Immunocytochemical localization of pendrin in intercalated cell subtypes in rat and mouse kidney

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Immunocytochemical localization of pendrin in intercalated cell subtypes in rat and mouse kidney. Am J Physiol Renal Physiol 283: F744–F754, 2002. First published May 22, 2002; 10.1152/ajprenal.00037.2002.—Recent studies have demonstrated that a novel anion exchanger, pendrin, is expressed in the apical domain of type B intercalated cells in the mammalian collecting duct. The purpose of this study was 1) to determine the expression and distribution of pendrin along the collecting duct and connecting tubule of mouse and rat kidney and establish whether pendrin is expressed in the non-A-non-B intercalated cells and 2) to determine the intracellular localization of pendrin in the different populations of intercalated cells by immunoelectron microscopy. A peptide-derived affinity-purified antibody was generated that specifically recognized pendrin in immunoblots of rat and mouse kidney. Immunohistochemistry and confocal laser scanning microscopy demonstrated the presence of pendrin in apical domains of all type B intercalated cells in mouse and rat connecting tubule and collecting duct. In addition, strong pendrin immunostaining was observed in non-A-non-B intercalated cells. There was no labeling of type A intercalated cells. Immunoelectron microscopy demonstrated that pendrin was located in the apical plasma membrane and intracellular vesicles of both type B intercalated cells and non-A-non-B cells; the latter was identified by the presence of H⁺-ATPase in the apical plasma membrane. The results of this study demonstrate that both pendrin and H⁺-ATPase are expressed in the apical plasma membrane of non-A-non-B intercalated cells, suggesting that these cells are capable of both HCO₃⁻ and proton secretion. Furthermore, the presence of pendrin in both the apical plasma membrane and the apical intracellular vesicles of type B intercalated cells and non-A-non-B intercalated cells suggests that HCO₃⁻ secretion may be regulated by trafficking of pendrin between the two membrane compartments.

acid-base metabolism; connecting tubule; collecting duct; bicarbonate secretion

THE COLLECTING DUCT PLAYS an important role in urine acidification. Acid secretion occurs along the entire collecting duct (2), and the cortical collecting duct (CCD) is capable of both proton and HCO₃⁻ secretion (17, 30). There is evidence from both structural and functional studies that intercalated cells are responsible for acid-base transport in the collecting duct (10, 32, 37).

At least two types of intercalated cells, type A and type B, are present in the CCD and the connecting tubule (CNT) of rats (1, 5, 13, 36), mice (12, 31), and rabbits (23, 24, 38, 39). Type A intercalated cells secrete protons mediated by a vacuolar type H⁺-ATPase, which is located in the apical plasma membrane and apical tubulovesicles (5, 12, 24, 31, 35). They reabsorb HCO₃⁻ by a truncated form of the erythrocyte Cl⁻/HCO₃⁻ exchanger AE1, which is located in the basolateral plasma membrane (1, 23, 31, 34, 38).

Type B intercalated cells secrete HCO₃⁻ by an apical Cl⁻/HCO₃⁻ exchanger that is functionally distinct from the basolateral Cl⁻/HCO₃⁻ exchanger in the type A intercalated cells (6, 18). Type B intercalated cells express H⁺-ATPase in the basolateral plasma membrane and in vesicles throughout the cytoplasm (5, 24, 31, 35). A third configuration of intercalated cell, which we have called the non-A-non-B cell, has been described in the CNT and CCD of both mouse (12, 31) and rat kidney (1, 12, 13, 16). They are characterized by the presence of H⁺-ATPase in the apical plasma membrane and do not express AE1 immunoreactivity. The non-A-non-B configuration is the most prevalent form of intercalated cell in the mouse CNT (12). The function of the non-A-non-B cells remains to be established, and it is not known whether they represent a distinct subtype of intercalated cell or a modified form of either the type A or the type B intercalated cell.

