Abstract. Although the inner medullary collecting duct (IMCD) plays a major role in urinary acidification, the molecular identification of many of the specific components of the transport system in this nephron segment are lacking. A cultured line of rat IMCD cells was used to characterize the mediators of cellular HCO₃⁻ exit. This cell line functionally resembles α-intercalated cells. Physiologic experiments document that HCO₃⁻ transport is a reversible, electroneutral, Cl⁻ dependent, Na⁺ independent process. It can be driven by Cl⁻ gradients and inhibited by stilbenes such as 4-acetamido-4'isothiocyanatostilbene-2,2'-disulfonic acid. Immunohistochemical analysis, using a rabbit polyclonal antibody against the carboxy-terminal 12 amino acids of anion exchanger 1 (AE1), revealed a distribution of immunoreactive protein that mediate HCO₃⁻ exit in these cultured cells and in α-intercalated cells identified in sections of rat kidney cortex. Immunoblot revealed two immunoreactive bands (approximately 100 and 180 kD in size) in membranes from cultured IMCD cells, rat renal medulla, and freshly isolated IMCD cells. The mobility of the lower molecular weight band was similar to that of AE1 in red blood cell ghosts and kidney homogenate and therefore probably represents AE1. The mobility of the 180-kD band is similar to that for rat stomach and kidney AE2 and therefore probably represents AE2. Selective biotinylation of the apical or basolateral membrane proteins in cultured IMCD cells revealed that both AE1 and AE2 are polarized to the basolateral membrane. Northern blot analysis documented the expression of mRNA for AE1 and AE2 but not AE3. Furthermore, the cDNA sequence of AE1 and AE2 expressed by these cells was found to be virtually identical to that reported for kidney AE1 and rat stomach AE2. It is concluded that this cultured line of rat IMCD cells expresses two members of the anion exchanger gene family, AE1 and AE2, and both of these exchangers probably mediate the electroneutral Cl⁻-dependent HCO₃⁻ transport observed in this cell line. (J Am Soc Nephrol 9: 746–754, 1998)

The inner medullary collecting duct (IMCD) plays an important role in urinary acidification. Previous studies have shown that 60 to 70% of net acid excretion occurs in this segment of the nephron (1,2). To characterize the cellular determinants of acidification by the tubular epithelial cells of the IMCD, we and others have developed a tissue culture model, using cells derived from the terminal third of this segment (3–5). The line of cultured cells developed in our laboratory functionally resemble α-intercalated cells. These cultured cells have an apical proton pump (H⁺- ATPase) that is, in part, regulated by exocytosis and endocytosis, cytoplasmic carbonic anhydrase, and Cl⁻-dependent HCO₃⁻ exit (6,7). Physiologic evidence for basolateral Cl⁻/HCO₃⁻ exchange has also been provided by other investigators in rat IMCD cells in primary culture (4) and in isolated perfused tubules (8). Two isoforms of a band 3-related protein that mediate Cl⁻/HCO₃⁻ exchange have been identified in kidney: anion exchanger 1 (AE1) and AE2 (9,10). AE1 has been localized by immunohistochemistry to the basolateral membrane of the acid-secreting, α-intercalated cells of cortical and medullary collecting ducts in rabbit, rat, human, and mouse kidney (11). AE2 expression has also been demonstrated by Western and Northern analysis in virtually all tubule segments along the length of the rat nephron, with enhanced expression in the IMCD (10). However, the subcellular localization of AE2 has not been characterized because this polypeptide has not been identified by immunohistochemical methods in kidney (10). Despite the functional demonstration of Cl⁻/HCO₃⁻ exchange and localization of AE2 expression in the intact IMCD segment, definitive identification of the isoform(s) of AE that are localized to the basolateral membrane and that mediate HCO₃⁻ exit in these cultured cells still has not been reported.

The purpose of this study was to characterize at a functional, protein, and molecular level the specific anion exchanger(s) expressed by our cultured IMCD cell line. The new finding in this study is the demonstration that AE1 and AE2, but not AE3, are expressed in a single cell type: rat cultured IMCD cells. In this study, we document the expression of AE1 and AE2 at both the protein and mRNA level and demonstrate that these two isoforms reside in the basolateral membrane. Finally, based on the cDNA sequence of these two transporters, we
deduce that they are identical to that reported previously for rat kidney band 3 and rat stomach AE2.

