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The human NBCe1-A mutant R881C, associated with proximal renal tubular acidosis, retains function but is mistargeted in polarized renal epithelia

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Toye, Ashley M., Mark D. Parker, Christopher M. Daly, Jing Lu, Leila V. Virkki, Marc F. Pelletier, and Walter F. Boron. The human NBCe1-A mutant R881C, associated with proximal renal tubular acidosis, retains function but is mistargeted in polarized renal epithelia. Am J Physiol Cell Physiol 291: C788–C801, 2006. First published May 17, 2006; doi:10.1152/ajpcell.00094.2006.—The human electrogenic renal Na-HCO3 cotransporter (NBCe1-A; SLC4A4) is localized to the basolateral membrane of proximal tubule cells. Mutations in the SLC4A4 gene cause an autosomal recessive proximal renal tubular acidosis (pRTA), a disease characterized by impaired ability of the proximal tubule to reabsorb HCO3− from the glomerular filtrate. Other symptoms can include mental retardation and ocular abnormalities. Recently, a novel homozygous missense mutant (R881C) of NBCe1-A was reported from a patient with a severe pRTA phenotype. The mutant protein was described as having a lower than normal activity when expressed in Xenopus oocytes, despite having normal Na+ affinity. However, without trafficking data, it is impossible to determine the molecular basis for the phenotype. In the present study, we expressed wild-type NBCe1-A (WT) and mutant NBCe1-A (R881C), tagged at the COOH terminus with enhanced green fluorescent protein (EGFP). This approach permitted semiquantification of surface expression in individual Xenopus oocytes before assay by two-electrode voltage clamp or measurements of intracellular pH. These data show that the mutation reduces the surface expression rather than the activity of the individual protein molecules. Confocal microscopy on polarized mammalian epithelial kidney cells [Madin-Darby canine kidney (MDCK;II) expressing nontagged WT or R881C demonstrates that WT is expressed at the basolateral membrane of these cells, whereas R881C is retained in the endoplasmic reticulum. In summary, the pathophysiology of pRTA caused by the R881C mutation is likely due to a deficit of NBCe1-A at the proximal tubule basolateral membrane, rather than a defect in the transport activity of individual molecules.

bicarbonate; intracellular pH; acidbase; SLC4A4; Na+−HCO3− cotransporter 1

THE ELECTROGENIC Na-HCO3 COTRANSPORTER (NBCe1; SLC4A4) was first described in the proximal tubule of the salamander (7) and first cloned from the salamander kidney (41). The cDNA has since been cloned from many other species, including rat (39) and human (2).

The mammalian renal electrogenic Na-HCO3 cotransporter (NBCe1-A) is expressed in the basolateral membrane of renal proximal tubule cells (43), where it exports Na+ and HCO3− with an apparent Na+−HCO3− stoichiometry of 1:3 (47). Transcription from an alternative promoter (3) results in the expression of a second SLC4A4 gene product (NBCe1-B) in pancreas (2) and heart (10) that has a different NH2 terminus. A third SLC4A4 gene product (NBCe1-C), with an alternative COOH terminus, has been demonstrated in rat brain (5). The membrane-spanning domain of NBCe1 is conserved among all three splice variants of NBCe1. NBCe1 protein has also been detected in many other human tissues including retina, cornea (6), and skeletal muscle (29).

The renal proximal tubule is responsible for the reabsorption of ~80% of the HCO3− filtered in the glomerulus. The reabsorption mechanism (reviewed in Ref. 23) relies on the secretion of H+ into the lumen by an apical Na/H exchanger (NHE3) and vacuolar-type H+ pump. In the lumen, protons combine with HCO3−, catalyzed by carbonic anhydrase (CA) IV on the brush border membrane, to produce CO2 and H2O that enter the cell. Cytosolic CA II catalyzes the hydration of this CO2 and H2O to H+ (which is extruded by NHE3 and the H+ pump) and HCO3−, which exits across the basolateral membrane via NBCe1-A.

Proximal renal tubular acidosis (pRTA or type 2 RTA) is a systemic disease caused by a defect in HCO3− reabsorption by the renal proximal tubule (33). In addition to impaired kidney function, certain forms of the disease can also be associated with growth and mental retardation, pancreatitis, ocular abnormalities, reduced bone density, and abnormal dentition (reviewed in Ref. 31). Three bases for pRTA have been identified (23): 1) sporadic, transient pRTA that is manifest during early infancy and disappears in later life after alkali therapy (the molecular basis of this form of pRTA is presently unknown); 2) inherited autosomal dominant pRTA, the molecular basis of which is also unclear; and 3) inherited autosomal recessive pRTA. One cause of autosomal recessive pRTA is a deficiency in CA II (46). In addition, studies of NHE3-null mice suggest that a deficiency of this protein could also produce a pRTA (44).

Recent genetic studies of patients with autosomal recessive pRTA have identified several missense and nonsense mutations in the SLC4A4 gene that encodes NBCe1, providing a further genetic basis for this disease (20). Three of these missense mutations appear to cause mistargeting of the protein. The first

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(R510H in NBCe1-A) causes the mistargeting of the NH2-terminally green fluorescent protein (GFP)-tagged protein to an unspecified intracellular compartment (32) of mammalian renal epithelial cells. Two other missense NBCe1-A mutants—R298S and S427L—have been demonstrated to have a reduced presence at the basolateral membrane, S427L being mistargeted to the apical membrane of renal epithelial cells (32). All three of these missense mutants exhibit reduced transport activity when heterologously expressed in Xenopus oocytes (13, 16, 20, 32), although it is not clear to what extent the deficiency is due to reduced surface expression.

Three nonsense mutations of NBCe1-A have also been reported in patients. The first two are predicted to result in the complete absence of functional NBCe1-A from proximal tubule cells. Q29X (21) produces a truncation early in the cytoplasmic NH2 terminus of the protein. The deletion of nt 2311 is predicted to produce a frameshift at codon 721 (24), resulting in the translation of 29 anomalous amino acids—beginning in the predicted extracellular loop between transmembrane segments 7 and 8—before a premature stop codon. The third nonsense mutation, which results from a 65-nt deletion at the exon 23/intron 23 boundary, would result in a product truncated in the cytoplasmic COOH terminus (22).