Despite intensive research, the protein responsible for apical Cl⁻/HCO₃⁻ exchange in the type B intercalated cell has long escaped identification. Recently, however, a novel anion exchanger, pendrin, was iden-
IMMUNOLOCALIZATION OF PENDRIN IN RAT AND MOUSE KIDNEY

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Methods

Membrane fractionation for immunoblotting. Kidneys from normal male Munich-Wistar rats were divided into three zones: cortex/outer stripe of the outer medulla, inner stripe of the outer medulla, and inner medulla. Kidneys from normal NMRI mice were divided into two parts only: cortex/outer medulla and inner medulla. The tissues were homogenized (0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, pH 7.2), containing 8.5 μM leupeptin, 1 mM phenylmethyl sulfonylfluoride), and incubated with Ultra-Turrax T8 homogenizer (IKA Laborteknik) at maximum speed for 30 s, and the homogenates were centrifuged in an Eppendorf centrifuge at 4,000 g for 15 min at 4°C to remove whole cells, nuclei, and mitochondria. The supernatant was then centrifuged at 200,000 g for 1 h to produce a pellet containing membrane fractions enriched for both plasma membranes and intracellular vesicles. The samples were prepared for gel electrophoresis by adding Laemmli sample buffer containing 2% SDS (final concentration) to the resuspended pellets.

Deglycosylation of membrane proteins. Twenty micrograms of membrane proteins from whole rat kidney, prepared as described above, were denatured by heating to 100°C in 1% SDS for 5 min, diluted 1.9 with 1% Triton X-100 in the buffer described above (without leupeptin and phenylmethyl sulfonylfluoride), and incubated with 4 U of N-glycosidase (PNGase F, Boehringer Mannheim, Mannheim, Germany) overnight at room temperature (RT). The incubation was stopped by addition of Laemmli buffer.

Antibodies. To identify pendrin in the kidney, polyclonal antibodies were generated against synthetic peptides corresponding to 22 amino acids, MEAENMAELDVDQEMAMB-LAS, of the COOH terminus of mouse pendrin conjugated to keyhole limpet hemocyanine. Immune sera against the erythrocyte Cl-/HCO3- exchanger AE1 (kindly provided by Dr. Philip S. Low, Purdue University, West Lafayette, IN) and the 70-kDa catalytic subunit of the vacuolar H+-ATPase (kindly provided by Dr. Dennis Stone, University of Texas Southwestern, Dallas, TX) were used to identify intercalated cells. These antibodies have been well characterized in previous studies and label intercalated cells in both rat and mouse kidney (12, 31, 35). Monoclonal mouse antibodies against the 31-kDa subunit of H+-ATPase (E11; kindly provided by Dr. S. Gluck, University of Florida, Gainesville, FL) were also used. Polyclonal rabbit antibodies against rat aquaporin-2 (AQP2; kindly provided by Dr. Mark Knepper, National Institutes of Health, Bethesda, MD) were used to identify principal cells (19).

Electrophoresis and immunoblotting. Samples of membranes from rat and mouse kidney (see Membrane fractionation for immunoblotting for details) were run on 9% polyacrylamide minigels (Mini Protein II, Bio-Rad). After transfer by electrophoresis to nitrocellulose membranes, blots were blocked with 5% milk in PBS-T (80 mM Na2HPO4, 20 mM NaH2PO4, 100 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 h and incubated overnight at 4°C with anti-pendrin antibodies. The labeling was visualized with horseradish peroxidase (HRP)-conjugated secondary antibodies (diluted 1:3,000; P448, DAKO, Glostrup, Denmark) by using an enhanced chemiluminescence system (Amersham International). Immunoblotting was used to test the specificity of the affinity-purified anti-pendrin antibodies by applying the immunizing peptide. All polyclonal antibodies were subsequently processed as described above. Immunolabeling controls were performed using preabsorption of the immune serum with the immobilizing peptide for 2 h at RT.

