A New Member of the HCO$_3^-$ Transporter Superfamily Is an Apical Anion Exchanger of β-Intercalated Cells in the Kidney*

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Received for publication, May 24, 2000, and in revised form, November 30, 2000
Published, JBC Papers in Press, December 1, 2000, DOI 10.1074/jbc.M004513200

The kidneys play pivotal roles in acid-base homeostasis, and the acid-secreting (α-type) and bicarbonate-secreting (β-type) intercalated cells in the collecting ducts are major sites for the final modulation of urinary acid secretion. Since the H$^+$/ATPase and anion exchanger activities in these two types of intercalated cells exhibit opposite polarities, it has been suggested that the α- and β-intercalated cells are interchangeable via a cell polarity change. Immunohistological studies, however, have failed to confirm that the apical anion exchanger of β-intercalated cells is the band 3 protein localized to the basolateral membrane of α-intercalated cells. In the present study, we show the evidence that a novel member of the anion exchanger and sodium bicarbonate cotransporter superfamily is an apical anion exchanger of β-intercalated cells. Cloned cDNA from the β-intercalated cells shows about 30% homology with anion exchanger types 1–3, and functional expression of this protein in COS-7 cells and Xenopus oocytes showed sodium-independent and 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid-insensitive anion exchanger activity. Furthermore, immunohistological studies revealed that this novel anion exchanger is present on the apical membrane of β-intercalated cells, although some β-intercalated cells were negative for AE4 staining. We conclude that our newly cloned transporter is an apical anion exchanger of the β-intercalated cells, whereas our data do not exclude the possibility that there may be another form of anion exchanger in these cells.

It is well known that the kidneys play very important roles in acid-base balance. Proximal convoluted tubules reabsorb bicarbonate from the primary urine, and the Na$^+/H^+$ exchanger and sodium bicarbonate cotransporter (NBC) are the key trans-

porter for this function. Cortical to medullary collecting ducts secrete protons and bicarbonate, whereas proton secretion occurs predominantly under usual conditions. Over the past 2 decades, the mechanisms of these acid-base transporters have been extensively studied. In the cortical collecting ducts, there are at least two types of intercalated cells, α and β, which secrete protons and bicarbonate, respectively (1). The α-intercalated cells possess H$^+$/ATPase and an anion exchanger on the apical membrane and the basolateral membrane, respectively (1, 2, 3). This basolateral anion exchanger in the α-intercalated cells is a truncated form of band 3 protein (4), anion exchanger (AE) type 1 (5), and its localization was revealed by immunohistochimical studies (3, 6). On the other hand, β-intercalated cells possess H$^+$/ATPase and an anion exchanger on the basolateral and the apical membranes, respectively. In the early work of Schuster et al. (3), it was suggested that peanut lectin binds exclusively to β-intercalated cells. Further immunohistochimical studies (7) revealed that a minority of the intercalated cells had basolateral band 3 protein labeling with apical peanut lectin binding, raising the possibility that there might be a third type of intercalated cell. Functional studies by Emmons and Kurtz (8) also suggested that about half of the peanut lectin-positive cells might be a variant of β-intercalated cells, which showed anion exchanger activities on both apical and basolateral membranes. The apical anion exchanger in the peanut-lectin-positive cells, however, exhibits several differences in its transporter characteristics, such as 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) sensitivity (8, 9), as compared with AE1. Furthermore, since immuno- histochimical studies with a specific antibody directed against the band 3 protein have not shown positive staining on the apical membranes of β-intercalated cells (3, 6, 7), it has been suggested that this apical anion exchanger is a transporter distinct from AE1. In contrast to these previous immunocytochemical and functional studies, van Adelsberg et al. (10) reported that purified and immortalized β-intercalated cells express AE1 and have apical anion exchanger activity, suggesting that the difference in the characteristics of the apical and basolateral membranes is responsible for the difference in the immunoreactivity to the anti-AE1 antibody in immunohistochimical studies. Fejes-Tóth et al. (11), however, reported that primary cultures of β-intercalated cells showed very low levels of AE1 mRNA and protein expression. In the present study, we examined whether there is indeed a novel anion exchanger in β-intercalated cells, using the polymerase chain reaction (PCR)-based cloning method. The obtained
cDNA was determined to be a new member of the AE and NBC superfamily, and this novel transporter is present on the apical membranes of the β-intercalated cells.