Materials and Methods

Solutions and Reagents

The following solutions were used: NaCl Hepes buffer (NHB): 110 mM NaCl, 33.3 mM Hepes, 16.7 mM Na+ -Hepes, 5 KH2PO4, 1 mM CaCl2, 1 mM MgCl2, and 5 mM glucose, pH 7.2; choline chloride Hepes buffer: 110 mM choline chloride, 50 mM Hepes acid, 1.47 mM KOH, 2 mM KH2PO4, 1 mM CaCl2, 1 mM MgCl2, and 5 mM glucose, pH 7.2; choline chloride-free Hepes buffer: identical to choline chloride Hepes buffer except that 110 mM choline methyl sulfate was substituted for choline chloride. HCO3/C02-sodium chloride buffer: 15 mM NaCl, 15 mM sodium bicarbonate, 2.5 mM KH2PO4, 1 mM CaCl2, 1 mM MgCl2, 5 mM glucose, and 5% CO2; BNC-chloride-free buffer: identical to BNC except that sodium gluconate was substituted for NaCl. Buffers were titrated to pH 7.2 with NaOH, KOH, or HCl. Calcium activity determined by an ion-selective electrode was adjusted in all solutions to 1.8 mEq. 2',7'-Bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was prepared as a stock solution in DMSO; the total DMSO content to which the cells were exposed was less than 0.7%. Nigericin was prepared in ethanol and used at a concentration of 10 μg/ml potassium Hepes buffer solution. Glicemicin was dissolved in ethanol and used at a concentration of 1 μg/ml. 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) was dissolved in distilled water and used at a concentration of 2 mM.

Cell Culture

IMCD cells were originally obtained from rat papillae as described previously (6). Aliquots of this isolation have been stored in a liquid N2 freezer and were used in these studies. Cells from passages 10 to 15 were grown to confluence in 75-cm2 plastic flasks or on 12-×-12-mm glass coverslips in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum and 1% antibiotic solution (Life Technologies) in an atmosphere of 95% air-5% CO2.

Cell pH

The monolayers were incubated for 1 h at 37°C in DMEM containing 20 μM of the acetoxymethyl ester of BCECF (5). The coverslip was then placed in a plastic cuvette containing 1 ml of NHB and secured by means of a device designed to hold the coverslip at a 35° angle to the excitation beam (6). The monolayer was washed three times with NHB and then perfused with various solutions, as described in Results, at a rate of 1 ml/min. Fluorescence intensity was measured in a Perkin-Elmer LS 650-105 fluorospectrophotometer equipped with a thermostatically controlled (37°C) cuvette holder at excitation wavelengths of 505 and 455 nm with a slit width of 5 nm, and emission wavelength of 560 nm with a slit width of 8 to 10 nm (6). At the end of each experiment, the fluorescence intensity ratio was calibrated to cell pH (pHi), using KCl buffer containing 10 mg/ml nigericin (5,6). The fluorescence intensity ratio varied linearly with pH over the range 6.5 to 7.5. Autofluorescence of probe-free monolayers was less than 10% of the fluorescent signal of BCECF-loaded monolayers at excitation of both 505 and 455 nm, and no correction was made. In preliminary studies, we evaluated the effect of substitution of Cl− by gluconate or methyl sulfate. Both ions induce a cellular alkalinization at similar rates.

Antibodies

A synthetic polypeptide (NH2-CGLDEYDEVPMPV) corresponding to the COOH-terminal 12 amino acid residues of the mouse band 3 sequence with the addition of an NH2-terminal Cys was prepared by Quality Controlled Biochemicals, Inc. (Hopkinton, MA) (12). The peptide was used to immunize New Zealand White rabbits. High-titer sera were obtained after four booster injections at 2-wk intervals. Sera were tested by peptide enzyme-linked immunosorbent assay and subsequently purified by thin-layer gel affinity chromatography. This antibody in preliminary immunoblots recognized a single 100-kD band from red cell ghosts (AE1) and a 180-kD band from stomach homogenates (AE2) (Figure 1). Similar sera have been shown to cross react with both rat AE1 and AE2 (10).

Preparation of Erythrocyte and Postnuclear IMCD Membranes

Erythrocytes from freshly drawn rat blood were washed three times in cold phosphate-buffered saline (PBS) and lysed in 4 vol of ice-cold 5 mM sodium phosphate, pH 8.0, containing 20 μg/ml phenylmethylsulfonyl fluoride (PMSF). Red cells were kept on ice for 10 min to allow lysis to occur. Ghost membranes were harvested by centrifugation at 19,000 × g for 20 min at 4°C and washed multiple times in the same lysis buffer until the membranes appeared white. These membranes were then solubilized in an equal volume of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer.

