Localization of pendrin in mouse kidney

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Pendrin is an anion exchanger expressed in type B intercalated cells of the cortical collecting duct (CCD). Whether pendrin localizes to other nephron segments with intercalated cells is unknown. Moreover, whether pendrin is expressed in proximal tubule is debated. Thus the distribution of pendrin mRNA and protein expression in mouse kidney was investigated by using light and electron microscopic immunohistochemistry and quantitative real-time PCR. We observed that pendrin mRNA is expressed mainly in cortex. Within cortex, pendrin mRNA is at least fivefold higher in CCD and the connecting tubule (CNT) than in the other segments. Pendrin protein was observed in a subset of cells within the distal convoluted tubule as well as in type B and in non-A-non-B intercalated cells of the CNT and CCD. In type B intercalated cells, pendrin immunoreactivity was highest in apical cytoplasmic vesicles with little immunolabel along the apical plasma membrane. In non-A-non-B intercalated cells, intense pendrin immunoreactivity was detected along the apical plasma membrane. These differences in the subcellular distribution of pendrin immunolabel were confirmed by morphometric analysis. In conclusion, pendrin is expressed in the mouse distal convoluted tubule, CNT, and CCD along the apical plasma membrane of non-A-non-B intercalated cells and in subapical cytoplasmic vesicles of type B intercalated cells.

intercalated cell; distal convoluted tubule; cortical collecting duct; connecting tubule; anion exchange

INTERCALATED CELLS MEDIATE transepithelial transport of net H+ equivalents along the collecting duct (24), a process mediated largely through vacuolar H+-ATPase. However, Brown et al. (3) observed that H+-ATPase has opposite polarity within subpopulations of intercalated cells. Thus these cells are thought to either secrete or absorb net H+ equivalents depending on whether H+-ATPase localizes to the apical or the basolateral plasma membrane. Intercalated cells are classified as type A, B, or non-A-non-B from immunological and ultrastructural characteristics (30). The immunohistochemical classification of these cells is based on the presence or absence of AE1 immunoreactivity and the distribution of H+-ATPase within the cell (16, 24, 30). The distribution and expression of each of these transporters in each intercalated cell subtype is displayed in Fig. 1. The ultrastructure of each cell type (A, B, and non-A-non-B) has been characterized (30). Type A intercalated cells have a central-nucleus, prominent apical plasma membrane microprojections, and prominent apical cytoplasmic membrane tubulovesicles. In the type A intercalated cell, H+-ATPase is expressed on the apical cytoplasmic vesicles and the apical plasma membrane, where it functions in series with the Cl-/HCO3- exchanger AE1 on the basolateral plasma membrane to mediate secretion of net H+ equivalents into the luminal fluid (1, 6, 23) in both the medullary and the cortical collecting duct (24). In the kidney, a truncated splice variant of erythrocyte AE1 or band 3 (17) is observed (kAE1). Both H+-ATPase and kAE1 are highly regulated by changes in acid-base status (21). For example, kAE1 and H+-ATPase are upregulated during metabolic acidosis (2, 21, 33), which increases secretion of H+ equivalents into the luminal fluid.

The non-A-non-B intercalated cell has been described in mice and rats (1, 16, 30). Non-A-non-B cells have a very high mitochondrial density, prominent apical plasma membrane microprojections, and sparse apical cytoplasmic vesicles (30). Similar to type A intercalated cells, this cell type has H+-ATPase in both the apical plasma membrane and in apical cytoplasmic vesicles; however, it does not express kAE1 (1, 16, 30). The physiological role of non-A-non-B intercalated cells in the regulation of acid-base homeostasis is, however, unknown (30).

Type B intercalated cells are distinguished from other intercalated cell subtypes ultrastructurally by the presence of a relatively smooth apical plasma membrane, an eccentric nucleus, clustered mitochondria, and cytoplasmic vesicles distributed throughout the cell. In the type B intercalated cell, H+-ATPase is expressed on the basolateral plasma membrane and in

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cytoplasmic vesicles throughout the cell (1, 16, 30). Type B intercalated cells are thought to mediate HCO₃⁻/H⁺ secretion into the luminal fluid (24). In rabbits and mice, the majority of non-A intercalated cells of the cortical collecting duct (CCD) display electroneutral Na⁺/H⁺-independent Cl⁻/HCO₃⁻/H⁺ exchange across the apical membrane (8, 10, 35). The gene product(s) responsible for apical anion exchange-mediated HCO₃⁻ secretion, thought to occur across type B intercalated cells, has been a matter of controversy. It has been proposed that kAE1 represents the putative apical anion exchanger of the type B intercalated cell (32). However, there is now abundant evidence that kAE1 does not represent the gene product of the apical anion exchanger of the type B intercalated cell (1, 9, 12, 16, 25, 30). More recent studies have shown that pendrin and AE4 represent other candidate genes for the putative apical anion exchanger.