**EXPERIMENTAL PROCEDURES**

**Isolation of β-intercalated Cells**—The β-intercalated cells were collected by a fluorescence-activated cell sorter, as previously reported (12). Female New Zealand White rabbits (1.2–1.8 kg) were anesthetized with pentobarbital (35 mg/kg, intravenously). Both kidneys were immediately removed and perfused through cannula inserted into the renal arteries with ice-cold solution A at 4 °C (which contained, in mM, NaCl 115, KCl 5.0, MgSO 4 1.0, CaCl 2 1.8, sodium acetate 10, NaHPO 4 1.6, NaHPO 4 0.4, Na-HEPES 5, H-HEPES 5, and n-glucose 8.3, at pH 7.4) containing 0.2% collagenase and 0.02% hyaluronidase. The kidney cortex was separated and cut into small pieces. The pieces were incubated in the enzyme-containing solution A at 37 °C for 5 min. After this enzymatic digestion, tubular fragments were obtained by centrifugation (50 × g) and washed three times with enzyme-free solution A. The fragments were suspended in 40% Percoll solution and centrifuged at 16,000 × g for 20 min. With this centrifugation, the tubular fragments were separated into two layers, and the distal tubular fragments became concentrated in the upper layer. This layer was resuspended and washed three times with solution A. These fragments were further incubated in solution A at 30 min under an O 2 atmosphere in solution A containing 0.2% collagenase, 0.02% hyaluronidase, and 500 units of DNase. The cells were then centrifuged at 50 × g for 5 min and incubated at 4 °C for 30 min in solution A containing fluorescein isothiocyanate (FITC)-labeled peanut lectin (50 μg/ml). The cell suspension was passed 2 times through a 26-μm stainless steel mesh. The final density of the cells was adjusted to 1–1.5 × 10 6 cells/ml with solution A. The cells were analyzed and sorted with an Epics cell sorter system (Coulter Electronics, Hialeah, FL) as reported previously (12). Living single cells were distinguished by forward and 90° light scatter, according to the criteria in the aforementioned report. The purity of the sorted β-intercalated cells was examined by flow cytometric analysis or counting of FITC-positive and -negative cells by fluorescence microscopy. Fluorescein isothiocyanate (FITC) coverslips with the GFP-transfected COS-7 cells were observed and the efficiency of the GFP transfection was determined by an epifluorescence microscope (Olympus Optical, Tokyo, Japan). In each of the transfection studies, 100 cells on the coverslips with the GFP-transfected COS-7 cells were observed and the ratio of GFP-positive to total cells was calculated.

**Reverse Transcription-PCR**—Total RNA from β-intercalated cells was extracted using an RNA extraction kit (Qiagen, GmbH, Hilden, Germany). One microgram of total RNA was reverse-transcribed with a random hexamer (Gene Amp RNA PCR Core kit; Applied Biosystems, Foster City, CA). PCR was performed with a pair of degenerate primers: 5′-AAAGGG(T/C)G(G/G)G(C/T)CCATTCG(T/C)CT-3′ (forward, F-1) and 5′-CGGACCTCGCGCCATCG(CT/C)GA-3′ (reverse, R-1), which were designed from the consensus sequences of AE1 to AE3 (GenBank accession numbers X12609, S45791, and AF031650). The PCR products were obtained and subcloned into the TA cloning vector. Sequencing analysis showed both clones to contain the 5′ region of this 1-kb fragment (5′-GGCTTCTGAGGATCAGCAGC-3′, probe-1) was used for the cDNA library screening. The kidney rabbit uni-ZAP cDNA library (Stratagene, La Jolla, CA) was purchased and mass-cultured in E. coli JM 109 cells. Two positive clones were isolated by a fluorescence-activated cell sorter, as previously reported (13). Female New Zealand White rabbits (1.2–1.8 kg) were anesthetized with pentobarbital (35 mg/kg, intravenously). Both kidneys were immediately removed and perfused through cannula inserted into the renal arteries with ice-cold solution A at 4 °C (which contained, in mM, NaCl 115, KCl 5.0, MgSO 4 1.0, CaCl 2 1.8, sodium acetate 10, NaHPO 4 1.6, NaHPO 4 0.4, Na-HEPES 5, H-HEPES 5, and n-glucose 8.3, at pH 7.4) containing 0.2% collagenase and 0.02% hyaluronidase. The kidney cortex was separated and cut into small pieces. The pieces were incubated in the enzyme-containing solution A at 37 °C for 5 min. After this enzymatic digestion, tubular fragments were obtained by centrifugation (50 × g) and washed three times with enzyme-free solution A. The fragments were suspended in 40% Percoll solution and centrifuged at 16,000 × g for 20 min. With this centrifugation, the tubular fragments were separated into two layers, and the distal tubular fragments became concentrated in the upper layer. This layer was resuspended and washed three times with solution A. These fragments were further incubated in solution A at 30 min under an O 2 atmosphere in solution A containing 0.2% collagenase, 0.02% hyaluronidase, and 500 units of DNase. The cells were then centrifuged at 50 × g for 5 min and incubated at 4 °C for 30 min in solution A containing fluorescein isothiocyanate (FITC)-labeled peanut lectin (50 μg/ml). The cell suspension was passed 2 times through a 26-μm stainless steel mesh. The final density of the cells was adjusted to 1–1.5 × 10 6 cells/ml with solution A. The cells were analyzed and sorted with an Epics cell sorter system (Coulter Electronics, Hialeah, FL) as reported previously (12). Living single cells were distinguished by forward and 90° light scatter, according to the criteria in the aforementioned report. The purity of the sorted β-intercalated cells was examined by flow cytometric analysis or counting of FITC-positive and -negative cells by fluorescence microscopy. Fluorescein isothiocyanate (FITC) coverslips with the GFP-transfected COS-7 cells were observed and the efficiency of the GFP transfection was determined by an epifluorescence microscope (Olympus Optical, Tokyo, Japan). In each of the transfection studies, 100 cells on the coverslips with the GFP-transfected COS-7 cells were observed and the ratio of GFP-positive to total cells was calculated.