In addition to the NBCe1 mutations discussed above, three novel NBCe1-A mutations—T485S, A799V, and R881C—have recently been reported in patients with autosomal recessive pRTA and ocular abnormalities (19). T485S failed to express in Xenopus oocytes and caused only half the expected rate of recovery of intracellular pH (pH7) from a nigericin-induced acid load when expressed in human endothelium-like ECV304 cells (19). When expressed in oocytes, A799V exhibited markedly reduced activity when assessed by two-electrode voltage clamp (19). As assessed by two-electrode voltage clamp in oocytes, R881C exhibited a 40% reduction in activity despite having normal values of the $K_{\text{a}}$ for Na$^+$ and reversal potential (19). However, these functional data were not corrected for relative levels of surface expression. Moreover, the trafficking of the mutants in mammalian epithelial cells was not reported. Thus it was not possible to attribute the pRTA to a specific molecular mechanism.

In the present study, we investigated the pathophysiology of the R881C mutation, using a combination of 1) electrophysiology on Xenopus oocytes expressing enhanced GFP (EGFP)-tagged, wild-type NBCe1-A (WT) and the comparable mutant (R881C); and 2) trafficking studies in Madin-Darby canine kidney (MDCK)I cells expressing the untagged proteins. Like WT, R881C exhibits qualitatively normal current-voltage (I-V) curves with a normal sensitivity to Na$^+$ removal or DIDS application. Moreover, like WT, R881C exhibits qualitatively normal pH7 recovery from CO2-induced acid loads, as well as normal sensitivity to Na$^+$ removal or DIDS application. The apparent Michaelis constant ($K_{\text{a}}$) for the NBCe1 inhibitor DIDS, magnitudes of NBCe1 currents, and rates of pH7 increase due to HCO$^-$ transport through NBCe1 show that—when corrected for surface expression—R881C exhibits normal activity in oocytes. Confocal microscopy shows that the untagged mutant protein is not delivered to the basolateral membrane of polarized MDCKI cells but substantially colocalizes with an endoplasmic reticulum (ER) marker. Thus the simplest explanation for the renal symptoms in the affected patient is that the defect lies solely with the trafficking of R881C.

**MATERIALS AND METHODS**

**Construction of an EGFP-Tagged NBCe1-A in a Xenopus Expression Vector**

Our starting material was NBCe1-A.pGH19, that is, NBCe1-A cDNA cloned into a pGEM-based vector between the 5’ and 3’ untranslated regions of the Xenopus β-globin gene (51). We introduced the sequence TCACCCGTA (i.e., a Ser codon plus an AgeI restriction site) at a position immediately prior to the NBCe1-A termination codon, using the Quikchange mutagenesis kit (Stratagene, Cedar Creek, TX) according to the manufacturer’s protocol. The resulting construct was NBCe1-A/AgeI.pGH19.

EGFP cDNA was supplied as the vector construct pEGFP-C1 (Clontech, Palo Alto, CA), in which the EGFP cDNA is flanked by a 5’ AgeI and a 3’ BspEI restriction site. Using the Quikchange mutagenesis kit, we mutated the 3’ BspEI restriction site into a second AgeI restriction site, enabling the isolation of the EGFP open reading frame from pEGFP-C1 cDNA by AgeI digestion.

The resulting AgeI/EGFP/AgeI was cloned nondirectionally into NBCe1-A/AgeI.pGH19 to create NBCe1-A-EGFP.pGH19 and resulted in the 5-amino acid linker “SPVAT” between NBCe1-A and EGFP. We abbreviate this clone as WT-EGFP. The sequence of the final construct was confirmed by automated sequencing performed by the Keck Sequencing Center at Yale University. Use of this construct was recently reported in Ref. 35.

**Mutagenesis**

The missense mutation was introduced into the Xenopus NBCe1-A expression construct with the Quikchange mutagenesis kit (Stratagene) according to the manufacturer’s protocol. The oligonucleotide primers used to introduce the R881C mutation into NBCe1-A were synthesized by the Keck Oligonucleotide Synthesis Facility at Yale University (sense: 5’-gtcagtcattgatTgtctgaagctgcttctg-3’; antisense: 5’-cagacgctctcagacAatccatgaagct-3’). The construct NBCe1-A(R881C)-EGFP.pGH19 is abbreviated as R881C-EGFP. The sequence of the final construct was confirmed by automated sequencing performed by the Keck Sequencing Center at Yale University. It should be noted that the C→T nucleotide substitution used to create the mutant constructs represents the actual nucleotide substitution present in the SLC4A4 gene of the affected patient (19).

**Expression in Xenopus Oocytes**

Capped mRNA was synthesized in vitro with the T7 mMessage mMachine kit (Ambion, Austin, TX). Stage V–VI oocytes from Xenopus laevis were isolated as described previously (41). One day after isolation, the oocytes were injected with 25 nl of a solution containing 1.0 ng/ml of mRNA encoding WT-EGFP or R881C-EGFP. Control oocytes were injected with 25 nl of sterile water. The oocytes were used in experiments 3–7 days after injection. All experiments were performed at room temperature (~22°C).

**Antibodies**

The COOH-terminal rabbit anti-NBCe1-A (K1A, orphanNBC) has been described previously (5, 9). Other primary antibodies used were a mouse monoclonal anti-EGFP (BD Biosciences, Palo Alto, CA), a mouse monoclonal anti-protein disulfide isomerase (PDI) (StressGen, San Diego, CA), and a mouse monoclonal anti-Na/K pump α-1 (clone C464.6; Upstate Biotech, Lake Placid, NY). The secondary antibodies were Alexa Fluor 488-conjugated goat anti-rabbit, Alexa Fluor 488-conjugated goat anti-mouse, and Alexa Fluor 594-conjugated goat anti-mouse (Molecular Probes, Eugene, OR) and tetramethylrhoda-
Preparation of Oocytes for Immunocytochemistry

Fixing. Individual oocytes were fixed at room temperature by washing twice for 10 min in 1 ml of phosphate-buffered saline (PBS) pH 7.4, once for 60 min in 1 ml of PBS containing 1% paraformaldehyde, and three times for 10 min in 1 ml of PBS. PBS was prepared from tablets according to manufacturer’s recommendations (catalog no. P-4417, Sigma, St. Louis, MO).