AE4 is an Na⁺/H⁺-independent Cl⁻/HCO₃⁻ exchanger first cloned in rabbit kidney by Tsuganezawa et al. (31). In rabbit CCD, AE4 localizes to the apical membrane of some type B intercalated cells (31). However, preliminary reports indicate that in rat CCD, AE4 resides along the basolateral membrane of both type A and type B cells (7). Like apical anion exchange in the type B intercalated cell, AE4 is insensitive to stilbene inhibitors when expressed in Xenopus laevis oocytes (31). However, because its distribution in intercalated cell subtypes is debated and because no AE4 mouse knock-out model exists, the physiological significance of AE4 in kidney is unknown.

Pendrin represents another Na⁺-independent Cl⁻/HCO₃⁻ exchanger (27, 28). The molecular structure of the pendrin gene was deduced by Everett et al. (11). RNA in situ hybridization, Northern blot analysis, and immunocytochemistry data have shown that pendrin mRNA and protein are highly expressed in the inner ear, thyroid, and kidney (11, 19, 20).

The distribution of pendrin in the mammalian kidney has been debated. Light and fluorescent microscopic immunolocalization studies of Royaux et al. (20) reported that pendrin protein is highly expressed in the apical region of a subpopulation of cells within the CCD of mice, rats, and humans in which H⁺-ATPase is either basolateral or apical (Fig. 1) but not in cells that express kAE1. Thus pendrin protein is expressed in the apical region of non-A intercalated cells. Localization of pendrin to the apical region of the type B intercalated cell raises the possibility that this transporter represents the putative apical anion exchanger of this cell type. However, other laboratories have reported a different distribution of pendrin expression within the cortex. Soleimani et al. (28) have investigated the distribution of pendrin mRNA and protein expression in rat kidney by using RT-PCR of individual nephron segments and immunoblots of brush-border membrane vesicles. They observed that pendrin message and protein are highly expressed not only in the CCD but also along the brush border of the proximal tubule. Pendrin mRNA expression was not measured in the other segments of the cortex. Localization of pendrin to the brush border suggests a different functional role for the transporter than is suggested with localization of the transporter to the apical membrane of the type B intercalated cell. The purpose of the present study was therefore to explore the cellular and subcellular distribution of pendrin message and protein in mouse kidney in greater detail.

METHODS

Animals. Nonalbino Swiss mice weighing 20–30 g were studied (Harlan, Ardmore, TX). Mice consumed a balanced rodent diet (Zeigler Brothers, Gardners, PA) and tap water. Mice were anesthetized with 100% O₂ at 1 l/min with 4% isoflurane before death.
Dissection of tubules. Mice were injected with 1.5 mg furosemide ip 30 min before death. The kidney was perfused initially with 10 ml of ice-cold dissection solution and then with 20 ml of the same solution containing 1 mg/ml collagenase B (0.2 U/mg; Roche, Indianapolis, IN) and 1 mg/ml BSA (Sigma). The dissection solution contained (in mM) 144 NaCl, 5 KCl, 1 Na2HPO4, 1.2 MgSO4, 2 CaCl2, 5.5 glucose, and 10 HEPES, pH 7.4. The kidneys were removed, and a coronal section was made that contained the entire corticopapillary axis. The cortex was separated from the rest of the section and incubated in collagenase solution for 5 min at 37°C. The tissue was transferred to the dissection solution with 1 mg/ml albumin but without collagenase. Tissue was dissected at 4°C for not more than 30 min. Nephron segments from the cortex were dissected as described previously (22). Cortical thick ascending limbs (cTALs), proximal straight tubules, and CCDs were dissected from the medullary rays. CCD segments were at least 0.5 mm in length, had no branched points, and displayed the typical "cobblestone" appearance. Proximal convoluted tubules, glomeruli, and connecting tubules (CNTs) were dissected from the cortical labyrinth. CNTs also displayed the typical cobblestone appearance and were cut to span two branched points. The distal convoluted tubule (DCT) was dissected between its juncture with the macula densa and the beginning of the CNT. Tubule length was measured with a calibrated optical micrometer. The tubules were then transferred to an Eppendorf tube containing 0.5 ml dissection solution plus 10 μl RNAlater (Qiagen, Valencia, CA) but without collagenase or albumin. Transfer of tubules was accomplished by using glass tubing coated with dissection solution containing 1% albumin and connected to a Hamilton syringe with Silastic tubing. Samples were centrifuged at 11,750 g for 1 min at 4°C. The supernatant of each sample was then snap frozen in liquid nitrogen and stored at −80°C.