**Northern Blotting**—Poly(A)+ RNA was extracted by the acid guanidinium thiocyanate/phenol chloroform method (13) using oligo(dT)-cellulose from various rabbit tissues. In each lane, 1–5 μg of RNA were electrophoresed in a 1% agarose gel in TBE buffer and stained for hydration with ethidium bromide solution at 63 °C, with a 32P-labeled probe prepared from the full-length cDNA of the newly cloned anion exchanger gene. A final high stringency wash was performed at 61 °C in 0.1× SSC, 0.1% SDS, and the blot was exposed to the Imaging Plate (Fuji Film, Tokyo, Japan). The blot was re-hybridized with a glyceraldehyde 3-phosphate probe, which was purchased from Novagen (Madison, WI).

**Measurement of Intracellular pH of Transfected COS-7 Cells**—COS-7 cells were grown and transfected in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and maintained in humidified incubators at 37 °C under 5% CO 2. The cells were seeded onto coverslips in 6-cm culture dishes, 18 h prior to transfection with 10 μg of plasmid DNA. pcDNA AE4a, pEGFP N3 (CLONTECH, Palo Alto, CA), or control vector pcDNA was transiently expressed in COS-7 cells by the LipofectAMINE method. After 36–42 h of incubation, the transfected COS-7 cells were used for functional studies, and the efficiency of the GFP transfection was determined by an epifluorescence microscope (Olympus Optical, Tokyo, Japan). In each of the transfection studies, 100 cells on the coverslips with the GFP-transfected COS-7 cells were observed and the ratio of GFP-positive to total cells was calculated.
performed on the same day. The rate of pH change (dpH/dt) during the initial 30 s after Cl− removal from the bath was calculated from the recording of pHb by a personal computer. The pHb and dpH/dt measurements were also performed with Cl−− and Na+-free solutions. The Na+-free solution contained (in mM) tetramethylammonium (TMA)-Cl 115, TMA-HCO3 25, KHEPES 5, NaCl 1.5, MgCl2 1, and n-glucose 10. The Na+- and Cl−-free solution contained (in mM) TMA hydroxide 115, NaCl 115, TMA-HCO3 25, KHEPES 5, KPO4 0.5, CaCl2 2, MgCl2 1, and n-glucose 10. Both solutions were gassed with 5% CO2 and 95% O2 and adjusted to pH 7.4 by Na-HEPES or H-HEPES.

In addition, the pHb and dpH/dt measurements were performed with Cl−− and HCO3-−-free solutions in another series of experiments with four independent transfections. The HCO3-−-free solution contained (in mM) NaCl 115, sodium gluconate 15, Na-HEPES 5, KCl 5, Na2HPO4 1.6, NaH2PO4 0.4, CaCl2 1, MgCl2 1, and n-glucose 10. The Cl−− and HCO3-−-free solution contained (in mM) sodium gluconate 130, Na-HEPES 5, H-HEPES 5, potassium gluconate 5, Na2HPO4 1.6, NaH2PO4 0.4, calcium gluconate 3.5, magnesium gluconate 1, and n-glucose 10. Both solutions were gassed with 100% O2 and adjusted to pH 7.4 by Na-HEPES or H-HEPES.

Patch Clamp Experiments on the Transfected COS-7 Cells—A coverslip to which cultured cells had adhered was placed in a recording chamber, and the chamber was mounted on the stage of an inverted microscope equipped with Nomarski optics (IX-70, Olympus, Japan) and an ×40 objective lens. The chamber was continuously superfused with solutions, gravity-fed at a rate of 1 ml/min at room temperature (−25 °C). Membrane voltages were recorded by a patch clamp method in the whole cell configuration. The patch pipette was made of Pyrex tubing pulled on a micropipette puller (P-87, Sutter Instrument, Novato, CA). The recording pipette was connected to the input stage of a patch clamp amplifier (Axopatch 200B, Axon Instruments, Foster City, CA). An Ag/AgCl wire connected to the bath via a ceramic bridge served as an indifferent electrode. The pipette resistance was ~10 MΩ when filled with pipette solution. The input capacitance and the series resistance were electrically compensated to the maximal extent possible. Signals were low-pass filtered (Bessel filter, cut-off frequency 5 kHz) and sampled at 10 kHz with a DigitData 1200 interface and pCLAMP 8 software (Axon Instruments) or a digital tape recorder (Sony Precision, Japan). Recorded data were analyzed with Igor Pro software (Wavemetrics, Lake Oswego, OR).

Three external solutions for the current clamp experiments were used. The CO2/HCO3-−-free external solution contained (in mM) NaCl 115, sodium gluconate 15, KCl 4, CaCl2 2, MgCl2 1, Na-HEPES 5, H-HEPES 5, and n-glucose 10 (pH 7.4, gassed with 100% O2). The CO2/HCO3-− external solution contained (in mM) NaCl 115, NaHCO3 25, KCl 4, CaCl2 2, MgCl2 1, and n-glucose 10 (pH 7.4, gassed with 5% CO2 and 95% O2). The Na+-free external solution contained (in mM) TMA-Cl 115, TMA-HCO3 25, KCl 4, CaCl2 2, MgCl2 1, and n-glucose 10 (pH 7.4, gassed with 5% CO2 and 95% O2). The pipette solution contained (in mM) KCl 20, potassium gluconate 110, MgCl2 2, CaCl2 1, EGTA 5, and HEPES 10 (pH 7.3).