Dehydration. Fixed oocytes were dehydrated by consecutive 10-min washes in 1 ml of each of the following: 70% EtOH, 95% EtOH, 100% EtOH, and polypropylene oxide.

Permeation and embedding. Embedding medium was prepared by combining 10 ml of Embed 812, 8 ml of dodecyl succinic anhydride (DDSA), 4 ml of nadic methyl anhydride (NMA), and 0.55 ml of benzylidimethylamine (BDMA) (according to manufacturer’s instructions; Embed 812 Resin Embedding Media Kit, Electron Microscopy Science, Hatfield, PA). Fixed and dehydrated oocytes were permeated by incubation for 60 min in a mixture of 50% embedding medium/50% polypropylene oxide. The oocytes were subsequently transferred to individual Easy Mold embedding capsules (size “00”; Electron Microscopy Science, Hatfield, PA). Fixed and dehydrated oocytes were permeated by incubation for 60 min in a mixture of 50% embedding medium/50% polypropylene oxide. The oocytes were subsequently transferred to individual Easy Mold embedding capsules (size “00”; Electron Microscopy Science) filled with 100% embedding medium, and the resin was cured overnight in an oven at 60°C.

Mounting. Oocytes were sliced to a thickness of 3 μm with a Leica Ultracut UCT (Leica-Microsystems, Milton Keynes, UK). Slices were mounted on Superfrost Plus microscope slides (Electron Microscopy Science) by laying each slice onto a drop of H2O on the slide and heating the slide on a Lab-Line Slide Warmer (Barnstead Instruments, Dubuque, IA) until the H2O had evaporated.

Immunocytochemistry on Oocytes

We chose not to detect EGFP by confocal microscopy on living oocytes because the laser would not penetrate deeply into the cell, and thus the images could create the false impression of a lack of EGFP deeper in the oocyte. Therefore, we fixed and sectioned oocytes. Because fixation eliminated the EGFP fluorescence, we performed indirect immunofluorescence using an antibody to detect the EGFP moiety of the fusion protein. A similar approach was used in Ref. 16.

Antigen retrieval. Five hundred milliliters of citrate buffer [pH 6.0, prepared by mixing 1:9 (vol/vol) 0.1 M citric acid and 0.1 M Na citrate] was preheated for 4 min at 100% power in a 700-W microwave. Slides placed in a glass rack were immersed in this solution and boiled at 40% power in the microwave for 2 × 10 min. Slides were allowed to cool in the solution at room temperature for 20 min.

Immunofluorescence. Before antibody staining, slides were washed in Tris-buffered saline (TBS) (3 × 5 min), 0.5 M NH4Cl (1 × 15 min), TBS (1 × 5 min), TBS + 1% SDS (1 × 5 min), and TBS (1 × 5 min). Slides were blocked by washing (1 × 15 min) in TBS + 0.1% BSA + 10% normal goat serum (Vector Laboratories, Burlingame, CA). The cells were incubated with mouse monoclonal anti-EGFP (1:100 in TBS + 0.1% BSA + 10% goat serum) overnight. The cells were then washed with TBS + high salt + 0.1% BSA (3 × 5 min) and incubated with the secondary antibody, Alexa Fluor 488-conjugated goat anti-mouse (1:200 in TBS + 0.1% BSA + 10% goat serum), for 1 h. Finally, the slides were washed in TBS + high salt + 0.1% BSA (2 × 5 min) and TBS (1 × 5 min) before being mounted in Vectashield (Vector Laboratories).

Confocal Microscopy on Oocytes

We qualitatively localized WT-EGFP and R881C-EGFP expression by obtaining confocal images of select, fixed oocyte slices with a Zeiss LSM 510 confocal laser-scanning microscope (Zeiss, Gottingen, Germany) equipped with an Ar/2 laser unit. The Alexa Fluor 488-labeled images were taken with excitation/emission filters set at band pass 488 nm/long pass 505 nm. Images are the product of eightfold scan averaging. Images were assembled with LSM Image Browser (version 3.5).

Solutions

Equilibrated oocyte solutions. Nominally CO2/HCO3–-free ND96 solution contained (in mM) 93.5 NaCl, 2 KCl, 1 MgCl2, 1.8 CaCl2, and 5 HEPES at a pH of 7.50 and was equilibrated with room air. HCO3–-containing solutions were prepared by replacing 33 mM NaCl in ND96 with 33 mM NaHCO3 and equilibrating the solution with 5% CO2–balance O2. Na+–free HCO3– solutions were prepared by replacing Na+ with N-methyl-D-glucamine (NMDG+). The osmolarities of all solutions were adjusted to ~200 mosmol/kgH2O by adding water or mannitol. DIDS concentrations are corrected for the 80% purity quoted by the manufacturer (Sigma, Poole, UK).

Out-of-equilibrium oocyte solutions. Nominally CO2/HCO3–-free NDxx solution contained (in mM) 42.4 NaCl, 33 Na-gluconate, 2 KCl, 1.8 CaCl2, 1 MgCl2, and 32.5 HEPES at a pH of 7.50. The nominally CO2–free “pure HCO3–” solution was prepared by rapidly mixing solutions A and B—each continuously flowing at 3 ml/min—according to the out-of-equilibrium (OOE) approach (54), which exploits the slow equilibrium HCO3– + H+ ⇌ CO2 + H2O. For oocytes, solution A contained (in mM) 76 NaCl and 66 NaHCO3 at a pH of 9.60, a pH at which the relative concentration of CO2 is extremely low. Solution B contained (in mM) 10 NaCl, 4 KCl, 3.6 CaCl2, 2 MgCl2, and 65 HEPES at a pH of 6.90. When the two solutions, A and B, are mixed at a T junction containing nylon mesh upstream of the oocyte chamber, the resulting “pure HCO3–” solution contains (in mM) 43 NaCl, 33 NaHCO3, 2 KCl, 1.8 CaCl2, 1 MgCl2, and 32.5 HEPES at pH 7.50. On the basis of the flow of the mixed solution into the chamber (i.e., 6 ml/min) as well as the volume of the tubing and chamber between the site of mixing and the location of the oocyte (i.e., 20 μl), we calculate that 200 ms elapses between mixing and the arrival of the newly mixed solution at the oocyte. Given the considerations presented in Refs. 54 and 55, we estimate that the oocyte is exposed to 20.3 μM CO2, which is equivalent to a gas mixture of 0.09%—less than 2% of the CO2 present in an equivalent, equilibrated solution at the same pH and HCO3– concentration.