Confluent IMCD cells were washed three times in cold PBS and harvested by centrifugation at 1000 × g for 10 min. The pellet of cultured cells or freshly isolated IMCD cells was suspended in 4 vol of ice-cold 5 mM sodium phosphate, pH 8.0, containing 20 μg/ml phenylmethylsulfonyl fluoride (PMSF). Red cells were kept on ice for 10 min to allow lysis to occur. Ghost membranes were harvested by centrifugation at 19,000 × g for 20 min at 4°C and washed multiple times in the same lysis buffer until the membranes appeared white. These membranes were then solubilized in an equal volume of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer.

Immunoprecipitation

IMCD postnuclear homogenate was immunoprecipitated using the C-terminal antipeptide AE antibody according to the following protocol. The homogenate was diluted to a protein concentration of 100 μg/ml with the homogenizing buffer also containing 0.5% deoxycholate. To a 900-μl aliquot of this diluted homogenate was added 2 μl of nonimmune serum and 30 μl of a 25% suspension of protein A-Sepharose 4B beads. This mixture was incubated at 4°C for 2 h, then centrifuged at 13,000 rpm in an Eppendorf centrifuge. The supernatant was incubated with 20 μl of anti-AE antibody and 50 μl of a protein A-Sepharose 4B bead suspension for 12 h at 4°C. The beads were pelleted by centrifugation and were washed three times and suspended in 60 μl of 2X SDS sample buffer. The immunoprecipitate in this suspension was analyzed for AE polypeptide by immunoblot.

Immunoblot

Red blood cell ghosts, whole-cell homogenates, and immunoprecipitated postnuclear IMCD samples, prepared as described above, were heated at 100°C for 5 min and 65°C for 10 min, respectively, before loading on a 7% polyacrylamide SDS gel and run under
reducing conditions (13). Protein was electrophoretically transferred to nitrocellulose filters that were washed in 150 mM NaCl, 100 mM Tris-HCl, pH 7.5, and 0.05% Tween 20 (TBST), and blocked for 1 h in TBST containing 5% wt/vol nonfat powdered milk (TBSTM) before incubation with AE antibody (1:1000 in TBSTM) at 4°C overnight. The filters were washed three times with TBST and incubated in secondary antibody (horseradish peroxidase-donkey anti-rabbit, 1:2000 in TBSTM) for 2 h at room temperature with agitation. After three washes, bound antibody was detected using the ECL enhanced chemiluminescence system (Amersham International).

**Immunohistochemistry**

IMCD cells grown on glass coverslips were fixed in paraformaldehyde-lysine-periodate, stored in PBS, and the detection of AE protein in these cells was performed by indirect immunofluorescence as described previously (6). The anti-AE antibody described above was used at a dilution of 1:100. Cortical sections of rat kidney were also examined for AE protein by indirect immunohistochemistry, using the same antibody at a dilution of 1:100 by methods described previously (11).

**Membrane Localization of AE**

IMCD cells were grown to confluence on permeable supports (2.5-cm Tanswell inserts, model 3412, Costar, Cambridge, MA). To metabolically label cellular proteins, monolayers of IMCD cells were incubated for 2 h in methionine-free DMEM followed by a 2-h incubation period in a similar medium that, in addition, contained 500 μCi/ml 35S-methionine. At the end of the labeling period, the cells were washed with DMEM that also contained 10 mM cold methionine. After metabolically labeling the proteins, the surface of the monolayers was labeled with biotin. These monolayers were incubated in NHB at 4°C. After 5 min, 0.3 mg/ml sulfosuccinimidyl-2-(biotinamido)-ethyl-1,3-dithiopropionate (NHS-SS)-biotin was added to either the apical or basolateral compartment. After 30 min in this solution, the biotin was washed away and quenched by rinsing the monolayers 3 times with a solution of 120 mM NaCl and 20 mM Tris-HCl. The cells were scraped from the surface of the inserts and homogenized in 1 ml of immunoprecipitation buffer (150 mM NaCl, 10 mM Tris-HCl, 5 mM ethylenediaminetetra-acetic acid, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 2 mg/ml bovine serum albumin, 4 mM PMSF, 0.5 μg/μl aprotinin, 2 μg/μl N-tosyl-l-phenylalanine chloromethyl ketone, and 5 μg/ml DNAase), and 5 μg/ml RNAase was added just before use. AE was immunoprecipitated from this homogenate as described above. After the wash of the Sepharose beads, 400 μl of 0.1 M glycine-HCl and 1% Triton X-100 was added to release the antigen-antibody complex from the bound protein A.