Cloning strategy for AE4 cDNA. (1) RT-PCR of total RNA from β-intercalated cells was performed with the degenerate primers, F-1 and R-1, and an ~300-bp clone was obtained. (2) RT was performed with an AE4-specific primer (RT-1). A specific reverse primer was designed from the sequence of the ~300-bp clone (R-2). With a forward primer designed from the consensus sequence of the AE family (F-2), PCR was again performed, and an ~1-kb clone was obtained. (3), a probe (probe-1) was designed from the sequence of the 1-kb clone, and a cDNA library was screened. An ~2.3-kb clone was obtained and sequenced. (4), to obtain the 5′-end of AE4, RT was performed with AE4-specific primer (GSP-31), and 5′-RACE was performed with a specific reverse primer (GSP-32) designed from the sequence of the 2.3-kb clone and the Abridged Anchor Primer (AAP). An ~1.1-kb clone was obtained and ligated with the 2.3-kb clone at the KpnI site.

Preparation of Specific Antibody to AE4—Rat polyclonal antibody was raised against a synthetic peptide corresponding to the 15 C-terminal amino acids of AE4 (residues LMYPKQPAEINSVN). A cysteine residue was attached to the N terminus of the peptide to introduce an SH residue for coupling. The 16-amino acid CLMYQPKAPEINISVN peptide was synthesized by the solid-phase synthesis method. A synthetic peptide conjugated with keyhole limpet hemocyanin by the sulfo-maleimidobenzoyl-N-hydroxysuccinimide ester method was used to immunize two Wistar rats with Freund’s complete adjuvant (first injection) or incomplete adjuvant (from the second injection). After five injections, a sufficient increase of the antibody titer was confirmed by enzyme-linked immunosorbent assay, and serum was collected. Specific antibodies were prepared from the antisera by affinity chromatography using the antigen peptide coupled to 2-fluoro-1-methylpyridinium-toluene-4-sulfonate-activated Cellulofine (Seikagaku Kogyo, Tokyo, Japan).

Immunoblotting—Membrane fractions were prepared from the kidneys of female New Zealand White rabbits. The kidneys were perfused with phosphate-buffered saline (PBS) via the renal artery. After washing as much blood as possible out of the kidney, the cortex, outer medulla, and inner medulla were dissected. Crude tissue homogenates were prepared by homogenization in ice-cold buffer (which contained (in mM) sucrose 320, Tris-HCl 10, EDTA 2, PMSF 0.1, pepstatin A 1 μg/ml, leupeptin 1 μg/ml) with a Teflon-glass homogenizer. Crude homogenates were centrifuged at 1,000 g for 20 min to remove nuclei and tissue debris, and a supernatant was obtained. The supernatant was further centrifuged at 105,000 × g for 20 min, and the pellet was suspended in the same buffer (membrane fraction). Total protein concentrations were determined by the Lowry method.

Fifty micrograms of the membrane fraction from each part of the kidney were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting. SDS-PAGE was performed as described by Laemmli (17) on 10% gels. Protein bands were transferred to a Hybond ECL nitrocellulose filter (Amersham Pharmacia Biotech) and probed with affinity-purified anti-AE4 antibody. Bound antibodies were visualized by an ECL Western blotting system (Amersham Pharmacia Biotech) with peroxidase-conjugated goat anti-rat IgG (Cappel, ICN Pharmaceuticals Inc., Aurora, OH) as the second antibody. To determine antigen specificity, the antibody solutions (0.5 μg/ml of IgG) were

FIG.1. Cloning strategy for AE4 cDNA. (1) RT-PCR of the 2.3-kb clone at the specific reverse primer (GSP-31) and the Abridged Anchor Primer (AAP) was sequenced. (2) PCR was again performed, and an ~1-kb clone was obtained. (3), a probe (probe-1) was designed from the sequence of the 1-kb clone, and a cDNA library was screened. An ~2.3-kb clone was obtained and sequenced. (4), to obtain the 5′-end of AE4, RT was performed with AE4-specific primer (GSP-31), and 5′-RACE was performed with a specific reverse primer (GSP-32) designed from the sequence of the 2.3-kb clone and the Abridged Anchor Primer (AAP). An ~1.1-kb clone was obtained and ligated with the 2.3-kb clone at the KpnI site.
preabsorbed with the antigen peptide (50 μg/ml).

Immunohistochemical Studies—Kidneys from female New Zealand White rabbits were fixed by perfusion of a periodate/lysine/paraformaldehyde mixture via the renal artery. The kidney pieces were embedded in OCT compound in dry ice and isopentane, and thin sections (6 μm) were prepared with a cryotome. The kidney sections were stained with affinity-purified polyclonal rat antibody raised against the C terminus of rabbit AE4 and post-stained with peroxidase-conjugated goat anti-rat IgG (Cappel) as follows. Endogenous peroxidase was blocked by incubation in 0.3% hydrogen peroxide in methanol. The sections were washed in PBS and incubated overnight at 4 °C with affinity-purified polyclonal rat antibody against rabbit AE4 (IgG concentration 4 mg/ml). The tissue sections were then washed in PBS (4 times) and incubated for 2 h peroxidase-conjugated goat anti-rat IgG diluted 1:200 in PBS. The sections were washed in PBS (4 times), and peroxidase was detected by incubation in solution containing 0.1% 3,3′-diaminobenzidine and 0.01% hydrogen peroxide. After color development, the samples were washed in PBS. For light microscopy, the sections were counterstained with methyl green, dehydrated, and coverslipped. For immunofluorescence observations, rhodamine-labeled goat anti-rat IgG (Cappel) was used as the second antibody. After incubation with the first antibody, the tissue sections were stained in PBS containing rhodamine-labeled antibody and FITC-labeled peanut lectin (10 μg/ml) for 2 h. The sections were washed in PBS (4 times) and viewed by confocal laser scanning microscopy (LM510, Carl Zeiss Co. Ltd., Jena, Germany). For FITC, the excitation and emission wavelengths were 488 and 505 nm, respectively. For rhodamine, the excitation and emission wavelengths were 568 and 585 nm, respectively. To determine antigen specificity, the antibody solutions were preabsorbed with the antigen peptide (100 μg/ml).