Preparation of total RNA from kidney slices. After death, the left kidneys were excised from the mice and coronal slices were made. Each slice was cut into three regions: cortex, outer medulla, and inner medulla. Each piece was snap frozen in liquid nitrogen and then weighed. Isolation of total RNA was performed by using a RNeasy mini kit (Qiagen). The kidney tissue was placed in RLT buffer (20 ml buffer/g body has been characterized previously and used for immunology utilizing a 7700 Sequence Detector (Applied Biosystems, Foster City, CA) (4, 15). Specific quantitative assays for mouse pendrin and β-actin were developed by using Primer Express software (Applied Biosystems) following the recommended guidelines on the basis of sequences from GenBank: I) mouse pendrin qRT-PCR assay (accession no. AF-167441), 1483 bp; GCTGGGTCTACTGACTG, 1552 bp; GCAAGGTTTCAGAAGCTT, 1504 bp: 6-carboxyfluorescein (FAM)/6-carboxy-tetramethylrhodamine (TAMRA): ATTGTTGTGGGTTGCC; and β-actin qRT-PCR assay (accession no. X03672), 1035 bp: GTCCTGCTCGACACCT, 1108 bp: CCACCGATCCACCAGAGTAC, 1059 bp: FAM/TAMRA: ATCAAGATCATGCTCTCTGCTTCCGTGCGC.

cDNA was synthesized in 10-μl total volume by the addition of 6 μl/well RT master mix consisting of 400 nM assay-specific reverse primer, 500 μM deoxynucleotides, SuperScript II buffer, DTT, and 10 U SuperScript II RT (Invitrogen, Carlsbad, CA), added to an ABI 7700 96-well plate followed by the unknown RNA sample (4 μl). Each sample was measured in triplicate plus a control without RT. Each plate also contained serial dilutions of an assay-specific sDNA (synthetic ampiclon oligonucleotides) standard spanning a 5-log range and a no-template control. Each plate was covered with Biofilm A (MJR, Waltham, MA) and incubated in a thermocycler (MJR) for 30 min at 50°C followed by 72°C for 10 min. Subsequently, 40 μl of a PCR master mix (400 nM forward and reverse primers, 100 nM fluorescent probe, 3 mM MgCl2, and 200 μM deoxynucleotides, PCR buffer, and 1.25 U Taq polymerase (Invitrogen)) were added directly to each well of the cDNA plate. RT master mixes and all RNA samples were pipetted by a Tecan Genesis RSP 100 robotic workstation (Tecan US, Research Triangle Park, NC). PCR master mixes were pipetted utilizing a Biomek 2000 robotic workstation (Tecan US, Research Triangle Park, NC). Each assembled plate was then capped and run in the ABI 7700 with the following cycling conditions: 95°C for 1 min and 40 cycles of 95°C for 12 s and 60°C for 1 min. The resulting data were analyzed by using SDS software (Applied Biosystems, Foster City, CA) with TAMRA as the reference dye.

Synthetic DNA oligonucleotides used as standards (sDNA) encompassed the entire ampiclon for the assay (Biosource International, Camarillo, CA). We have shown in several assays that in vitro transcribed RNA amplicon standards (sRNA) and sDNA standards have the same PCR efficiency when performed as described above (data not shown). As a negative control, β-actin transcript levels were measured in each sample assayed for pendrin mRNA. β-Actin and pendrin mRNA are expressed as pendrin transcript per million. Samples were pipetted on a 2× master mix with β-actin (per 10 μl). Antisense.

Antibody. The primary anti-pendrin antibody was a polyclonal antibody raised in rabbit that recognizes amino acids 766–780 of the human pendrin protein sequence. This antibody has been characterized previously and used for immunolocalization of pendrin in normal mouse kidney (20). For
immunohistochemical localization of the thiazide-sensitive Na-Cl cotransporter (TSC), we used a polyclonal antibody raised in rabbit against a 110-amino acid segment of the NH₂ terminus of rTSC1, which corresponds to amino acids 2–112 of the rat rTSC1. This antibody has been characterized previously (18) and was a gift from Dr. Steven C. Hebert (Yale University School of Medicine, New Haven, CT).

Tissue preparation for light microscopy. For light microscopic studies, –2-mm-thick transverse sections of kidney from each animal were embedded in polyester wax (polyethylene glycol 400 distearate, Polysciences, Warrington, PA), and 5-μm-thick sections were cut and mounted on gelatin-coated glass slides.