After an incubation of 25 min in this extraction solution, the samples were centrifuged at 12,000 × g, and the supernatant was removed and neutralized with 5 μl of 1N NaOH, 40 μl of 1 M Tris-HCl, and 25 μl of 10% bovine serum albumin. The biotin-labeled AE immunoprecipitate was removed from this solution by the addition of 60 μl of avidin-Sepharose beads. After 1 h of incubation at 4°C, the beads were washed three times and resuspended in 100 μl of SDS sample buffer and heated for 5 min to 65°C. The samples were subjected to SDS-PAGE electrophoresis, and the gels were processed for autoradiography. As positive controls, similar studies were also performed with two other membrane proteins: E-cadherin, which in confluent monolayers is restricted to the basolateral membrane, and GP-135, which is restricted to the apical membrane (14,15).

**RNA Isolation and cDNA Synthesis**

Total RNA was isolated from rat kidney and IMCD cells by using Tri Reagent® (Molecular Research Center, Inc.), and poly(A)+ RNA was isolated by oligo(dT) selection, using the polyA Spin® mRNA isolation kit (New England BioLabs, Beverly, MA). First-strand cDNA was synthesized using the GeneAmp RNA PCR Kit (Perkin Elmer Cetus, Norwalk, CT). After denaturation of the RNA at 95°C for 5 min, reverse transcription was carried out at 42°C for 60 min by using oligo(dT)16 primers, and the reaction was terminated by heating the tubes to 95°C for 5 min.

**Northern Blot Analysis**

Northern blotting was performed using standard protocols (16). A total of 2.5 μg of poly(A)+RNA derived from both rat kidney and IMCD cells was fractionated on 1.0% agarose gel containing 5.9% formaldehyde. The RNA were transferred to a nylon membrane (0.45 μm; Millipore, Bedford, MA) and probed with either full-length cDNA probes or PCR products. Full-length mouse cDNA probes for AE1 from pBL (17), AE2 from pΔX (18), or AE3 were used (19). In addition, for AE1, a 1506-bp PCR fragment was used as a probe that corresponds with nucleotides 1448 to 2954. For AE2, a 1376-bp product was used as a probe that corresponds to nucleotides 2705 to 4081. Each of these probes had no more than a 63% homology with other AE isoforms. The probes were labeled with 32P, using the Prime-a-Gen Labeling System kit (Promega, Madison, WI). Labeled probe was separated from unlabeled probe using the Push Column Beta Shield Device and NucTrap Probe Purification Columns (Stratagene, La Jolla, CA). The nylon membrane was prehybridized at 68°C for 2 h in Rapid-Hyb Hybridization Solution (Stratagene). Hybridization was performed for 2 h to overnight at 68°C. Two low-stringency washes were performed at 20°C for 10 min each with 2× SSC, 0.1% SDS, followed by two high-stringency washes with 0.1× SSC, 0.1% SDS at 67°C for 30 min. The membranes were exposed to x-ray film overnight at −70°C.

** Primer Selection**

Sense and antisense PCR oligonucleotide primers were designed (Oligo 4.1, National Biosciences, Plymouth, MN) based on the published cDNA sequences of rat kidney AE1 (3) and rat stomach AE2 (8), and were synthesized by Center for Advanced Biological Research (CABR) DNA/Protein Analysis Core (Boston University). The primer selection for the AE1 PCR product used as a probe for Northern hybridization was (shown 5' to 3') CCA AGC TCC AGT CAG GTC GAT (primer 5) and CCT CAG GTC CGA GAA GAA CTA (primer 3). The AE1 primers anneal to nucleotides 406 to 420 (primer 5) and 1048 to 1066 (primer 3) on the rat kidney and IMCD cell AE1 and bracket a 661-bp sequence. The PCR primers used for extension of the remaining sequences of AE1 and AE2 were designed according to the above-referenced sequence information, and were 22 bp in length.

**PCR Amplification and DNA Sequencing**

The AmpliTaq DNA Polymerase kit (Perkin Elmer Cetus) was used with 2 μl of cDNA. After an initial denaturation at 95°C for 5 min, PCR was carried out for 30 cycles with denaturation at 95°C for 1 min, annealing at 58 to 68°C for 1 min, and primer extension at 72°C for 1 to 1.5 min. The reaction mixtures were incubated for a final extension at 72°C for 10 to 12 min. The products were analyzed on 1.0% agarose gels and visualized with 0.5 μg/ml ethidium bromide. After glass-milk purification (GeneClean kit), the PCR products were subjected to automated sequencing (CABR DNA/Protein Analysis...
Core, Boston University), and the homology between these PCR products and corresponding DNA sequences of rat kidney AE1 and stomach AE2 was compared using NCBI BLAST program (National Institutes of Health, Washington, DC).