**Fig. 2.** The deduced amino acid sequence of AE4a and its homology to AE1 to AE3. In the multiple sequence alignment, AE sequences (GenBank™ accession numbers X12609, S45791, and AF031650) identical to AE4a are highlighted by the shaded areas. The deduced amino acid sequence of AE4b lacks 16 residues in the N-terminal region (underlined). The amino acid sequence for the antibody is double underlined. The predicted membrane-spanning domains are indicated by the dotted line. The GenBank™ accession numbers for AE4a and AE4b are AB038263 and AB038264, respectively.

β-Intercalated Cell-specific Anion Exchanger

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AE4 has an extensive hydrophilic N-terminal region with about 400 amino acids and a C-terminal region of about 500 amino acids, which is rich in hydrophobic residues. Twelve membrane-spanning domains were predicted from the primary amino acid sequence using a Kyte-Doolittle algorithm. The topology showed a marked similarity to that of the AE and NBC superfamily (18–20). In the region homologous to the AE-consensus DIDS-binding motif (21), KLXX (where X is I, V or Y), AE4 has the sequence KMLN (amino acids 517–520), and there are a few protein kinase A and protein kinase C sites in the cytoplasmic domain (Fig. 3b).

Northern blots of rabbit tissue poly(A)+ RNA hybridized with an AE4a cDNA probe detected a major transcript of ~3.2 kb with three additional minor bands in the kidney cortex (Fig. 4a) at the lengths of ~3.8, ~3.4, and 2.8 kb. These minor bands might be products of alternative splicing, although we identified only one shorter transcript by cDNA sequencing, which lacked 16 residues in the N-terminal region. We detected no expression of the ~3.2-kb AE4a mRNA from the kidney medulla, spleen, liver, skeletal muscle, lung, brain, heart, stomach, and small intestine, whereas a very faint band at ~3.2 kb was observed in the case of the large intestine. In the inner medulla, small intestine, and spleen, however, faint bands at ~3.8 kb, also seen in the kidney cortex, were revealed. In addition, in the heart and skeletal muscle, faint bands were observed at ~4 kb. It is possible that these ~4-, ~3.8-, ~3.4-, and 2.8-kb length bands may reflect cross-hybridization of the AE4a probe with isoforms of the AE and NBC superfamily. It is also possible that they are novel isoforms of AE4.

Expression of the AE4a cDNA in COS-7 cells caused ~60% of the cells on the coverslips to undergo an increase in pHi in response to Cl− removal from the bath, whereas ~40% of the cells showed a small increase in pHi (Fig. 5b). Cell histograms, which are classified based on the dpHi/dt value, are shown in Fig. 6. All of the cells with mock transfection showed dpHi/dt below 3.0 × 10−3 pH units/s, whereas about 60% of the cells with AE4a transfection showed dpHi/dt higher than 4.0 × 10−3 pH units/s. In each transfection, the frequency of the cells with dpHi/dt values higher than 4.0 × 10−3 pH units/s was calculated. The frequency of cells with higher dpHi/dt was 63.2 ± 6.3%, whereas GFP transfection indicated transfection efficiency to be 68.8 ± 7.2%. Since the transfection efficiencies determined by functional and GFP studies were in good agreement, it is assumed that the high dpHi/dt values observed in a portion of the transfected cells reflect the function of transfected AE4. Rapid alkalization after bath Cl− removal was also seen in the Na+−free medium (Fig. 5d), indicating that cells showing transient expression of AE4a possess sodium-independent anion exchanger activity on their membranes.

Mock-transfected cells, on the other hand, showed very little alkalization in response to Cl− removal from the bath in either Na+−containing or Na+−free solution (Fig. 5g). In Fig. 5, the basal pHi of mock-transfected cells and cells with lower dpHi/dt was higher than that of cells with higher dpHi/dt. The basal pHi in the mock-transfected and AE4-transfected cells are summarized in Table I. Theses values did not differ significantly among three groups of cells in either Na+−free or Na+−containing solutions.

In a different series of experiments, the dpHi/dt values were measured with or without bath CO2/HCO3−. In the absence of CO2/HCO3−, dpHi/dt values were still significantly higher in AE4a-transfected cells (2.2 ± 0.32 × 10−3 pH units, n = 98 cells of all examined cells on the coverslips) than in mock-transfected cells (0.29 ± 0.31 × 10−3 pH units, n = 54 cells, p < 0.05), although, in AE4a-transfected cells, the dpHi/dt values in the presence of CO2/HCO3− (12.0 ± 0.68 × 10−3 pH unit, n = 56
absence of CO2/HCO3

These data, however, strongly suggest that AE4 mediates Cl− exchange in the mock-transfected cells. As shown in the figure, dpHi indicates the AE4-transfected cells. In this experiment, about 60% of the pcDNA AE4a-transfected cells on the coverslip showed rapid alkalization, and 40% showed a slight degree of alkalization after the removal of Cl− from the bath. The representative pHi changes in the cells with rapid alkalization (closed circles) and a slight degree of alkalization (open circles) on the same coverslip were shown. Each plot denotes the mean values of the three cells of each type on one coverslip. Each plot denotes the mean pHi changes divided in two types, as shown in c. About 40% of the cells showed slow and slight alkalization, whereas about 60% showed rapid alkalization after Cl− removal from the bath in the absence of Na+. The representative pHi changes in the cells with rapid alkalization (closed circles) and a slight degree of alkalization (open circles) on the same coverslip were shown. Each plot denotes the mean values of three cells of each type on one coverslip.