Immunocytochemistry. TBS for oocyte immunocytochemistry contained 100 mM Tris-base and 150 mM NaCl. TBS + high salt contained 100 mM Tris-base and 430 mM NaCl.

Fluorometric Quantification of WT-EGFP or R881C-EGFP in Intact Oocytes

We used a POLARstar Fluorescence Polarization Microplate Reader (serial no. 403–0513, BMG Labtechnologies, Durham, NC) to quantitate the EGFP fluorescence in intact oocytes that were expressing WT-EGFP or R881C-EGFP. Data were acquired with FLUOstar Galaxy Software (BMG Labtechnologies). Briefly, each oocyte was placed into a well of a black-sided 96-well plate (Corning, Corning, NY), each well containing 300 μl of ND96. Fluorescence due to EGFP was measured while exciting the fluorophore at 485 nm and monitoring the emission at 510 nm. The “gain” was adjusted to 80% by calibration using a single well containing an oocyte expressing WT-EGFP. For each experimental oocyte, we obtained the average of 10 separate second-long readings of 10 flashes each, with a “positioning delay” between individual well readings of 0.2 s. The background fluorescence of wells containing 300 μl of ND96 (but no oocyte) was subtracted from the total fluorescence of the well containing the oocyte. Water-injected oocytes were used as a control. A typical EGFP fluorescence signal of 5,000 for an oocyte expressing WT-EGFP represents a value ~10% greater than background.

Electrophysiological Measurements

Chamber. An oocyte was placed in a plastic perfusion chamber into which was machined a channel that was 3 mm wide and 30 mm long.
The floor of the chamber was a glass coverslip. Solutions were contained in 140-ml plastic syringes (Sherwood Medical, St. Louis, MO) and delivered to the chamber with syringe pumps (Harvard Apparatus, South Natick, MA). Solutions were carried via Tygon tubing (Ryan Herco Products, Burbank, CA; formulation R3603-3; OD 4.8 mm, ID 1.6 mm) to the chamber and flowed from one end of the channel to the other at a rate of 4 ml/min in non-OOE experiments and 6 ml/min in OOE experiments. Solutions were switched with pneumatically operated valves (Clippard Instrument Laboratory, Cincinnati, OH). In all experiments, the oocyte was initially superfused with the ND96 solution, which is nominally CO2/HCO3− free.

Measurement of pH. We assayed acid-base transport of oocytes expressing WT-EGFP or R881C-EGFP by measuring pH1 with pH-sensitive microelectrodes. The electrodes were fabricated and used as described previously (39, 41, 45). Briefly, the oocyte was impaled with two microelectrodes, one for measuring the membrane potential (Vm) and the other for measuring pH1. The silanized tip of the pH electrode contained a liquid membrane (Hydrogen Ionophore I-Cocktail B; catalog no. 95293, Fluka Chemical, Ronkonkoma, NY) across which the voltage depends on pH1 and Vm. A 725I Oocyte Bath Clamp (Warner Instruments, Hamden, CT) was used to set the bath potential to 0 mV and to measure the signal for the voltage electrode. Voltages for the pH electrode were measured with an FD 223 electrode meter (World Precision Instruments, Sarasota, FL). The voltage due to pH1 alone was obtained by using a model V3.1 Subtraction Amplifier (Yale University) to subtract the signal of the Vm electrode from that of the pH electrode and to amplify the difference for input into the analog-to-digital converter of a computer. Data were acquired with customized software. We obtained the pH-slope of the pH/Vm electrode system by placing the tips of the two electrodes in the channel of the chamber, which was filled with stationary standards at pH 6.0 and 8.0. An additional single-point calibration was performed in our standard ND96 solution of pH 7.50, which flowed through the bath before the oocyte was impaled.

Two-electrode voltage clamp. We used a two-electrode voltage clamp to measure whole cell ionic currents of oocytes expressing WT-EGFP or R881C-EGFP or injected with water (control). Currents and voltages were recorded with a model OC-725C oocyte clamp (Clampex module of a computer). We obtained the pH-slope of the pH/Vm electrode system by placing the tips of the two electrodes in the channel of the chamber, which was filled with stationary standards at pH 6.0 and 8.0. An additional single-point calibration was performed in our standard ND96 solution of pH 7.50, which flowed through the bath before the oocyte was impaled.

Choice of Polarizable Cell Line

Two polarizable MDCK cell lines are commonly used as cell models for polarized trafficking, MDCKI and MDCKII. Both are thought to be derived from the canine duct (38). MDCKI cells (high resistance) exhibit properties akin to intercalated or principal cells, whereas MDCKII cells (low resistance) have properties exhibited by β-intercalated cells (e.g., apical peanut lectin binding). In preliminary studies on nonpolarized cells, we found that plasma membrane expression of WT was more robust in MDCKI than MDCKII cells. Moreover, we polarized the MDCKI cells on coverslips because the sustained expression of NBCe1-A was substantially higher than when we polarized the cells on filters. MDCKI cells have the machinery necessary for basolateral trafficking of NBCe1-A and Na/K pump (see Fig. 10); thus they are a useful tool for investigating the effects of NBCe1-A mutations on polarized trafficking. We did not attempt expression of NBCe1-A in LLC-PK1 cells, which are deficient in μ1B (34), an adapter protein that promotes cell polarization and is also necessary for basolateral localization of some proteins (17). We do not imply that the MDCKI cell, or any other cell line, is an exact model of a proximal tubule cell.