Statistical Analyses
Data are means ± SEM. Differences of statistical significance (P < 0.05) were determined using the paired and unpaired t test where appropriate.

Results
AE Protein Expression
To characterize the isoform(s) of AE expressed by our IMCD cell line, we analyzed homogenates of these cultured cells (cells from the rat renal medulla and papilla, stomach, and red cell ghosts) by immunological methods (immunoblot and immunoprecipitation). With immunoblotting, using a polyclonal AE antibody directed against the carboxy-terminal 12 amino acids of mouse AE1, two immunoreactive protein bands were identified in membranes from IMCD cultured cells, rat renal medulla, and freshly isolated IMCD cells from the renal papilla (Figure 1A). These immunoreactive bands were approximately 100 and 180 kD in size. The migration of the lower band was virtually identical to that of control red blood cell ghost AE1 (Figure 1A, lane 2) and kidney homogenate AE1 (Figure 1A, lane 3), and therefore probably represents AE1 (11). The approximately 180-kD band has a mobility similar to both the rat stomach AE (lane 5) (12) and kidney (lane 6) (10), and therefore this higher molecular weight band of protein probably represents AE2. Proteins immunoprecipitated from either IMCD or renal medulla homogenate with this same antibody (Figure 1B) also identified two proteins with similar molecular weights of approximately 100 kD (AE1) and approximately 180 kD (AE2) when analyzed by immunoblot. The 12 amino acid peptide used to generate this antibody blocked visualization of the bands observed in Figure 1A and the immunoprecipitation of the proteins noted in Figure 1B.

Immunohistochemical Localization of AE Protein
Immunocytochemistry of IMCD cells and rat kidney was performed using the same anti-AE-peptide antibody that was used for immunoblot and immunoprecipitation studies. All IMCD cells had detectable AE (Figure 2A). The immunoreactive AE in monolayers of IMCD cells grown on permeable supports was observed primarily in a pattern consistent with localization of AE at the lateral borders of cultured IMCD. There was no staining of the apical membrane of these cells. Rat kidney intercalated cells in the cortical collecting duct also displayed only basolateral staining (Figure 2B), a pattern similar to that noted in a prior publication using a different antipeptide antibody to AE1 (11). In contrast to the cortex, we were unable to demonstrate positive immunocytochemical staining of the terminal 2/3 of IMCD in the intact renal papilla (data not shown).

Selective Labeling of Apical and Basolateral Proteins
To selectively label apical or basolateral surface proteins, IMCD monolayers were grown to confluence on polycarbonate filter inserts, and after the proteins were metabolically labeled with 35S-methionine, they were exposed to NHS-SS-biotin added to the basolateral or apical compartments. To test the specificity of the labeling, we first examined the labeling of surface proteins known to have a polarized distribution to the apical surface (GP-135) (14), or to the basolateral surface (E-cadherin) (15). After apical addition, biotin GP-135 was labeled, but E-cadherin was not (Figure 3, lanes 2 and 4), whereas after basolateral addition of biotin, E-cadherin was labeled but GP-135 was not (Figure 3, lanes 1 and 3). Thus, this method in IMCD monolayers can be used to selectively

![Figure 1](image-url)

Figure 1. Immunoblot (A) and immunoprecipitate (B) of anion exchanger (AE). (A) Lane 1, molecular weight markers; lane 2, red blood cell ghosts; lane 3, postnuclear membrane homogenates from rat kidney; lane 4, inner medullary collecting duct (IMCD) cells; and lane 5, rat stomach. (B) Lane 1, red blood cell ghosts; lane 2, kidney homogenate; and lane 3, IMCD cell homogenate. For the Western blot, 1 μg of protein of red blood cell ghosts and 50 μg of protein of kidney, IMCD cell, or stomach homogenate were loaded into each lane; for immunoprecipitation, 10 μg of protein of red blood cell ghosts and 200 μg of protein kidney or IMCD homogenates were used. The blots were visualized with enhanced chemiluminescence.
membrane localization of AE polypeptides by selective surface biotinylation of plasma membrane proteins. IMCD cells were grown to confluence on polycarbonate membranes, metabolically labeled with 35S-methionine, and either the basolateral surface (lanes 1, 3, 5, and 6) or apical surface (lanes 2, 4, 7, and 8) was biotinylated. Homogenates were first immunoprecipitated with anti-GP-135 (lanes 1 and 2), anti-E-cadherin (lanes 3 and 4), or anti-AE antibodies (lanes 5 through 8). The immunoprecipitates were then solubilized, and the biotinylated proteins were isolated by precipitation with avidin-agarose beads and analyzed by gel electrophoresis and fluorography.