Co-localization of pendrin and TSC immunoreactivity. Co-localization of pendrin and thiazide-sensitive cotransporter immunoreactivity was accomplished by using sequential immunoperoxidase procedures. Five-micrometer sections were dewaxed in ethanol, rehydrated, and then rinsed in PBS. Endogenous peroxidase activity was blocked by incubation of the sections in 0.3% H₂O₂ for 30 min. The sections were rinsed in PBS, treated for 20 min with 5% goat serum in PBS, and then incubated at 4°C overnight with the anti-pendrin primary antibody diluted 1:1,000 in PBS. The sections were again rinsed in PBS and incubated for 20 min with 5% goat serum in PBS, then exposed to diaminobenzidine (peroxidase substrate kit, Vector Laboratories, Burlingame, CA) diluted 1:200 in PBS and then washed with PBS. The sections were then exposed to diaminobenzidine (peroxidase substrate kit, Vector Laboratories, Burlingame, CA), the sections were washed in glass-distilled water and then in PBS and incubated in 0.3% H₂O₂ for 30 min. The sections were again washed in PBS and incubated for 20 min with 5% normal goat serum in PBS. The sections were treated for 60 min with the anti-TSC antibody diluted 1:8,000 in PBS, washed in PBS, incubated for 30 min with the peroxidase-conjugated anti-rabbit secondary antibody, and then again washed with PBS. For detection of TSC immunoreactivity, Vector SG (Vector Laboratories) was used as the chromogen to produce a blue label. This label was easily distinguishable from the brown label produced by the diaminobenzidine used for detection of pendrin immunoreactivity. The sections were washed with glass-distilled water, dehydrated with xylene, mounted with Permount (Fisher Scientific, Fair Lawn, NJ), and observed by light microscopy. In each colocalization experiment, three control slides were included in which PBS only was substituted for the anti-pendrin primary antibody, the anti-TSC primary antibody, or both primary antibodies.

Tissue processing for immunoelectron microscopy. Mice were anesthetized and the kidneys were preserved by in vivo cardiac perfusion with 3% paraformaldehyde, 0.12% picric acid in PBS, pH 7.4, followed by overnight immersion at 4°C. The tissue was rinsed in PBS, and samples from the outer and inner cortex were immersed in 0.1 M NH₄Cl for 1 h at 4°C. The tissue samples were then dehydrated in a graded series of alcohols and processed and embedded in Lowicryl K4M (Electron Microscopy Sciences, Ft. Washington, PA). Lowicryl polymerization was carried out under ultraviolet light for 24 h at 20°C and then for 48 h at room temperature. Samples containing well-preserved connecting segment and collecting duct were selected after light microscopic examination of 1-μm-thick sections stained with toluidine blue. Ultrathin sections of these were mounted on Formvar/carbon-coated nickel grids for immunogold cytochemistry.

Immunogold labeling. Briefly, the immunogold labeling procedure was performed by exposure of the ultrathin tissue sections to the primary antibody and then to a goat anti-rabbit IgG secondary antibody conjugated to 0.8-nm colloidal gold particles (Aurion UltraSmall gold conjugate, Electron Microscopy Sciences), followed by silver enhancement (Aurion R-Gent SE-EM, Electron Microscopy Sciences). Unless

[Fig. 2. Pendrin transcript in mouse kidney. Pendrin mRNA was measured in cortex (COR), outer medulla (OM), and inner medulla (IM) of mouse kidney. Pendrin mRNA was expressed as the percentage of β-actin transcript measured in the same sample. The pendrin/β-actin transcript ratio was greater in cortex than in either inner or outer medulla, P < 0.05. β-actin mRNA/100 ng total RNA was (×10⁶), inner medulla, 2.0 ± 0.4; outer medulla, 2.7 ± 0.2; and cortex, 2.5 ± 0.09 (n = 6, P = not significant).

Fig. 3. Pendrin transcript abundance in nephron segments of the mouse cortex. A: pendrin mRNA/mm tubule length was measured in individual nephron segments of mouse cortex. B: in each of these nephron segments, β-actin mRNA was also measured. Pendrin transcript was higher in CCD and connecting tubule (CNT) than in cortical thick ascending limb (cTAL), proximal straight tubule (PST), or proximal convoluted tubule (PCT) (P < 0.05). GLM, glomeruli.]

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noted otherwise, all steps were done by floating the grids on droplets of solution at room temperature. The following solutions were used: incubation solution, 0.2% acetylated BSA (Aurion BSA-c, Electron Microscopy Sciences) and 10 mM NaN₃, in PBS, pH 7.4; and blocking solution, 5% BSA, 0.1% sh-skin gelatin, and 5% normal goat serum, in PBS. The sections were exposed to 0.05 mM glycine in PBS for 15 min, incubated with the blocking solution for 30 min, washed with PBS, postfixed with 1.25% glutaraldehyde in PBS, washed with PBS, and finally washed with glass-distilled water. The sections were then exposed to the silver-enhancement reagent for 40 min, washed with glass-distilled water, and counterstained with saturated uranyl acetate and lead citrate. Each group of sections subjected to the immunogold procedure included a control section that was exposed to incubation buffer in place of the primary antibody.

Electron microscopy. Ultrathin sections were examined with a Zeiss-EM10 transmission electron microscope. The CCD was identified by its characteristic heterogeneous epithelial cell population, which included principal cells and intercalated cells, and its location parallel to a cTAL of Henle's loop in the medullary ray. Connecting segments and initial collecting tubules (ICTs) were located in the cortical labyrinth between medullary rays. ICTs were identified by their epithelial cell morphology, which is similar to that described for the CCD. Connecting segments were distinguished from ICTs by the increased height of the epithelial cells and presence of tall vertical mitochondria in the connecting segment cells.