Transient Transfection of MDCKI cells and Mammalian Cell Confluent Microscopy

MDCKI cells were grown in Dulbecco’s modified Eagle’s medium containing 25 mM HEPES supplemented with 10% (vol/vol) fetal bovine serum and maintained at 37°C in 5% CO2. MDCKI cells were seeded onto poly-lysine-coated coverslips (Sigma) either at low density (5–10 × 104 cells/well) to obtain dense cells or at high density (1–10 × 104 cells/well) to obtain polarized cells (49). Cells were transfected with 2 μg of WT.pcDNA3 or R881C.pcDNA3, using Fugene 6 (Roche) according to the manufacturer’s instructions. The cells on coverslips were then examined by immunofluorescence microscopy after 48 h, as described below. Transfection efficiency was typically 1–2% under these conditions.

The cells were washed with PBS (pH 7.4) and fixed in methanol-acetone (6/4 vol/vol) at −20°C for 5 min. The coverslips were washed twice with PBS and blocked with 4% BSA in PBS (4% BSA-PBS) for 15 min. The cells were incubated with the rabbit polyclonal K1A antibody (1 μg in 4% BSA-PBS) for 1 h. The coverslips were then washed with PBS three times and incubated with either rat anti-ZO-1 (0.8 μl/100 μl 4% BSA-PBS) or mouse monoclonal anti-PDI (1 μg in 100 μl 4% BSA-PBS) for 1 h. The cells were washed three times with PBS and then incubated with the suitable secondary antibodies: Alexa Fluor 488-conjugated goat anti-rabbit and either TRITC-conjugated donkey anti-rat antibody (to detect ZO-1) or Alexa Fluor 594-conjugated goat anti-mouse (to detect PDI). The cells were washed a further three times and then mounted in Vectashield. Fluorescence imaging was done with a Leica TCS-NT confocal laser-scanning microscope (Leica-Microsystems) equipped with a Kr/Ar laser. The dual-labeling Alexa Fluor 488 or TRITC/Alexa Fluor 594 images were taken sequentially with excitation/emission filters set at short pass 510 nm/band pass 530 nm for Alexa Fluor 488 and band pass 568 nm/long pass 590 nm for TRITC/Alexa Fluor 594. Images were assembled with Adobe Photoshop (version 8.0) and Adobe Illustrator CS (version 11.0).

Data Analysis

Data were analyzed with Clampfit 8.0, Microsoft Excel 97, and SigmaPlot 4.01. Values are given as means ± SE and the number of replicate experiments (n) in the data set from which they were cloned into the pcDNA3 vector as directed by the manufacturer (Invitrogen). The sequences of the final constructs were confirmed by automated sequencing (Oxford University DNA sequencing facility, Oxford, UK).

Cloning WT and R881C into a Mammalian Expression Vector

WT and R881C cDNAs were cloned into the pcDNA3 vector (Invitrogen, Paisley, UK) to make WT.pcDNA3 and R881C.pcDNA3, respectively, as follows. Our starting materials were NBCe1-A.pGH19 and NBCe1-A(R881C).pGH19. The two NBCe1-A constructs were used as templates for PCR, using a sense primer (CCACC-CATGTCCTGAATGTTGGAA) that incorporates a Kozak sequence (CCACC) before the initiating methionine, an antisense primer (TCAGCATGATGTTGGC) that contains a termination codon as shown by underline, and Expand Taq polymerase (Roche, Mannheim, Germany). The coding sequences were then TOPO-TA cloning into the pcDNA3 vector as directed by the manufacturer (Invitrogen). The sequences of the final constructs were confirmed by automated sequencing (Oxford University DNA sequencing facility, Oxford, UK).
calculated. Slopes of data were calculated with Microsoft Excel. Statistical analyses were performed on data with Microsoft Excel.

RESULTS

Immunolocalization of WT-EGFP and R881C-EGFP in Xenopus Oocyte Sections

Immunocytochemistry on sectioned oocytes, using confocal microscopy and an antibody directed against EGFP, demonstrated that both wild-type NBCe1-A, tagged at its COOH terminus with EGFP (WT-EGFP; Fig. 1A), and the mutant R881C-EGFP (Fig. 1B) are concentrated at the plasma membrane of Xenopus oocytes. On the other hand, we observed no signal in oocytes injected with H2O rather than cRNA (Fig. 1C).

Fluorescence detection of WT-EGFP and R881C-EGFP in living Xenopus oocytes

To obtain a more quantitative index of the expression of the EGFP constructs, we used a fluorescence plate reader to measure the average EGFP signal of a large number of individual, intact oocytes 6 days after injection with cRNA. As summarized in Fig. 2A, we found that—compared to oocytes expressing WT-EGFP (Fig. 2A, left)—oocytes expressing R881C-EGFP (Fig. 2A, middle) had an EGFP signal ~40% lower. However, the signals from both constructs were substantially higher than that from H2O-injected oocytes, the fluorescence of which was virtually the same as the background. Figure 2B shows how the fluorescence of oocytes expressing EGFP-tagged NBCe1-A increased with time. Typically the fluorescence of a healthy oocyte expressing WT-EGFP would gradually rise to a value no greater than 15,000 units (defined as described in MATERIALS AND METHODS) and then sharply decline with time, or the oocyte would die. The fluorescence of an oocyte expressing R881C-EGFP rarely rose beyond 10,000 before the oocyte reached the end of its life span. Thus R881C-EGFP oocytes were underrepresented at high fluorescence levels, and those R881C-EGFP oocytes that reached modestly high fluorescence levels tended to be older than the WT-EGFP counterparts (see Fig. 2B).