**Figure 2.** Immunofluorescence photomicrograph of AE in an IMCD monolayer (A) and section of rat renal cortex (B). The primary antibody used was a rabbit antipeptide antibody that reacts with both AE1 and AE2. The secondary antibody was a goat anti-rabbit IgG conjugated to rhodamine. (A) Immunoreactive protein in these IMCD cells was localized predominantly to the periphery of the cell, a pattern consistent with a basolateral membrane localization. Magnification, ×200. (B) Using the same antibody as in A, AE immunoreactivity was observed in rat cortical collecting duct cells in the basal and lateral membranes of intercalated cells. Magnification, ×1000.

Northern Blot Analysis

Northern blot analysis was performed to confirm that the two bands identified by immunoblot corresponded to AE1 and AE2 mRNA from rat kidney (control) and IMCD cells was electrophoretically transferred to a nylon membrane and sequentially exposed to three probes specific for AE1, AE2, or AE3. A single hybridization band of approximately 4.4 kb was identified in both kidney and IMCD cells when probed with a full-length AE1 cDNA (Figure 4, left panel) (3). When the same membrane was stripped and reprobed with a second full-length AE2 cDNA (Figure 4, right panel), another single hybridization band was visualized that was somewhat smaller (approximately 400 bp) than that for AE1 (4.4 kb) (10,19). No hybridization band was noted when these membranes were probed with a full-length cDNA for AE3. Similar results were obtained when either PCR fragment was used as cDNA probe for AE1 and AE2 (data not shown). These findings indicate that IMCD cells express two distinct AE mRNA.

Reverse Transcription-PCR Analysis for AE1 and AE2 in IMCD Cells and Rat Kidney

To confirm that IMCD cells expressed two different AE isoforms, AE1 and AE2 but not AE3, the sequences of AE1 and AE2 mRNA were determined. Using six different sets of primers for AE1, six different PCR overlapping products spanning 3656 nucleotides were generated (Figure 5). Automated sequencing revealed 96 to 100% homology of these products with the published sequence of rat kidney band 3. Similarly, using five different sets of primers for AE2, the full-length AE2 was sequenced and found to be 95 to 100% homologous to the published sequence of rat stomach AE2 (Figure 6). The major cause for mismatching of sequences between the PCR products and the published data was related to indeterminate bases (errors) by the automatic sequencer. If these are excluded, then the homology was approximately 99%. To confirm that our studies were not the result of contamination of the mRNA samples with genomic DNA, PCR was performed
Figure 4. Northern blot analysis of poly(A)+ RNA from rat kidney and IMCD cells. The blot was probed sequentially at high stringency with a full-length cDNA probe for AE1 and then, after being stripped with a full-length cDNA probe, for AE2. The size (in kilobase pairs) of the positive bands in the left panel is approximately 4400, and in the right, 4000.

Figure 5. Gel electrophoresis of reverse transcription (RT)-PCR amplification products of AE1. Lane 1 contains DNA standards, and lanes 2 through 7 contain PCR products corresponding to the following AE1 nucleotide sequences: lane 2, 2081 to 3172 (1091 bp); lane 3, 1448 to 2954 (1506 bp); lane 4, 1424 to 2975 (1551 bp); lane 5, 15 to 551 (536 bp); lane 6, 3963 to 4426 (463 bp); and lane 7, 298 to 1194 (896 bp).

without prior reverse transcription RT). In these studies, no appropriate reaction products were observed (data not shown). When primers for AE3 were used, no products were obtained when IMCD mRNA was used, but an appropriate product was obtained when mRNA from heart or brain was used (results not shown).

Functional Characterization of Cl-/HCO3- Exchange
To test whether HCO3 exit is a Cl-dependent process, pH of monolayers equilibrated in a Cl-containing (110 mM) solution buffered to extracellular pH (pHe) 7.2 with 25 mM HCO3/5% CO2 was continuously monitored during removal and restoration of Cl from the bathing solution. The pHe of the Cl-free and Cl-replete solutions was identical (pH = 7.2). In seven experiments, the steady-state pH in the presence of Cl was 7.17 ± 0.21 (Figure 7). Removal of Cl (Cl substituted isosmotically with gluconate) resulted in intracel-