The mouse CCD and CNT contain at least three morphologically distinct intercalated cell subtypes: type A, type B, and a third cell type that has been identified in both rat and mouse kidney and referred to as non-A-non-B (30). The morphological characteristics used to identify these intercalated cell subtypes were established in morphological and immunocytochemical studies of both rat and mouse collecting duct (16, 30). Type A intercalated cells typically contain a centralized nucleus, mitochondria that are distributed throughout the cell, moderate apical plasma membrane microprojections, and prominent apical cytoplasmic membrane tubulovesicles that have relatively electron-dense limiting membranes. Type B intercalated cells typically exhibit a rounded cell outline, eccentric nucleus, clustered mitochondria, relatively smooth apical plasma membrane, and cytoplasmic vesicles throughout the cell. The cytoplasmic vesicles of type B intercalated cells are typically smaller in profile, and the limiting membranes are less electron dense than those in type A intercalated cells. Type B intercalated cells frequently exhibit a vesicle-free band of cytoplasm along the apical plasma membrane. The third intercalated cell subtype, non-A-non-B, has distinctive morphological features, including a very high mitochondrial density, prominent apical plasma membrane microprojections, and relatively few cytoplasmic vesicles, which are apical.

Morphometric analysis. The boundary length of the apical plasma membrane, cytoplasm area, number of gold particles along the apical plasma membrane, and number of gold particles over the cytoplasm, including cytoplasmic vesicles,
were quantified in type B and non-A-non-B intercalated cells (34) in three individual mice. A minimum of five of each intercalated cell type in each animal were selected randomly and photographed at a primary magnification of $\times 6,200$. Individual photomicrographs were examined at a final magnification of approximately $\times 23,400$. The exact magnification was calculated by using a calibration grid with 1,134 lines/mm.

The boundary length of the apical plasma membrane was determined by intersection counting using the Merz curvilinear test grid and the formula for area ($A$)

$$A = P \times D^2$$

in which $P$ is the number of points over the cytoplasm, and $A$ was expressed in square millimeters.

Gold particles that were touching the apical plasma membrane and those over the cytoplasm, including cytoplasmic vesicle membranes, were counted and were related to either the apical plasma membrane boundary length (gold particles/mm of apical plasma membrane boundary length) or the cytoplasm area (gold particles/mm$^2$ cytoplasm), respectively. In addition, the ratio of gold particles associated with the apical plasma membrane to gold particles over the cytoplasm was determined for each cell type.

Statistical analysis. For the RT-PCR, data comparisons among three or more groups were made by using ANOVA with Tukey's posttest. For comparisons of gold label density, repeated-measures ANOVA with Tukey's posttest was used. Statistical significance was achieved with a $P < 0.05$. Data are displayed as means ± SE.

RESULTS

Distribution of pendrin message in mouse kidney. Pendrin message abundance was examined in the cortex, outer medulla, and inner medulla of mouse kidney. As shown in Fig. 2, expression of pendrin relative to $\beta$-actin mRNA was low in both the outer and the inner medulla. However, pendrin transcript expression was 6- to 10-fold higher in the cortex than in the medulla, similar to previously published results in rats (28). However, $\beta$-actin mRNA/100 ng total RNA was the same in all three regions of the kidney (Fig. 2). Because pendrin mRNA is highly expressed in mouse cortex, the distribution of pendrin message within this region of the kidney was studied in greater detail.

Figure 3 shows pendrin mRNA expression in individual nephron segments of mouse cortex. As shown, pendrin message expression was detected in glomeruli, proximal convoluted tubule, proximal straight tubule, and CTAL. However, pendrin mRNA expression was at least fivefold higher in CNT and CCD ($P < 0.05$). To determine whether this distribution resulted from variation in RNA integrity in these segments, $\beta$-actin mRNA abundance was measured in each sample studied. In contrast to the distribution of pendrin expression, $\beta$-actin message was not lower in glomeruli, cTAL, and proximal tubule than in CCD and CNT (Fig. 3). Therefore the relatively low levels of pendrin mRNA detected in glomeruli and proximal tubule were not the result of RNA degradation. We conclude that nephron segments containing type B intercalated cells, such as CNT and CCD, express high levels of pendrin mRNA.