Immunohistochemistry. Male Sprague-Dawley rats and C57BL/6 mice were used. The animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt). The kidneys were initially perfused briefly through the abdominal aorta or through the left ventricle with PBS to rinse away all blood. This was followed by perfusion with a periodate-lysine-paraformaldehyde for 10 min. The kidneys were removed and cut into 1- to 2-mm-thick slices that were fixed additionally by immersion in the same fixative for 2 h at RT and then overnight at 4°C. Sections of tissue were cut transversely through the entire kidney on a Vibratome (Pelco 101, sectioning series 1000, Ted Pella, Redding, CA) at a thickness of 50 μm and processed for immunohistochemical studies by using an HRP preembedding technique. To identify the intercalated cell populations that express pendrin, a multiple labeling procedure was used. AE1 and pendrin were labeled simultaneously by a double-labeling technique using a preembedding method, which was followed by labeling for H+-ATPase using a postembedding method.

Preembedding method for AE1 and pendrin or AE1 alone. Sections of periodate-lysine-paraformaldehyde-fixed tissue were cut transversely through the kidney on a Vibratome at a thickness of 50 μm and processed for immunohistochemistry by using an indirect immunoperoxidase method. All sections were washed with 50 mM NH4Cl in PBS three times for 15 min. Before incubation with the primary antibodies, the tissue sections were incubated for 3 h with PBS containing 1% bovine serum albumin, 0.05% saponin, and 0.2% gelatin (solution A). The sections were then incubated overnight at 4°C in a mixture of antisera against AE1 (1:2,000) and pendrin (1:5,000) or with the antibody against AE1 (1:2,000) alone in PBS containing 1% bovine serum albumin (solution B). After several washes with solution A, the tissue sections were incubated for 2 h in HRP-conjugated donkey anti-rabbit IgG, Fab fragment (Jackson ImmunoResearch Laboratories), diluted 1:100 in solution B. The tissues were then rinsed, first in solution A and subsequently in 0.05 M Tris buffer, pH 7.6. For the detection of HRP, the sections were incubated in 0.1% 3,3′-diaminobenzidine in 0.05 M Tris buffer for 5 min, after which H2O2 was added to a final concentration of 0.01% and the incubation was continued for 10 min. After washing with Tyrode buffer three times, the sections were dehydrated in a...
graded series of ethanol and propylene oxide and embedded in TAAB812 between polyethylene vinyl sheets.

Postembedding method for \( H^+ -ATPase \), pendrin, or AQP2. From the flat-embedded 50-\( \mu \)m-thick sections processed for double immunolabeling of pendrin and AE1 or AE1 alone, sections from the renal cortex were excised and glued onto empty blocks of TAAB812, and consecutive 1.5-\( \mu \)m sections were cut for postembedding immunolabeling. The sections were treated for 15 min with a saturated solution of sodium hydroxide in absolute ethanol to remove the resin. After three brief rinses in absolute ethanol, the sections were hydrated with graded ethanol and rinsed in tap water. The sections were rinsed with PBS, incubated in normal donkey serum for 1 h, and subsequently incubated overnight with antibody against \( H^+ -ATPase \) (1:2,000), pendrin (1:3,000), or AQP2 (1:1,000) at 4°C. The sections were rinsed with PBS and incubated for 2 h in HRP-conjugated donkey anti-rabbit IgG, Fab fragment, and washed again with PBS. For detection of antibodies, Vector SG (Vector Laboratories) was used as the chromogen to produce a gray-blue label, which is easily distinguished from the brown label produced by 3,3′-diaminobenzidine in the first immunolocalization procedure for pendrin and AE1 by the preembedding method. The sections were washed with distilled water, dehydrated with graded ethanol and xylene, mounted in Canada balsam, and examined by light microscopy.

