layers of the epidermis (12). Similarly, claudin-4 is found along the basolateral surfaces of enterocytes in the small and large intestine (33), in the second/third layers of the stratum granulosum of the epidermis (12), and in cultured MDCK cells (7). Finally, claudin-7 is localized to the basolateral surface of the aldosterone-sensitive distal nephron and in the thin descending limb of Henle (24a).

Localization at sites of cell-cell contact was also noted for ZO-1 in mouse bladders and the dome region of rabbit bladders.
and for occludin in rat and mouse bladders. The localization of occludin to the lateral margins of the cells was surprising as in most epithelial tissues this protein localizes exclusively to the tight junctions (12, 13); however, occludin staining was recently observed in the suprabasal cell layers of some stratified epithelia (21). Why association of ZO-1 and occludin at the sites of cell-cell contact showed species and regional differences is unknown, but it may reflect species and regional differences in factors such as rates of bladder filling, hydrostatic pressure, bladder tone, or innervation.

One potential function for tight junction protein association at sites of cell-cell contact is to promote cell adhesion. Such interactions would be important in tissues like the skin, gut, and bladder that are constantly exposed to mechanical forces such as compression, distention, and hydrostatic pressure. Consistent with this hypothesis is the observation that ZO-1 is associated with adherens junction proteins during the initial steps of epithelial cell polarization (34). Furthermore, VAB-9, a tetraspanning claudin-like protein in Caenorhabditis elegans, associates with HMR1 (C. elegans cadherin), localizes to adherens junctions, may modulate the association of the actin cytoskeleton with the adherens junction, and may also regulate some aspects of cell adhesion (38). At present, it is unknown if claudins interact directly with cadherin complexes in mammalian cells.

Our results better define the proteins that may comprise the high-resistance paracellular barrier associated with the uroepithelium. In addition to their established roles as tight junction proteins, occludin, claudin–4, and ZO-1 may have additional functions in bladder epithelium, including a possible role in cell-cell adhesion. This information not only serves as a launching point for study of the uroepithelial barrier under cell-cell adhesion. This information not only serves as a launching point for study of the uroepithelial barrier under cell-cell adhesion. This information not only serves as a launching point for study of the uroepithelial barrier under cell-cell adhesion.

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TIGHT JUNCTION PROTEINS IN BLADDER