Light and electron microscopic immunolocalization of pendrin protein in mouse kidney cortex. Because pendrin protein and mRNA (20, 28) are most highly expressed in cortex, the distribution of pendrin immunoreactivity in mouse cortex was studied in greater detail by using light microscopic immunohistochemistry of mouse kidney cortex labeled for pendrin. We observed that within the mouse kidney, the distribution of pendrin protein was similar to the distribution
of pendrin mRNA described above. As reported previously (20), pendrin immunoreactivity was not observed in the glomerulus, proximal tubule, or thick ascending limb of Henle’s loop (Fig. 4). Figure 4 shows labeling of a subset of cells within the collecting duct, as described previously (20). Because the DCT contains intercalated cells (16), pendrin expression in this segment was explored. To determine whether pendrin is expressed in the DCT, sections were labeled with antibodies specific for pendrin and for the TSC, a marker of DCT. Although the majority of DCT profiles did not exhibit pendrin-positive cells, a minority of cells with pendrin immunoreactivity were present in occasional tubules that expressed TSC (Fig. 4). In rare profiles, continuous apical TSC immunoreactivity was interrupted by a few pendrin-positive cells (Fig. 4d). Thus, pendrin is expressed in a subset of cells within the DCT. Furthermore, some tubule profiles were observed that contained the transition from the DCT to the CNT (Fig. 4b). In these tubules, the DCT portion contained nearly continuous apical TSC immunoreactivity with rare pendrin-positive cells, whereas the CNT portion contained primarily cells that were negative for TSC immunoreactivity and a minority of cells with apical pendrin immunoreactivity (Fig. 4e). In addition, occasional CNT profiles were observed that had negative cells, pendrin-positive cells, and TSC-positive cells interspersed (Fig. 4c).

To further characterize the distribution of pendrin, immunoelectron microscopy was performed. Again, no labeling was observed in the glomerulus, proximal tubule, or thick ascending limb (Fig. 5). However, in all sections observed, significant immunogold label was present in a subset of cells within the CCD, ICT, and CNT identified morphologically as intercalated cells (see below). As reported previously with light microscopic techniques (20), principal cells and CNT cells were consistently negative (not shown), exhibiting no more than background levels, which was the level of gold particles also observed over tubule lumens or intercellular spaces.

Three distinct types of intercalated cells, type A, type B, and non-A-non-B, were observed in mouse CCD, ICT, and CNT (30). Type A intercalated cells were observed frequently in the CCD and ICT and were consistently negative for pendrin immunoreactivity (Fig. 6). However, all other intercalated cells throughout the CCD, ICT, and CNT were positive for pendrin. Although both type B and non-A-non-B intercalated cells exhibited pendrin immunoreactivity (see below), the subcellular distribution of the immunolabel differed qualitatively between these cell types.

In type B intercalated cells, pendrin immunolabel was predominantly associated with apical cytoplasmic vesicles (Fig. 7). Although type B cells have cytoplas-
mic vesicles distributed throughout the cell, only vesicles in the apical region were labeled (Fig. 7, a and b); vesicles in the basal region were negative (Fig. 7c). The apical plasma membrane of type B intercalated cells was also labeled. However, in most type B cells, the amount of label on the apical plasma membrane was low. In these cells, the apical plasma membrane surface was small and smooth, with few apical microprojections. However, occasional type B intercalated cells displayed prominent apical plasma membrane microprojections, and these cells also exhibited increased pendrin immunolabel on the apical plasma membrane (Fig. 7d). Sections that were exposed to incubation buffer in place of the primary antibody exhibited only rare gold particles in any location (Fig. 8). Type B intercalated cells were frequently identified in the CCD and the ICT and were rarely found in the CNT, as reported previously (1, 30).

In non-A-non-B intercalated cells, which typically exhibited extensive apical plasma membrane microprojections, pendrin immunolabel was predominantly located on the apical plasma membrane (Fig. 9). Fewer gold particles were associated with apical cytoplasmic vesicles (Fig. 9). As we reported previously (30), non-A-non-B intercalated cells were prevalent in the CNT. They were detected less often in the ICT and were not found in the CCD.

The distribution of gold label in type B and non-A-non-B intercalated cells was quantified. In these two cell types, we compared the total amount of gold label and the density of label in the apical membrane and in the cytoplasm and cytoplasmic vesicles (Table 1). Both the density of gold label and the total gold label in the apical plasma membrane were markedly greater in non-A-non-B cells compared with type B intercalated cells. In contrast, the density of gold label in the cytoplasm was greater in type B cells than in non-A-non-B cells; however, there was no difference in the total cytoplasmic label in the two cell types. Furthermore, the ratio of label in the apical plasma membrane to