Confocal laser scanning microscopy and immunoelectron microscopy. Kidneys from Munich-Wistar rats and NMRI mice were fixed by retrograde perfusion via the aorta with 4% paraformaldehyde, in 0.1 M cacodylate buffer, pH 7.4, as previously described (9). For immunofluorescence microscopy, kidney blocks containing all kidney zones were dehydrated and embedded in paraffin. The paraffin-embedded tissues were cut at 2 \( \mu \)m on a rotary microtome (Leica, Heidelberg, Germany). The sections were dewaxed and rehydrated. To reveal antigens, sections were placed in 1 mM Tris buffer (pH 9.0) supplemented with 0.5 mM EGTA and were heated by a microwave oven for 10 min. Nonspecific binding of Ig was prevented by incubating the sections in 50 mM NH\(_4\)Cl for 30 min followed by blocking in PBS supplemented with 1% BSA, 0.05% saponin, and 0.2% gelatin. Sections were incubated overnight at 4°C with pendrin antibodies. In double-labeled fluorescence studies, the vacuolar \( H^+ -ATPase \) was localized with mouse monoclonal antibodies that were mixed with the antibody against pendrin. The labeling was visualized by using a rhodamine-conjugated goat anti-mouse antibody (diluted 1:200; Alexa 546, Molecular Probes) mixed with a fluorescein-conjugated goat anti-rabbit antibody (diluted 1:200; Alexa 488, Molecular Probes). The microscopy was carried out by using an SP2 laser confocal microscope (Leica). For immunoelectron microscopy, the frozen samples were freeze substituted in a Reichert AFS freeze substitution unit (9, 15). In brief, the samples were sequentially equilibrated over 3 days in methanol containing 0.5% uranyl acetate at temperatures gradually raised from −80 to −70°C, rinsed in pure methanol for 24 h while increasing the temperature from −70 to −45°C, and infiltrated with graded Lowicryl HM20 and methanol solutions (1:1, 2:1) and pure Lowicryl HM20 before ultraviolet polymerization for 2 days at −45°C and 2 days at 0°C. For immunolabeling, Lowicryl HM20 sections were pretreated with the saturated solution of NaOH in absolute ethanol (2–3 s), rinsed, and preincubated for 10 min with 0.1% sodium borohydride and 50 mM glycine in 0.05 M Tris, pH 7.4, containing 0.1% Triton X-100. Sections were rinsed and incubated overnight at 4°C with anti-pendrin antibodies diluted 1:200. After being rinsed, sections were incubated for 1 h at RT with goat anti-rabbit IgG conjugated to 10-nm colloidal gold particles (1:50; GAR.EM10, BioCell Research Laboratories, Cardiff, UK). The sections were stained with uranyl acetate and lead citrate before examination in a Philips CM100 electron microscope.

RESULTS

Immunoblotting of pendrin in rat and mouse kidney membrane. To establish the presence and examine the cellular distribution of pendrin in the kidney, a rabbit antibody raised against a peptide corresponding to mouse pendrin was produced. The antibody recognized the peptide on immunoblots (not shown) and specifically recognized a ~126-kDa band on immunoblots using membrane fractions from mouse and rat kidney (Fig. 1). Labeling was observed in membrane fractions from the rat cortex/outer stripe of the outer medulla but not in the inner stripe of the outer medulla or the inner medulla (Fig. 1A). A band of the same size was also seen in immunoblots of membrane fractions from mouse kidney cortex/outer medulla, but no band was detected in membranes from the inner medulla (Fig. 1A). The specificity of the labeling was confirmed by using anti-pendrin antibody preabsorbed with the immunizing peptide (Fig. 1B). The size of the recognized protein is larger than the predicted size of ~87 kDa, suggesting that pendrin is glycosylated or posttranslationally modified in other ways. Consistent with this, immunoblotting using membrane fractions subjected to deglycosylation by N-glycosidase (PNGase F) treatment revealed a marked reduction in molecular mass corresponding to ~95 kDa (Fig. 1C), which is closer to the predicted molecular size of pendrin.

Cellular and subcellular localization of pendrin in mouse kidney determined by double- and triple-labeling immunocytochemistry and confocal laser microscopy. To evaluate the overall distribution of pendrin in mouse kidney, immunohistochemistry was performed by using paraffin sections or preembedding immuno-
staining of 50-μm-thick vibratome sections. Immunohistochemistry revealed abundant labeling of the CNT and CCD in the outer and inner cortex (inset, Fig. 2A). Confocal laser scanning microscopy revealed strong apical pendrin immunoreactivity (green, Fig. 2A) in type B intercalated cells with basolateral H^+-ATPase (red, Fig. 2A). In contrast, no labeling was observed in type A intercalated cells. As shown in Fig. 2, B–D, pendrin immunolabeling was observed in a minority of intercalated cells expressing apical H^+-ATPase (arrows, Fig. 2), strongly suggesting that pendrin is expressed in non-A-non-B cells. To confirm this and identify the three subtypes of intercalated cells with certainty, the distribution of pendrin was determined in 50-μm-thick vibratome sections processed for preembedding immunostaining, followed by sectioning and postembedding double labeling (see METHODS).