To investigate whether this protein transports Cl−, AE4a was expressed in Xenopus oocytes, and the 36Cl− uptake was measured. As shown in Fig. 7a, 36Cl− uptake was linear up to 15 min of incubation. Therefore, the oocytes were incubated for 15 min in subsequent studies. As shown in Fig. 7b, the oocytes injected with the cRNA of AE4a showed significantly higher 36Cl− uptake than that in the control oocytes, and this increase in 36Cl− uptake by cRNA injection of AE4a was not significantly changed by 200 μM DIDS. Furthermore, in the oocytes injected with the cRNA of AE4a, 36Cl− uptake in Na+-free medium was similar to that in control flux medium. These 36Cl− uptake data reveal that this anion exchanger is sodium-independent and DIDS-insensitive.

Since AE4 showed significant homology to NBC, we investigated the effect of Na+ removal from the bath on membrane voltage in the presence of HCO3− by the patch clamp method (Figs. 8, a and b). The basal membrane voltage of the mock-transfected cell was approximately −20 mV (−23 ± 1.6 mV, n = 6 cells) and was not significantly different from the membrane voltage of the AE4-transfected cells (−25 ± 1.3 mV, n = 32 cells). The membrane voltage of mock-transfected cells was not significantly changed by bath Na+ removal (Fig. 8a), and an AE4-transfected cell showed a similar response (Fig. 8b). Examining 32 AE4-transfected and 6 mock-transfected cells from four independent transfections, no cells showed significant membrane voltage changes in response to bath Na+ removal. In the patch clamp study, the cell viability was verified by the response to high K+ -external solution, and all cells were shown to retain K+ sensitivity. In these four transfection stud-
ies, since the efficiency of GFP transfection and the frequency of cells with high dpH/dt were 68.8 and 63.2%, respectively, it is extremely unlikely that all of the cells examined by patch clamp expressed no AE4 protein. These patch clamp studies confirmed that the AE4a-transfected COS-7 cells do not possess electronegic NBC activity.

We generated a polyclonal antibody specific for AE4 to examine the localization of this protein in the kidney. The specificity of the antibody was examined by immunoblot analysis with membrane fractions prepared from rabbit kidneys (Fig. 9). Immunoblotting showed three bands in the cortex and two bands each in the outer medulla and the inner medulla. Among the three bands in the cortex, those at 96 and 90 kDa were also seen in the outer medulla and the inner medulla, whereas the band at 110 kDa was seen only in the cortex. The band at 110 kDa was abolished by preabsorption of the antibody with the synthetic peptides used for immunization. The 96- and 90-kDa bands were considered to be nonspecific, since these bands were not affected by the preabsorption of the antibody with the antigen peptides. As shown in Fig. 10, a and b, the immunohistological study revealed AE4 immunoreactivity only in certain types of cells in the kidney. Moreover, AE4 immunostaining was detected only on apical cell membranes (Fig. 10b). This staining was completely abolished by preabsorption of the antibody with an excess of the synthetic peptide used for immunization (Fig. 11). In this Fig. 11, the serial sections of the same kidney were used. Since we used 6-μm sections of the kidney, it was difficult to determine precisely which cells were preserved in the two consecutive sections. The same collecting duct seen in the left panel of Fig. 11, however, was preserved in the right panel, and the AE4 labeling in this tubule was not seen by preabsorption of the antibody with an excess of the synthetic peptide used for immunization. The immunohistochemical studies with preabsorbed antibody were performed three times, and all three experiments gave the same results.

Double staining with FITC-labeled peanut lectin and rhodamine-labeled anti-rat IgG was performed to characterize the AE4-positive cells. In the rabbit kidney, peanut lectin is known to bind exclusively to the apical membrane of β-intercalated cells (1, 3). As illustrated in Fig. 12, double staining studies clearly showed that all of the AE4-positive cells (red) were also peanut lectin-positive (green), indicating that AE4 is only expressed in β-intercalated cells. There were also a few cells that were peanut lectin-positive and AE4-negative. These cells may represent variants of the intercalated cells (7). Rhodamine labeling is shown to be localized exclusively on the apical membrane by confocal microscopic observation, whereas the subapical region is also weakly stained in some cells. The immunostaining by anti-AE4 antibody, however, was not detected in the basolateral membrane of any cells. On the other hand, the double staining studies with preabsorbed antibody were also performed three times, and all three experiments gave no significant staining in the tubules except red blood cells (data not shown). In these experiments, red blood cells showed rhodamine fluorescence even without the first antibody, indicating that this staining is nonspecific binding of the second antibody to red blood cells.