Figure 7. Cl dependence and 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS) sensitivity of base exit from IMCD cells. Removal of extracellular Cl- (gluconate) resulted in alkalinization. Reintroduction of the Cl-replete solution induced cellular acidification. Experiments done in the presence of the SITS (2 mM) showed a 55 and 75% inhibition, respectively, of the alkalinization and acidification responses to Cl removal and reintroduction. *P < 0.01 for effect on fluorescence intensity ratio calibrated to cell pH (pHi) induced by changes in Cl-.
lular alkalinization at an initial rate of 0.11 ± 0.02 pH units/min. The new steady-state pH was 7.48 ± 0.14 (P < 0.007) and was stable for at least 5 min. In some experiments, pH was monitored for as long as 10 min and did not change. Cl⁻ restoration to the bathing solution resulted in a decrease in pH at an initial rate of 0.15 ± 0.02 pH units/min (P < 0.008, n = 7) to a new steady-state value (7.20 ± 0.15) that was not significantly different from the initial value in the presence of Cl⁻. In these studies, the initial rate of acidification that occurred with Cl⁻ addition (0.15 ± 0.02 pH units/min) to the external solution was greater than the rate of alkalinization (0.11 ± 0.02 pH units/min) after Cl⁻ removal (P < 0.05, n = 7). This difference in rate is probably due to the pH sensitivity of the exchanger. The cellular alkalinization-acidification induced by the outward and inward Cl⁻ gradients, respectively, indicates that base exit/entry is a Cl⁻-dependent process.

AE are characteristically inhibited by stilbenes. Therefore, we examined the effects of Cl⁻ removal and addition on pH changes in the presence of the stilbene AE inhibitor SITS. The addition of 2 mM SITS had no statistically significant effect on the steady-state pH. However, cell alkalinization was reduced by 50% with Cl⁻ removal in the presence of 2 mM SITS (0.05 ± 0.01 pH units/min), and acidification with the readdition of Cl⁻ was reduced by 92% (0.01 ± 0.01 pH units/min, P < 0.01 compared with control for effect on ΔpHi induced by removal/addition of Cl⁻; n = 6) (Figure 7). To examine whether the alkalinization and acidification responses induced by the imposed Cl⁻ gradient were related to changes in the cell membrane potential, similar experiments were performed in the presence of the Na⁺/K⁺ ionophore gramicidin to clamp the electrical potential (14). The experiments done in the presence of 1 μM of gramicidin resulted in pH changes similar to those observed in control monolayers. Cl⁻ removal with gramicidin in the external solution induced an alkalinization of 0.13 ± 0.02 pH units, and Cl⁻ readdition induced an acidification of 0.14 ± 0.03 pH units. These results indicate that the pH changes were not induced by alterations in cell electrical potential and are likely due to an electroneutral process, Cl⁻/HCO₃⁻ exchange.

Both Na⁺-dependent and Na⁺-independent Cl⁻/HCO₃⁻ transport have been reported in primary culture of IMCD cells. To test Na⁺ dependence of Cl⁻/HCO₃⁻ transport in our IMCD cell line, similar experiments were performed in the nominal absence of Na⁺ (Figure 8). In these experiments, baseline pH in a Na⁺-replete, HCO₃⁻/CO₂ solution was 7.11 ± 0.09. Removal of Na⁺ and Cl⁻ (substituted isosmotically with gluconate and choline, respectively) resulted in alkalinization (0.16 ± 0.03 pH units/min; P < 0.05) to a pH of 7.82 ± 0.09 and remained virtually unchanged for at least 5 min. This alkalinization, in part, could be related to the removal of Na⁺, Cl⁻, or both ions. However, the readdition of Cl⁻ to the bathing solution in the continued absence of Na⁺ in the extracellular solution resulted in acidification at a rate of 0.17 ± 0.03 pH units/min. Finally, Cl⁻ was removed from the external solution in the continued absence of Na⁺, and the cells were realkalinized at a rate of 0.14 ± 0.03 pH units/min. The similarity of the degree of alkalinization and acidification with Cl⁻ removal/addition in the presence or absence of Na⁺ indicates that Cl⁻/HCO₃⁻ transport in these cells is a Na⁺-independent process.

Discussion

Although there are several publications (2,3,8,20,21) in diverse model systems that document that the terminal portion of rat IMCD participates in urinary acidification, the molecular identification of all of the specific components of the transport system in this nephron segment are lacking. In this study, we have characterized on a physiologic, immunochromic, and molecular level the AE responsible for base exit from our cultured line of rat IMCD cells. By immunoblot, immunoprecipitation, and Northern blot analysis, we have shown that these cells express an mRNA and protein level both AE1 and AE2, and by immunohistochemistry that AE protein is localized to the basolateral membrane. In addition, we confirm in these cells that Cl⁻/HCO₃⁻ exchange in IMCD cells is a Cl⁻-dependent, Na⁺-independent, and SITS inhibitable process. These observations document for the first time the expression of two AE isoforms in a single type of renal epithelial cell.