To establish that pendrin is expressed in non-A-non-B cells, we used two consecutive sections from the mouse CNT, in which non-A-non-B cells are the major type of intercalated cells. The first section was labeled for H^+-ATPase and AE1 (Fig. 3A), and the second section was labeled for pendrin and AE1 (Fig. 3B). Pendrin immunoreactivity was observed in the apical region of the AE1-negative intercalated cells, with strong apical H^+-ATPase immunoreactivity that identified them as non-A-non-B cells. In contrast, there was no pendrin immunoreactivity in the AE1-positive type A intercalated cells. The expression of pendrin in the mouse CCD was examined on three consecutive sections labeled for H^+-ATPase and AE1 (Fig. 3C), pendrin and AE1 (Fig. 3D), and AQP2 and AE1 (Fig. 3E). There was strong apical pendrin immunoreactivity in type B intercalated cells with basolateral H^+-ATPase and no AE1 immunoreactivity (Fig. 3, C and D). However, pendrin immunoreactivity was never observed in principal cells with apical AQP2 (Fig. 3, D and E).

**Immunoelectron microscopic localization of pendrin in mouse kidney.** Immunoelectron microscopy was conducted by using immunogold labeling of sections prepared from mouse kidney embedded in Lowicryl HM20 by cryosubstitution. In sections from the CNT, abundant immunogold labeling was associated with apical plasma membrane domains (arrows, Fig. 4A) as well as subapical intracellular vesicles of non-A-non-B intercalated cells (Fig. 4, A and B), which is consistent with
the results of immunoperoxidase and immunofluorescence microscopy. There was no labeling of the basolateral plasma membrane of non-A-non-B intercalated cells (Fig. 4C). Immunolabeling controls using antibody preabsorbed with excess immunizing peptide produced no labeling (not shown). Type A intercalated cells, which are characterized by prominent microvilli and abundant subapical tubulovesicular structures (Fig. 5, A and B) or CNT cells (not shown), exhibited no immunogold labeling of pendrin, which is consistent with immunoperoxidase and immunofluorescence microscopy. In the CCD, strong immunogold labeling was observed in the apical plasma membrane and apical intracellular vesicles of type B intercalated cells (not shown) as well as non-A-non-B intercalated cells (Fig. 6B). There was no labeling of the basolateral plasma membrane (not shown). The identity of the cells was confirmed by H⁺-ATPase labeling of the same cells in serial sections (Fig. 6, E–G). There was no pendrin immunolabeling in principal cells (Fig. 6C) or type A intercalated cells (Fig. 6D). Immunolabeling controls using antibody preabsorbed with excess immunizing peptide produced no labeling (not shown).