Functional Detection of WT-EGFP and R881C-EGFP at the Plasma Membrane of Xenopus Oocytes

Figure 3 shows representative steady-state I-V data obtained from two-electrode voltage-clamp experiments on intact oocytes expressing WT-EGFP (Fig. 3, A and D), expressing R881C-EGFP (Fig. 3, B and E), or injected with H2O (Fig. 3, C and F). Figure 3, A–C, summarizes experiments in which we sequentially exposed the oocytes to 1) the nominally CO2/HCO3-free ND96 solution at pH 7.50, 2) 5% CO2/33 mM HCO3 at pH 7.50, and 3) 5% CO2/33 mM HCO3 at pH 7.50,
but with Na\(^+\) replaced by NMDG\(^+\). Figure 3, D–F, summarizes experiments in which we sequentially exposed the oocytes to 1) ND96 as in Fig. 3, A–C; 2) CO\(_2\)/HCO\(_3\)^\(-\) as in Fig. 3, A–C; and 3) 160 μM DIDS dissolved in 5% CO\(_2\)/33 mM HCO\(_3\)^\(-\) at pH 7.50. The data show that, qualitatively, oocytes expressing R881C-EGFP display the same I-V relationships as those expressing WT-EGFP, namely, adding CO\(_2\)/HCO\(_3\)^\(-\) markedly increased the slope conductance and subsequently removing Na\(^+\) or adding DIDS substantially reduced the slope conductance. In each experiment, we determined the intersection of the I-V curves in CO\(_2\)/HCO\(_3\)^\(-\) and CO\(_2\)/HCO\(_3\)^\(-\) + DIDS to obtain the reversal potential of the DIDS-sensitive current. The mean reversal potential was \(-148 \pm 3\) mV (n = 17) for R881C-EGFP (as in Fig. 3E), which is similar to the mean value of \(-141 \pm 3\) mV (n = 15) obtained for WT-EGFP (as in Fig. 3D).

Although the currents in oocytes expressing WT-EGFP and R881C-EGFP are qualitatively similar, we noted a tendency for the absolute currents to be larger in the WT-EGFP oocytes, as noted by Igarashi et al. (19). Below in this section the currents are normalized to EGFP fluorescence levels. It should be noted that oocytes injected with H\(_2\)O (Fig. 3, C and F) had very small and invariant currents.

Our laboratory previously demonstrated (5, 9, 41) that when expressed in Xenopus oocytes the non-EGFP-tagged renal electrogenic NBC mediates an electrogenic, HCO\(_3\)^\(-\)-dependent pHi recovery from a CO\(_2\)-induced acid load. Moreover, this activity requires Na\(^+\) and is inhibited by DIDS. Figure 4, A and D, demonstrates that these properties also hold for the EGFP-tagged version of human NBC\(_{e1}\)-A. As illustrated in Fig. 4A, the switch to CO\(_2\)/HCO\(_3\)^\(-\) elicits a relatively rapid pHi decline (due to CO\(_2\) entry into the cell) from which the cell recovers...
more slowly (due to NBCe1-dependent HCO₃⁻ transport into the cell). The switch also elicits an instantaneous hyperpolarization to about −150 mV in oocytes expressing WT-EGFP (due to NBCe1-dependent HCO₃⁻ transport into the cell). The removal of extracellular Na⁺ reverses the pHᵢ recovery and elicits a large depolarization. As illustrated in Fig. 4D, application of 160 μM DIDS inhibits the pHᵢ recovery.

Figure 4, B and E, repeats, for R881C-EGFP, the protocols already illustrated for WT-EGFP. It is clear that R881C-EGFP expressed in Xenopus oocytes mediates a Na⁺-dependent, DIDS-sensitive, electrogenic Na-HCO₃ cotransport with the same qualitative traits as WT-EGFP. Figure 4, C and F, repeats these protocols for H₂O-injected oocytes.

**DIDS Inhibition of WT-EGFP and R881C-EGFP**

The most reliable measure of the functional expression of NBCe1-A at the plasma membrane of the oocyte is the magnitude of the DIDS-sensitive current carried by the wild-type transporter in the presence of Na⁺ and HCO₃⁻. However, comparisons of the DIDS-sensitive currents mediated by WT-EGFP vs. R881C-EGFP would be valid only if the chosen DIDS concentration produced similar levels of blockade of cotransporter activity for the two proteins. To this end, we determined the Kᵢ(app) and maximal inhibition of DIDS for WT-EGFP and R881C-EGFP. Briefly, as part of a voltage-clamp protocol like those in Fig. 3, we measured the HCO₃⁻-dependent current at +20 mV, first in the absence of DIDS and then in the presence of 16, 32, 64, or 128 μM DIDS. We computed the fractional DIDS inhibition as the DIDS-sensitive current at +20 mV divided by the HCO₃⁻-dependent current at +20 mV. We fitted the Michaelis-Menten equation to these data (r² > 0.99; Fig. 5) and obtained a Kᵢ(app) of 27 ± 2 μM for WT-EGFP and 27 ± 2 μM for R881C-EGFP. The maximal inhibition was 101 ± 3% for WT-EGFP and 100 ± 2% for R881C-EGFP. Thus the R881C mutation affects neither the apparent potency nor the maximal efficacy of DIDS toward NBCe1-A.

**The Relationship Between EGFP-Fluorescence and Functional Cell Surface Expression of WT-EGFP**

If the EGFP signal in the plate reader experiments (see Fig. 2) is truly proportional to the density of NBCe1-A chimeras at the oocyte surface, then the DIDS-sensitive current ought to be proportional to the fluorescence of individual oocytes. From data such as those shown in Fig. 3D for oocytes expressing WT-EGFP, we computed the DIDS-sensitive current at +20 mV (i.e., the maximal outward current in our protocol). In Fig. 6A, we plot DIDS-sensitive current as a function of the EGFP signal in 18 individual oocytes. The plot shows that the magnitude of the DIDS-sensitive current at +20 mV positively correlates with EGFP fluorescence. Thus EGFP fluorescence correlates well with the functional surface expression of WT-EGFP.

**Comparison of the Conductance-Fluorescence Relationship for WT-EGFP and R881C-EGFP**

The filled gray circles and regression line in Fig. 6B are a reproduction of the WT-EGFP data from Fig. 6A; the open circles are comparable data for 22 oocytes expressing R881C-EGFP. The relationship between the DIDS-sensitive current
and the fluorescence of oocytes expressing R881C-EGFP suggests that, molecule for molecule, this mutant carries as much current as WT-EGFP.