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**Fig. 7.** Transmission electron micrograph of a type B intercalated cell from the mouse CCD subjected to immunogold labeling for pendrin. a: Typical morphological features of the type B intercalated cell, including a relatively small and smooth apical surface, cytoplasmic vesicles throughout the cell, an eccentric cell nucleus, and prominent basolateral plasma membrane infoldings. Pendrin immunolabeling is primarily associated with apical cytoplasmic vesicles. Magnification, ×11,800. Higher magnification transmission electron micrographs of the apical (b and d) and basal (c) regions of a type B intercalated cell from mouse CCD subjected to immunogold labeling for pendrin are shown. b: In the majority of type B intercalated cells, numerous gold particles were associated with cytoplasmic vesicles in the apical region (arrowheads), whereas only occasional particles are present along the apical plasma membrane (arrows). c: No significant labeling was present in the basolateral region of type B intercalated cells, over either the basolateral plasma membrane (arrows) or the cytoplasmic vesicles (arrowheads). Magnification, b and c, ×21,000. d: Occasional type B intercalated cells contained more prominent apical plasma membrane microprojections and more pendrin immunolabeling along the apical plasma membrane (arrows) compared with the typical type B intercalated cell illustrated in a and b. Prominent labeling of apical cytoplasmic vesicles was also present (arrowheads). Magnification, ×26,400.
label in the cytoplasm was markedly greater in non-A-non-B cells than in type B cells.

We also observed profiles of intercalated cells that were positive for pendrin immunoreactivity that did not exhibit the full complement of morphological features that are characteristic for either type B or non-A-non-B intercalated cells and thus could not be definitively identified. However, the pattern of pendrin immunolabel was similar to that observed in cells identified as type B and non-A-non-B in that they exhibited variable degrees of pendrin immunolabel in the apical cytoplasmic vesicles and apical plasma membrane.

**DISCUSSION**

The kidney has a tremendous capacity to excrete alkaline loads. For example, after drinking hypertonic NaHCO₃ for 5–8 days, rats develop only a mild metabolic alkalosis (14). During metabolic alkalosis apical Cl⁻/HCO₃⁻ exchange is upregulated, which augments secretion of OH⁻ equivalents along the CCD (29). Thus
Thus both pendrin and apical Na\textsuperscript{+} non-A intercalated cell of the mouse CCD (30).

acid-base balance. Either through direct transport of HCO\textsubscript{3}\textsuperscript{-} or formate exchange in heterologous expression systems (26, 28) and because it localizes to the apical membrane of type B intercalated cells, pendrin represents a candidate gene for the putative apical anion exchanger of the type B intercalated cell (20). Pendrin could mediate HCO\textsubscript{3}\textsuperscript{-} secretion in native mouse CCD, either through direct transport of HCO\textsubscript{3}\textsuperscript{-} by pendrin or through pendrin-mediated transport of another base such as OH\textsuperscript{-} or formate\textsuperscript{-}. However, the physiological role of pendrin may be more for regulation of halide balance, such as Cl\textsuperscript{-} or I\textsuperscript{-}, rather than for regulation of acid-base balance.

Pendrin is expressed in type B cells, the predominant non-A intercalated cell of the mouse CCD (30). Thus both pendrin and apical Na\textsuperscript{+}-independent Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchange are found in a large percentage of intercalated cells of the mouse CCD. However, virtually all of the pendrin immunolabel was restricted to apical cytoplasmic vesicles, even though type B cells have cytoplasmic vesicles distributed throughout the cell. Very little immunolabel was detected on the apical plasma membrane of the type B intercalated cell. However, under the conditions of previous studies, pendrin is sufficiently expressed along the apical plasma membrane of type B cells to modulate transepithelial transport of HCO\textsubscript{3}\textsuperscript{-} in the mouse CCD (20). During metabolic alkalosis, CCD from wild-type, Pds (+/+), mice secrete HCO\textsubscript{3}\textsuperscript{-} (20). However, CCDs from mice with a genetic disruption of the pendrin gene, Pds (−/−), absorbed HCO\textsubscript{3}\textsuperscript{-} when studied under the same treatment conditions (20). Thus it is likely that pendrin represents a gene product that contributes to the apical anion exchange process of the type B intercalated cell. However, low levels of HCO\textsubscript{3}\textsuperscript{-} secretion reported in wild-type mice (20) might reflect low levels of pendrin protein expressed along the apical plasma membrane of the type B intercalated cell. It is also possible that under the conditions of the perfusion studies, pendrin is trafficked to the apical membrane of the type B cell.

In mice, cells with the morphological characteristics of intercalated cells of the B type express H\textsuperscript{+}-ATPase along the basolateral plasma membrane and diffusely within cytoplasmic vesicles (30). The presence of pendrin in the subapical space of cells known to express H\textsuperscript{+}-ATPase within the cytoplasm raises the question of whether these cells have the capacity to up- or down-regulate transepithelial transport of net H\textsuperscript{+} or OH\textsuperscript{-} equivalents or Cl\textsuperscript{-} through trafficking of these transporters between the cytosol and the plasma membrane following changes in acid-base balance. These questions, however, will remain the subject of future studies.