**DISCUSSION**

In the present study, we identified the apical anion exchanger in β-intercalated cells of the kidney as a new member of the bicarbonate transporter superfamily. The newly cloned transporter showed homology to members of the anion exchanger family and the NBC family, and the homology to the NBC family was higher than that to the anion exchanger family.
The expressed protein in COS-7 cells and Xenopus oocytes, however, showed anion exchanger activity. Immunohistological study confirmed this protein to be localized to the apical membranes of β-intercalated cells.

We have cloned a novel anion exchanger from the mRNA of sorted peanut lectin-positive cells, assuming that peanut lectin is one of the best markers of the β-intercalated cells. The nomenclature for the subtypes of the intercalated cells is somewhat confusing, although it is common for the acid-secreting

FIG. 8. Representative recording of the membrane voltage by the whole cell patch clamp technique. a, a sample recording of a mock-transfected cell. The cell did not show any significant changes in membrane voltage by the removal of bath Na⁺, whereas it was depolarized by high K⁺ solution. The other five cells also showed similar behavior. b, a sample recording of an AE4-transfected cell. Similar to the mock-transfected cells, this cell did not show any voltage changes by the removal of bath Na⁺, whereas it was depolarized by high K⁺ solution. Thirty two cells in four independent transfections were examined, and no cell showed significant changes in membrane voltage with the removal of bath Na⁺. In these four independent transfections, the pH changes after bath Cl⁻ removal were also examined, and 63.2% of the cells had anion exchanger activity, as shown in Fig. 5.

FIG. 9. Representative Western blot analysis. Western blot analysis was performed on the membrane fractions prepared from rabbit kidney cortex (C), outer medulla (OM), and inner medulla (IM). Left panel, the affinity-purified anti-AE4 antibody was used as the first antibody. There were three bands in the cortex and two bands each in the outer medulla and the inner medulla. Among the three bands in the cortex, the bands at ~96 and ~90 kDa were also seen in the outer medulla and the inner medulla, whereas the band at ~110 kDa was seen only in the cortex. Right panel, the affinity-purified anti-AE4 antibody was preincubated with the synthetic peptides used for immunization (50 μg/ml). The band at ~110 kDa in the cortex was abolished by this preincubation, whereas the ~96- and ~90-kDa bands were not affected by preabsorption of the antibody with antigen peptides, indicating that these bands are nonspecific.

FIG. 10. Immunohistological study with affinity-purified antibody directed against the C terminus of AE4. a, AE4-positive-cells are seen only in the cortex in a punctate pattern. b, at higher magnification, the labeling is mainly localized to the apical membrane (arrowheads).
H^-ATPase for bicarbonate secretion. The immunohistochemical study by Schuster et al. (3) revealed the basolateral membrane of a certain type of intercalated cells to be labeled by anti-AE1 antibody. Deducing from the functional studies, it was suggested that α-type intercalated cells possess AE1 on their basolateral membranes. On the other hand, the intercalated cells without AE1 labeling showed apical peanut lectin binding, suggesting that peanut lectin is a marker for β-intercalated cells. Consistent with this immunohistochemical study, electrophysiological analysis of the intercalated cells (22, 23) indicated that there were two types of the intercalated cells, the cells with apical anion exchanger and the cells with basolateral anion exchanger, and that both types of the cells had basolateral Cl^- conductance. On the other hand, with the measurement of the intracellular pH, Emmons and Kurtz (8) showed the existence of a third type of intercalated cells, γ-type cells, which possess anion exchanger activity on the both apical and the basolateral membranes. In their microperfusion study, the response of pH_i to Cl^- removal from the bath or luminal perfusate was determined in all of the peptide lectin-positive cells. In the rat outer cortical collecting ducts, 4% of the intercalated cells were considered to be α-type, whereas 96% were peptide lectin-positive. Among the intercalated cells, 57% showed peptide lectin labeling and anion exchanger activities on both the apical and the basolateral membranes, and this type of the cell was defined as γ-type. Thirty nine percent of the intercalated cells showed anion exchanger activity only on the apical membrane as well as apical peptide lectin binding, and this type of the cell was defined as β-type. The anion exchangers on basolateral and apical membranes in β- and γ-type cells, however, shared common characteristics, i.e. they were both sodium-independent and DIDS-insensitive. Moreover, the functional studies by Weiner et al. (24) revealed all peptide lectin-positive cells with apical anion exchanger activity to also have anion exchanger activity on their basolateral membranes, whereas basolateral anion exchanger was also insensitive to DIDS and was Na^-independent. The reason for the discrepancy between these two in vitro microperfusion studies is not clear, although the authors raised the possibility that a sex difference of the rabbits, male in Emmons' study and female in Weiner's study, might have resulted in a difference in the proportion of intercalated cells with anion exchanger activity on both the apical and the basolateral membranes. Both functional studies, however, demonstrated that all peptide lectin-positive cells had apical anion exchanger activity, which is required for bicarbonate secretion. It is likely that peptide lectin-positive cells including γ-type cells work as bicarbonate secreting, i.e. β-intercalated, cells. Consistent with the functional studies of peptide lectin-positive cells (8, 9), our newly cloned anion exchanger, AE4, was sodium-independent and DIDS-insensitive, and this result strongly indicates that AE4 is an apical anion exchanger of β-intercalated cells. The results of the present immunohistological study with anti-AE4 antibody and peanut lectin (Fig. 12) also support this possibility.