Comparison of Normalized Acid Extrusion Rates Mediated by WT-EGFP and R881C-EGFP

We monitored the rate of increase of pH_i (dpH_i/dt) for individual oocytes expressing WT-EGFP or R881C-EGFP during exposure to an OOE “pure HCO_3^-/H_2CO_3” solution that contained 33 mM HCO_3^- at pH 7.50 but virtually no CO_2. Figure 7 demonstrates that for oocytes expressing WT-EGFP (Fig. 7A) and R881C-EGFP (Fig. 7B), application of an OOE “pure HCO_3^-” solution elicits an increase in pH_i and a rapid hyperpolarization of the oocyte. These responses, characteristic of electrogenic HCO_3^-/H_2CO_3 transporter activity, did not occur in the case of H_2O-injected oocytes (Fig. 7C). This approach has the advantages that 1) the pH_i increase begins soon after the application of HCO_3^-, so that cell composition should have changed minimally; 2) it is easy to assess dpH_i/dt at a specified pH_i; and 3) dpH_i/dt is not affected by the acidifying influence of a potentially lingering CO_2 influx.

Figure 8A shows the dpH_i/dt values computed from pH_i traces—such as those shown in Fig. 7—at a pH_i of 7.55 and plotted against the EGFP fluorescence of individual oocytes. Although the data are consistent with a trend toward greater dpH_i/dt values in oocytes with greater fluorescence (i.e., those expressing more WT-EGFP or R881C-EGFP at the cell surface), the correlations are weak, perhaps because of variations in surface-to-volume ratio and buffering power among individual oocytes. However, as illustrated in Fig. 8B, the mean dpH_i/dt per unit values of EGFP fluorescence for oocytes expressing WT-EGFP and R881C-EGFP are statistically indistinguishable.

Immunolocalization of WT and R881C in Transiently Transfected Nonpolarized MDCKI Cells

To examine the effects of the R881C mutation on the trafficking of NBCe1-A in nonpolarized kidney cells, we transiently expressed WT and R881C mutant NBCe1-A—both without a COOH-terminal EGFP tag—in MDCKI cells. We determined the localization of NBCe1-A by confocal immunofluorescence microscopy, using a rabbit polyclonal antibody that detects the COOH terminus of NBCe1-A. The antibody did not detect NBCe1-A in untransfected MDCKI cells (not shown). In nonpolarized MDCKI cells transiently transfected with the WT construct, the antibody demonstrated obvious plasma membrane localization of the NBCe1-A protein, with additional immunoreactive protein visible in the interior of the cell (Fig. 9, A and B).

In nonpolarized MDCKI cells transiently expressing the R881C construct, the antibody staining demonstrated that the mutant protein fails to reach the plasma membrane and the majority of the R881C immunolocalizes in the interior of the cell (Fig. 9, C and D). To confirm the intracellular localization of R881C in MDCKI cells, we double labeled the transiently transfected cells with the rabbit polyclonal anti-NBCe1-A...
antibody and a monoclonal antibody to PDI, an ER marker protein that does not label the plasma membrane. Figure 9, E–G, shows that the majority of the R881C immunoreactive protein overlaps with the localization of PDI, confirming the retention of R881C in the ER of nonpolarized MDCKI cells.

The incubation of R881C-transfected MDCKI cells at 27°C did not influence the subcellular distribution of the mutant protein (data not shown). This result suggests that the intracellular retention of R881C mutant is not temperature sensitive, unlike another ER-retained mutant membrane protein, CFTR (H9004F508), which correctly traffics to the plasma membrane with incubation at low temperatures (14).

**Immunolocalization of WT and R881C in Transiently Transfected Polarized MDCKI Cells**

In addition to examining the localization of WT and R881C in nonpolarized MDCKI cells, we also examined the localization by transiently expressing the proteins in polarized MDCKI cells. Figure 10, A and B, shows X-Y and X-Z views of polarized MDCKI cells expressing WT, double-labeled with the anti-NBCe1-A antibody and either an antibody to PDI (Fig. 10A) or an antibody to the Na/K pump (Fig. 10B). The Na/K pump is a common marker for the basolateral membrane. WT does not overlap with PDI distribution (Fig. 10A) but colocalizes with the Na/K pump at the basolateral membrane (Fig. 10B). Therefore, in polarized MDCKI cells, the wild-type NBCe1-A protein is targeted to the basolateral membrane.

Figure 10, C and D, shows R881C images taken under conditions similar to those of WT in Fig. 10, A and B. In contrast to the obvious basolateral localization of the wild-type protein, the R881C mutant is distributed throughout the cytoplasm (Fig. 10, C and D), with a localization that overlaps substantially with that of PDI (Fig. 10C). Conversely, the mutant protein does not overlap with Na/K pump distribution (Fig. 10D). Therefore, the R881C mutant does not reach the basolateral membrane but is retained in the ER of polarized MDCKI cells.

**DISCUSSION**

*The R881C Mutation Does Not Affect the Function of Individual NBCe1-A Molecules in Xenopus Oocytes*

Although R881C-EGFP, like WT-EGFP, can traffic to the oocyte plasma membrane, R881C-EGFP has a markedly reduced surface expression (Fig. 1). In the present study, we demonstrated that the R881C mutation does not significantly affect the activity of individual NBCe1-A molecules. This conclusion is borne out by six observations.

1) The expression of NBCe1-A in *Xenopus* oocytes, estimated by EGFP fluorescence in excess of that in H2O-injected oocytes, suggests that compared with WT-EGFP, R881C-EGFP protein levels are reduced 40–70%, depending on the time after cRNA injection (Fig. 2, A and B). This observation would account for the average 60% reduction in apparent NBCe1 functional activity of the untagged mutant protein in oocytes, as reported by Horita et al. (19).