As described previously in ultrastructural studies of the mouse (30), we observed that non-A-non-B cells are most prevalent in the CNT, less frequently observed in the ICT, and not detected in the CCD. The subcellular

![Fig. 8. Transmission electron micrograph of the apical region of a type B intercalated cell from the mouse CCD subjected to the immunogold labeling procedure with buffer substituted for the anti-pendrin primary antibody. Virtually no gold particles are present. Magnification, ×15,500.](http://ajprenal.physiology.org/)

**Table 1. Distribution of gold label in type B and non-A-non-B intercalated cells**

<table>
<thead>
<tr>
<th></th>
<th>Type B</th>
<th>Non-A-Non-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apical plasma membrane label density, gold particles/mm</td>
<td>1.226 ± 0.374</td>
<td>4.707 ± 0.518</td>
</tr>
<tr>
<td>Gold label along apical plasma membrane, gold particles/cell</td>
<td>5.38 ± 2.49</td>
<td>81.8 ± 14.2*</td>
</tr>
<tr>
<td>Apical plasma membrane label density, gold particles/mm</td>
<td>400 ± 64</td>
<td>1,746 ± 253*</td>
</tr>
<tr>
<td>Gold label over cytoplasm and cytoplasmic vesicles, gold particles/cell</td>
<td>53.1 ± 10.6</td>
<td>36.3 ± 4.5</td>
</tr>
<tr>
<td>Cytoplasmic density, gold particles × 10\textsuperscript{3}/mm\textsuperscript{2}</td>
<td>1,489 ± 111</td>
<td>680 ± 7*</td>
</tr>
<tr>
<td>Subcellular label distribution ratio, apical plasma membrane gold/cytoplasmic gold</td>
<td>0.0937 ± 0.0260</td>
<td>2.241 ± 0.194*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 3 animals/group. *P < 0.05, significantly different compared with type B by ANOVA with Tukey’s posttest.
distribution of pendrin differs markedly in type B vs. non-A-non-B cells. In non-A-non-B cells, pendrin protein is highly expressed along the apical plasma membrane. These observations predict that in mice under basal conditions, apical anion exchange, due to pendrin, occurs to a greater extent in non-A-non-B cells than in type B cells. Thus in untreated mice, pendrin-mediated anion exchange is likely greater in the CNT.
than in the CCD. However, this hypothesis cannot be tested directly because the CNT is not easily perfused in vitro.

Occasional intercalated cells are found in the late DCT of mouse kidney (16). In the DCT, the vast majority of non-A intercalated cells in mouse DCT are non-A-non-B intercalated cells (16). Because pendrin labeling was observed by light microscopic immunohistochemistry in a subset of cells within occasional DCT profiles, it is likely that pendrin is expressed in non-A-non-B intercalated cells. However, this could not be confirmed by ultrastructural observation because DCT profiles containing intercalated cells were not observed by electron microscopy. The failure to observe by electron microscopy DCT profiles containing intercalated cells is not surprising, because the great majority of DCT profiles did not contain pendrin-positive cells.

In mice, intercalated cells that display the morphological characteristics of non-A-non-B intercalated cells express H^+–ATPase in the apical plasma membrane and diffusely within cytoplasmic vesicles (30). Expression of both pendrin and H^+–ATPase along the apical plasma membrane of non-A-non-B cells is surprising because pendrin is thought to mediate HCO_3^- secretion (20), whereas H^+–ATPase mediates H^+ secretion (13). It is possible that through upregulation of one transporter, in parallel with downregulation of the other, the cell can be converted from an H^+–to an HCO_3^- secreting cell and vice versa, depending on the acid-base derangement of the animal. Alternatively, H^+–ATPase and pendrin may act in tandem to modulate transepithelial Cl^- transport.

We observed the distribution of pendrin protein and mRNA to be similar in mouse kidney. Previous studies have detected pendrin mRNA in proximal tubule and CCD of rats by using Northern blots and RT-PCR (28). Use of quantitative real-time PCR has the advantage over Northern blots and other RT-PCR techniques in that it allows pendrin mRNA expression to be quantified over a 5-log range (4, 15). Using this technique, we observed expression of pendrin in the proximal tubule and the CCD, as reported by Soleimani et al. (28). However, in mice, pendrin mRNA expression is more than fivefold higher in CNT and CCD than in both proximal tubule and cTAL.

In conclusion, pendrin is highly expressed in the CNT and the CCD of mouse kidney. Pendrin is expressed in intercalated cells in a minority of DCT profiles, likely in the late portion of the DCT. Pendrin protein and mRNA expression are lower in the other structures of the mouse cortex. Expression of pendrin in the apical plasma membrane is greater in non-A-non-B intercalated cells than in type B intercalated cells. The observed differences in the apical plasma membrane of pendrin immunoreactivity in type B intercalated cells and non-A-non-B intercalated cells suggest that under the conditions of these experiments, non-A-non-B intercalated cells are more actively involved in pendrin-mediated anion exchange than are type B intercalated cells. However, the presence of pendrin immunoreactivity in the apical cytoplasmic vesicles in both type B and non-A-non-B intercalated cells suggests that vesicle trafficking may occur in both cell types to regulate pendrin-mediated anion exchange under different physiological conditions.

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