Cellular and subcellular localization of pendrin in rat kidney determined by double- and triple-labeling immunocytochemistry, confocal laser microscopy, and immunoelectron microscopy. The distribution of pendrin in rat kidney was determined in paraffin sections and in 50-μm-thick vibratome sections processed for immunolabeling. At low magnification, pendrin immunolabeling was observed in the CNT and CCD (inset, Fig. 7A), consistent with results from previous studies (21). Confocal laser scanning microscopy revealed strong apical immunostaining for pendrin in type B intercalated cells (green, Fig. 7A), which were identified by basolateral H⁺-ATPase labeling (red, Fig. 7A). There was no labeling in intercalated cells with apical H⁺-ATPase in the rat CCD (Fig. 7A). In the CNT (Fig. 7, B–D), pendrin and H⁺-ATPase were colocalized in the apical region of a minority of intercalated cells (arrows, Fig. 7D), suggesting that pendrin was expressed in non-A-non-B intercalated cells. To establish this interpretation, the distribution of pendrin was determined by triple immunolabeling in 50-μm-thick vibratome sections processed for preembedding immunolabeling, followed by sectioning and postembedding labeling (see METHODS) or by immunoelectron microscopy. The triple labeling for pendrin, AE1, and H⁺-ATPase demonstrated that apical pendrin labeling was seen in AE1-negative intercalated cells corresponding to type B intercalated cells with basolateral H⁺-ATPase in the CCD (⁎, Fig. 8A) and CNT as well as in non-A-non-B intercalated cells with apical H⁺-ATPase (not shown). In contrast, AE1-positive cells were associated with apical H⁺-ATPase labeling, corresponding to type A intercalated cells (arrows, Fig. 8A). Immunoelectron microscopy confirmed the presence of strong labeling of the apical plasma membrane and apical intracellular vesicles of type B intercalated cells (Fig. 9) and non-A-non-B intercalated cells, identified by apical H⁺-ATPase labeling (not shown), whereas no labeling was seen in the basolateral plasma membrane.

DISCUSSION

The results of the present study demonstrate that pendrin is expressed in the apical domain of all type B intercalated cells as well as in non-A-non-B intercalated cells in the CNT and CCD of both mouse and rat kidney. The demonstration that pendrin is expressed in type B intercalated cells is in agreement with obser-
vations by Royaux et al. (21) and provide further support for pendrin representing the apical anion exchanger of type B intercalated cells as indicated by their elegant transport studies in isolated CCD segments from pendrin-deficient mice (21). The results of our immunoelectron microscopic studies revealed strong labeling for pendrin in the apical plasma membrane as well as in apical intracellular vesicles of both type B and non-A-non-B intercalated cells. As reported previously (21), there was no expression of pendrin in the AE1-positive type A intercalated cells. Taken together, these observations indicate that both type B and non-A-non-B intercalated cells are capable of pendrin-mediated HCO₃⁻ secretion in the CCD and CNT and suggest that HCO₃⁻ secretion may be regulated by trafficking of pendrin between intracellular vesicles and the apical plasma membrane.

Pendrin is a novel anion exchanger that is closely related to a family of sulfate transporters. Mutations in the gene that encodes pendrin are known to cause pendred syndrome, a genetic disorder associated with goiter and deafness (7). Previous studies have demonstrated strong expression of pendrin in both the thyroid gland (20) and the inner ear (8), and there is...
that type B intercalated cells are involved in HCO$_3$-$^-$ mouse, and human kidney. It is generally accepted that type B intercalated cells, in the CCD and CNT of rat, are rich in mitochondria and have numerous apical tubulovesicular structures. M, mitochondria. Magnifications: A, \times 3,200; B, \times 34,000.

Fig. 5. Immunoelectron microscopy of ultrathin Lowicryl HM20 sections, demonstrating absence of pendrin in the type A intercalated cell from the mouse CNT (A and B). There is no immunogold labeling for pendrin in the apical plasma membrane or the subapical tubulovesicular structures. M, mitochondria. Magnifications: A, \times 3,200; B, \times 34,000.

evidence from studies in Xenopus laevis oocytes that pendrin functions as an iodide transporter (28) and a chloride/formate exchanger (27). In addition, Soleimani et al. (29) have provided evidence that pendrin expressed in human embryonic kidney HEK-293 cells can function as a Cl$^-$/OH$^-$ or a Cl$^-$/HCO$_3$-$^-$ exchanger.