The antibody to AE4, however, failed to label some of the peptide lectin-positive cells. These peptide lectin-positive and AE4-negative cells may represent a variant of β-intercalated cells (7). The results from the further immunohistochemical studies with anti-H^-ATPase and anti-AE1 antibodies and peptide lectin by Schuster et al. (7) were somewhat different from their previous report (3). In cortical collecting ducts, double staining with anti-H^-ATPase and anti-AE1 antibodies showed that ~40% of the intercalated cells had diffuse or apical H^-ATPase staining with basolateral AE1 labeling, indicating that these cells are α-type intercalated. In the rabbit kidney, the H^-ATPase staining was diffuse in the remaining of the intercalated cells but not exclusively present on the basolateral membrane, and this staining pattern in the rabbit kidney was different from that in the rat kidney (2). The results of double staining with peptide lectin and anti-H^-ATPase antibody yielded results different from those of their previous report (3). Seventy five percent of the intercalated cells showed diffuse H^-ATPase staining and apical peptide lectin labeling, and these are considered to be β-type intercalated cells. Seventeen percent of peptide lectin-positive cells showed apical H^-ATPase staining or no labeling by anti-H^-ATPase antibody. Furthermore, fluorescence-activated cell sorting showed 6–18% of the peptide lectin-positive cells to also be positive for AE1. The functions of these variants of peptide lectin-positive cells were not clarified in their study, although the authors raised the possibility that there may be hybrids of α- and β-intercalated cells. In these studies and the immunohistological study by Madsen et al. (6), however, it is consistent that apical membrane of the intercalated cells has never been labeled by anti-AE1 antibody.

Considering these functional (8, 9, 24) and immunohistological studies (3, 7), AE4 is considered to be an apical anion exchanger in β-intercalated cells, which are defined as cells with anion exchanger activity on the apical membrane, whereas we cannot deny a possibility that AE4 may be also the apical anion exchanger of γ-intercalated cells in the study by Emmons et al. (8) or of the hybrid cells reported by Schuster et al. (7). Furthermore, from our present immunohistological study, it is highly likely that there is another form of apical anion exchanger in β-intercalated cells. On the other hand, it is unlikely that AE4 is a basolateral anion exchanger of β- and

**Fig. 11.** When affinity-purified anti-AE4 antibody was preabsorbed with an excess of the synthetic peptide used for immunization (100 μg/ml), apical staining by the antibody (arrowheads, left panel) was completely abolished (right panel, a serial section of the left panel).
γ-intercalated cells, since our immunohistological data did not show any basolateral labeling by anti-AE4 antibody. These possibilities and molecular characteristics of the basolateral anion exchanger in β-intercalated cells remain to be determined in future studies.

The amino acid sequence of AE4 showed 30–48% homology with the known members of the sodium bicarbonate cotransporter family. Among these transporters, AE4 showed the highest homology to human NBC1, which induces electrogenic bicarbonate efflux in a sodium-dependent manner (19). In contrast to the homology with NBC, when AE4 was expressed in COS-7 cells and Xenopus oocytes, AE4 revealed sodium-independent and DIDS-insensitive anion exchanger activity, as described under “Results.” The experiments with and without CO2/bicarbonate in the superfusion solution indicated that high dpH/dt values in AE4a-transfected cells with CO2/bicarbonate were caused by Cl−/bicarbonate exchanger activity of AE4. On the other hand, dpH/dt values were small but significantly higher in AE4a-transfected cells than those in mock-transfected cells without bath CO2/bicarbonate, suggesting that AE4 mediates Cl−/base exchange. This transporter activity of AE4, Cl−/base exchanger activity in the absence of CO2/bicarbonate, is consistent with the previous report in the apical anion exchanger of the β-intercalated cells (25), and Cl−/base exchanger activity has been also reported in the other types of anion exchangers in various cells (26–28). To confirm that AE4 is not an electrogenic sodium bicarbonate cotransporter, we performed patch clamp experiments. With sodium removal from the bath in the presence of bicarbonate, the transfected cells did not show changes in membrane voltage, suggesting that AE4 does not possess electrogenic sodium-dependent transporter activity, which is a characteristic feature of NBC1 (19). Regarding NBCn1 (29), NBC3 (20), and nDAE1 (30), the sodium-independent feature of AE4 transporter activity distinguishes it from these transporters. These results indicate that AE4 is a transporter distinct from the previously known members of the NBC family. Since the transporter function of this novel protein is a sodium-independent anion exchanger, we named the newly cloned transporter as AE4, despite its homology being highest to NBC1.

Based on its localization, AE4 probably mediates HCO3− efflux from β-intercalated cells into the luminal fluid. In regard to the cell polarity change theory (31, 32), since the immunohistological study with anti-AE4 antibody showed no labeling of the basolateral membranes in the collecting ducts, a total cell polarity change of intercalated cells is unlikely. Instead, it is possible that the two types of intercalated cells interchange by switching their expressions of the AE1 and AE4 genes and by sorting of H+−ATPase. The cloning of AE4 should shed light upon the molecular mechanisms underlying the cell polarity change.

In conclusion, we have succeeded in the molecular cloning of an apical anion exchanger from β-intercalated cells of the kidney collecting ducts, and this finding should provide insight into both the molecular and the cell biological mechanisms that regulate acid-base balance in the kidney.

Acknowledgment—We thank Dr. Shingo Kato for technical support.

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