Our physiologic experiments in general are in agreement with similar studies done in IMCD cells in primary culture (4) and in isolated perfused tubules (8). These studies document that HCO₃⁻ transport is a reversible, electroneutral, Cl⁻-dependent, Na⁺-independent process. It can be driven by Cl⁻ gradients and inhibited by stilbenes such as SITS. It also appears that the exchanger rate is regulated by pH. At alkaline pH, base exit occurred at a higher initial rate with Cl⁻ addition than the initial rate of base entry at a lower pH with Cl⁻ removal. Thus, these cultured IMCD cells express an AE that is functionally similar to that predicted for cells that express either AE1 or AE2.

Two distinct AE immunoreactive proteins have been iden-
tified in these cells by both immunoprecipitation and Western analysis. The antibody used in these studies was a rabbit polyclonal antibody directed against the COOH-terminal 12 amino acid residues of mouse AE1. Similar polyclonal antibodies have been shown to cross react with rat AE2 (10,12). In the immunoblots depicted in Figure 1, A and B, we believe that the lower protein band is AE1. This protein has a mobility that corresponds to a protein with a molecular weight of approximately 100 kD, and glycosylated AE1 is reported to have a similar molecular weight (9,17). Also, the migration pattern of this band was virtually identical to that of a protein derived from red blood cell ghosts, further identifying this band as AE1. The higher molecular weight band (approximately 180 kD) described in these immunoblots has a migration pattern similar to that observed with other carboxy-terminal anti-AE1 peptide antibodies (11). The proteins identified in this study were restricted to the basolateral membrane of rat kidney intercalated cells (Figure 2B). The AE immunoreactive protein expressed in these intercalated cells is believed to be AE1. Thus, it is likely that our antibody can be used to localize AE1. Because in rat kidney this antibody did not identify any other distribution of immunoreactivity, it is possible that AE2 in tissue sections is not recognized by the antibody. On the basis of these observations, we are confident that, at a minimum, AE1 is expressed in the basolateral membrane.

We also propose that AE2 is localized to the basolateral membrane. This latter conclusion is based on the physiologic observations that Cl-/HCO3- exchange in these cells is relatively resistant to the effect of SITS and the rate of exchanges is substantially stimulated by cell alkalinity. Both of these characteristics are features of AE2. Because all Cl- -dependent HCO3- transport occurs across the basolateral membrane of the IMCD cell (4), AE2 must also be localized to this site.

To confirm our hypothesis that both AE1 and AE2 are polarized to the basolateral membrane of these cultured IMCD cells, we determined whether these polypeptides are labeled apically or basolaterally in these cultured IMCD cells in culture. We biotinylated after addition of NHS-SS-biotin to the basolateral membrane. This latter conclusion is based on the physiologic characteristics of AE. It is uncertain whether the protein(s) localized by this antibody. On the basis of these observations, we are confident that, at a minimum, AE1 is expressed in the basolateral membrane of these cultured IMCD cells. However, we have documented in this study by Western blot analysis that noncultured, freshly isolated IMCD cells express a 180- and a 100-kD AE immunoreactive peptide. This observation confirms a recent study that also documents expression of both AE1 and AE2 by rodent renal papilla (10). In this latter study, expression of AE could only be detected when high concentrations of protease inhibitors were used during the isolation procedure. Furthermore, they noted that neither AE1 nor AE2 could be detected by immunohistochemical methods. The lack of immunohistochemical detectability of AE in this segment may result from rapid degradation of AE during fixation by proteases. These proteases may not be sufficiently inactivated by the perfusion fixation methods used because of the relative low flow to this inner segment of the nephron.

There may be a functional advantage of expressing both AE1 and AE2 in the terminal IMCD cells. The epithelial cells of the IMCD in vivo are exposed to a highly variable environment in which the extracellular osmolality, [NH4]/[NH4+], Pco2, and pH may vary over wide ranges. To maintain stable rates of H+ secretion and base exit across the basolateral surface, despite these highly variable conditions, it may be necessary to express two distinct isoforms of AE with distinct regulatory patterns. AE2 has a steep pH activity relationship, and it is activated even at low pH by a high osmolality in some systems (10,18). AE1 is less pH-sensitive and may not be affected by osmolality. The combination of expressing both isoforms would allow continued anion exchange activity over a wider range of pH than would occur with either isoform alone.

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