Recently it was reported that pendrin mRNA (29) and protein (21) are expressed in the kidney. Immunofluorescence studies by Royaux et al. (21) demonstrated strong expression of pendrin in the apical domain of AE1-negative intercalated cells, presumably type B intercalated cells, in the CCD and CNT of rat, mouse, and human kidney. It is generally accepted that type B intercalated cells are involved in HCO$_3$-$^-$ secretion, which is mediated by an apical Cl$^-$/HCO$_3$-$^-$ exchanger that is distinct from AE1. To establish whether pendrin might represent the apical anion exchanger in the type B cells, Royaux et al. (21) examined HCO$_3$-$^-$ transport in isolated perfused CCD segments from pendrin-deficient mice (pendrin-knockout mice). In contrast to tubules from wild-type mice, CCD segments from pendrin-deficient mice did not secrete HCO$_3$-$^-$ in response to an alkali load. Those findings indicated that pendrin is responsible, at least in part, for HCO$_3$-$^-$ secretion in the CCD and thus may correspond to the apical Cl$^-$/HCO$_3$-$^-$ exchanger in the type B intercalated cells. However, until data from the present study became available, it was not clear from the studies by Royaux et al. whether pendrin was expressed in non-A-non-B intercalated cells, which constitute the majority of intercalated cells in the CNT of mice; nor was it clear whether all type B intercalated cells were labeled. Furthermore, there was no information about the subcellular distribution of pendrin at the electron microscopic level.

Soleimani et al. (29) have examined the expression of pendrin in microdissected tubule segments by using RT-PCR and found that pendrin mRNA was present in both the proximal tubules and the CCD. In contrast, there was no evidence of pendrin immunoreactivity in the proximal tubule in the present study or in the study by Royaux et al. (21). The reason for this discrepancy is not known. However, it is possible that the primers used for PCR reacted with the cDNA for another member of the same anion exchanger family to which pendrin belongs. In this regard, it is of interest that a recent study by Knauf et al. (14) identified a homolog of pendrin, a chloride/formate exchanger, in the brush-border membrane of the mouse proximal tubule. It should be pointed out that recent studies have reported the expression of a second anion exchanger, AE4, in the apical region of type B intercalated cells in rabbits (33). Whether this transporter is also expressed in mouse and rat kidney remains to be established.

The non-A-non-B intercalated cell was first described in rat kidney by Kim et al. (13) and Madsen et al. (16), who reported that a few intercalated cells in the CNT exhibited ultrastructural characteristics that were distinct from those of type A and type B intercalated cells. The non-A-non-B intercalated cells are larger than type A and type B intercalated cells; they are rich in mitochondria and have numerous apical microprojections similar to those described in type A intercalated cells. It was suggested that they might correspond to the AE1-negative intercalated cells with apical H$^+$-ATPase that according to a previous study by Alper et al. (1), constituted \textasciitilde 1% of the intercalated cells in the renal cortex of rats. Subsequent studies revealed that non-A-non-B cells were also present in mice and confirmed that these cells exhibit strong labeling for H$^+$-ATPase in the apical plasma membrane but do not express AE1 (12, 31). Moreover, the prevalence of the non-A-non-B intercalated cells in the CNT and CCD was found to be higher than previously anticipated, and it was demonstrated that the majority of intercalated cells in the CNT of the mouse belongs to the non-A-non-B subtype (12).

The function of the non-A-non-B intercalated cells remains to be established. It is not known whether they represent a distinct subtype of intercalated cell or a modified form of either the type A or the type B intercalated cell. Moreover, the response of the non-A-non-B intercalated cells to changes in acid-base balance has not been investigated in detail, and there are no studies of acid-base transport in the CNT of mice, in which non-A-non-B cells constitute a major proportion of the cells. However, the demonstration that these cells express both H$^+$-ATPase and the anion exchanger...
Fig. 6. Immunoelectron microscopy of ultrathin Lowicryl HM20 sections illustrating pendrin and H\(^+\)-ATPase in the mouse CCD. A: survey view of the CCD with three different cell types; principal cells (PC), type A intercalated cell (IC-A), and non-A-non-B cell (IC-nAnB). Rectangles, areas presented at higher magnification in B–G. B–D: there is strong labeling of pendrin (arrows) in the apical plasma membrane and intracellular vesicles in the apical part of the non-A-non-B intercalated cell (B), whereas no labeling is seen in the principal cell (C) or the type A intercalated cell (D). E–G: adjacent section showing abundant labeling of vacuolar H\(^+\)-ATPase (arrows) in the apical plasma membrane and apical intracellular vesicles of both the non-A-non-B and the type A intercalated cell (E and G), whereas no labeling is seen in the principal cell (F). Magnifications: A, ×16,000; B–G, ×36,000.
pendrin in the apical plasma membrane suggests that the non-A-non-B intercalated cell represents a unique cell type capable of both apical proton secretion and apical \( \text{HCO}_3^- \) secretion. From a functional point of view, this is difficult to explain. However, it is possible that a main function of the non-A-non-B cell is chloride reabsorption mediated by pendrin in the apical plasma membrane. By simultaneous secretion of both \( \text{HCO}_3^- \) and protons, mediated by an electrogenic \( \text{H}^+/-\text{ATPase} \), chloride reabsorption would be accomplished without changes in acid-base balance.

It has been previously suggested that intercalated cells might be able to change their polarity depending on the acid-base status of the animal (25). Consistent with this, a recent study demonstrated an adaptive remodeling of type B intercalated cells to functionally resemble type A intercalated cells from the CCD of rabbit kidney in response to acid incubation, and this process was associated with deposition of the hensin in the extracellular matrix of these cells (26). However, the percentage of intercalated cells with basolateral AE1 immunoreactivity appears to be constant during various experimental conditions (11, 22), and AE1 has never been observed in the apical plasma membrane of any cells in the collecting duct in vivo. Thus experimental evidence that intercalated cells can change their polarity in vivo is lacking. However, it should be pointed out that a recent study by Bagnis et al. (3) has reported striking changes in the prevalence of the different cell populations in the collecting duct after treatment with the carbonic anhydrase inhibitor acetazolamide. Rats treated with acetazolamide for 2 wk showed a significant increase in the percentage of type A intercalated cells in both the CCD and the outer cortex.
medullary collecting duct. This was associated with a decrease in the percentages of type B intercalated cells and principal cells in the CCD and outer medullary collecting duct, respectively. Whether the reduction in the prevalence of type B intercalated cells and principal cells represents a remodeling or an elimination of the cells remains to be established.

The results of this study demonstrate that pendrin is expressed in the apical plasma membrane and apical intracellular vesicles of both type B and non-A-non-B intercalated cells. Moreover, non-A-non-B intercalated cells share features of both type A and type B intercalated cells, expressing apical pendrin similar to type B intercalated cells and apical H^+\text{-}ATPase similar to type A intercalated cells. On the basis of these characteristics, we suggest that non-A-non-B intercalated cells represent a separate cell type with unique transport properties. Furthermore, on the basis of the observation that pendrin is located in both the apical plasma membrane and the apical intracellular vesicles, we propose that HCO_3^- secretion in both type B and non-A-non-B intercalated cells may be regulated through intracellular trafficking. Future studies are warranted to establish whether the expression and subcellular localization of pendrin are subject to regulation in response to changes in systemic acid-base status.

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Figure 8. DIC microscopy of serial sections of the CCD from rat kidney illustrating triple immunostaining for pendrin, AE1, and H^+\text{-}ATPase (A) or pendrin, AE1, and AQP2 (B and C). Pendrin and AE1 are shown in brown, and H^+\text{-}ATPase (A) and AQP2 (B and C) are shown in blue. A: pendrin is expressed in apical domains of the AE1-negative intercalated cells with basolateral H^+\text{-}ATPase (*). B and C: pendrin is expressed only in AE1-negative cells (*). Principal cells with apical AQP2 (blue) do not express pendrin. Arrows, type A intercalated cells with apical H^+\text{-}ATPase (blue) and basolateral AE1 (brown) but without apical pendrin expression. Magnifications: A–C, ×600.

Figure 9. Immunoelectron microscopy of ultrathin Lowicryl HM20 sections illustrating labeling for pendrin in the CCD of rat kidney. A: survey view of section of a CCD showing two different cell types, principal cell (PC), and type B intercalated cell (IC-B). Rectangle, area presented at higher magnification in B. B: there is strong labeling in the apical plasma membrane (arrows) and apical intracellular vesicles (arrowheads) in the type B intercalated cell. M, mitochondria. Magnifications: A, ×1,750; B, ×34,000.
REFERENCES