2) The DIDS-sensitive reversal potential for NBCe1-A in HCO3−-containing solutions is not significantly affected by the R881C mutation, in agreement with the findings of Horita et al. (19), who further demonstrated that the Na+ affinity of Na+-HCO3− cotransport is unaffected by the
Fig. 10. Immunolocalization of wild-type or mutant NBCe1-A in polarized MDCKI cells. MDCKI cells were seeded at high density, transiently transfected with WT or R881C, and then allowed to polarize as described in MATERIALS AND METHODS. The fixed cells were doubly stained with rabbit anti-NBCe1-A (green, A–D) and either a mouse anti-PDI (red, A and C) or a mouse anti-Na/K pump (red, B and D). The images on left (X-Y) represent the focal plane parallel to the epithelium near the center of the cells, whereas the 3 stacked images on right (X-Z) represent focal planes perpendicular to the same epithelium, as represented by the blue lines. The majority of the mutant protein overlapped with PDI (yellow, see merged panels in C) but not at all with Na/K pump (see merged panels in D). Scale bar, 25 μm.
mutation. 3) I-V relationships (Fig. 3) and pH$_i$ data (Fig. 4) obtained from *Xenopus* oocytes expressing R881C-EGFP demonstrate that the mutant protein is capable of mediating the electrogenic, Na$^+$-dependent, DIDS-sensitive HCO$_3^-$ transport activity characteristic of the wild-type protein. 4) The $K_{i(app)}$ for reversible DIDS inhibition of NBCe1-A activity is unaffected by the R881C mutation (Fig. 5). 5) The DIDS-sensitive, HCO$_3^-$ dependent current carried by NBCe1-A is undiminished by the R881C mutation, once the data are normalized for expression levels in individual oocytes (Fig. 6). 6) The rate of NBCe1-mediated pH$_i$ increase in *Xenopus* oocytes superfused with a “pure HCO$_3^-$” solution is the same whether the oocytes are expressing WT-EGFP or R881C-EGFP, once data are normalized for expression levels in individual oocytes (Fig. 8).

If the behavior of NBCe1-A in oocytes is predictive of its behavior in humans, defects in the transport properties of individual NBCe1-A molecules are unlikely to be responsible for the observed pRTA phenotype exhibited by the patient with the R881C mutation. Rather, the reduced surface expression of R881C-EGFP in *Xenopus* oocytes suggests that the pathophysiology is related to a trafficking defect.

The R881C Mutation Causes Retention of NBCe1-A in the ER

We have shown that untagged, wild-type NBCe1-A is localized to the plasma membrane in nonpolarized, transiently transfected MDCKI cells and that it is localized to the basolateral membrane in polarized MDCKI cells. This basolateral localization is consistent with that in the kidney and with that previously reported for an NBCe1-A that was GFP tagged at the NH$_2$ terminus and exogenously expressed in polarized previously reported for an NBCe1-A that was GFP tagged at the NH$_2$ terminus and exogenously expressed in polarized

of autosomal dRTA mutants in polarized kidney cells (12, 15, 42, 49) can cause autosomal dominant and recessive forms of distal renal tubular acidosis (dRTA).

In the case of autosomal dominant dRTA, heterodimerization between mutant and wild-type AE1 leads to the mistargeting of both proteins away from the basolateral membrane, a mechanism first suggested by Toye et al. (50). A detailed study of autosomal recessive dRTA mutants in polarized kidney cells has yet to be conducted, but studies in nonpolarized cells indicate that delivery of mutant AE1 to the plasma membrane may be restored in heterozygotes by heterooligomerization with wild-type AE1 (28). To date, all pRTA cases associated with mutations in NBCe1 are reported to be recessive.

A Severe pRTA Phenotype in Heterozygotes is Avoided, Possibly by WT/R881C Heterooligomerization

Horita et al. (19) note that both parents of the proband were heterozygous for the R881C mutation. Although the authors make no direct reference to the RTA status of the parents, we assume that the parents do not have severe pRTA. Given that the minimal active unit of AE1—and therefore probably NBCe1 as well—is a dimer, it is probable that some mutant protein is expressed as WT-R881C heterodimers in the parents. Indeed, preliminary data (Parker MD and Boron WF, unpublished observations) from coexpression of WT-EGFP and R881C-EGFP in *Xenopus* oocytes are consistent with a model in which expression of WT-EGFP is able to rescue/stabilize the surface expression of R881C-EGFP. Recall that we have demonstrated that the R881C protein is fully functional once it reaches the plasma membrane. We predict that—if the trafficking of the WT-R881C heterodimer to the proximal tubule basolateral membrane is normal, and if the trafficking of R881C-R881C is nil—human heterozygotes would have ~75% of the normal NBCe1-A activity. This level presumably would be sufficient to avoid a severe pRTA phenotype.

A Mutation Identical to R881C—but in AE1—Causes Hereditary Spherocytosis

A fascinating “coincidence”—not previously noted in the literature—is that the R808C mutation in human AE1, which causes hereditary spherocytosis, corresponds directly to the R881C mutation in human NBCe1 (Fig. 11A). This pair of R→C mutations is the first example of cognate mutations demonstrated to result in two distinct disease phenotypes by virtue of their independent occurrence in two members of the SLC4 gene family. The codons for both arginines contain CpG dinucleotides, CGT in NBCe1 and CGC in AE1. CpG dinucleotides tend to create “mutational hot spots” (reviewed in Ref. 11) because the cytosine in the dinucleotide is susceptible to methylation. Subsequent deamination of this methylated cytosine, either by environmental factors or errant cellular processes, creates a thymine. In the case of NBCe1, this process would create TGT at position 881 (encoding a cysteine), as found in the affected patient. Sritippayawan et al. (48) invoked this mechanism to explain another mutational hot spot in AE1 at position 859.

Despite normal expression levels of total protein, functional surface expression of R808C AE1 could not be detected in the plasma membranes of HEK cells because of retention of the overexpressed protein in an intracellular compartment (37, 56). This lack of surface expression prevented functional characterization of R808C AE1. Reduced binding of the solubilized mutant protein to an inhibitor affinity resin suggests that the R808C mutation results in some structural perturbation of AE1 (37).

R881 is located in the COOH-terminal portion of the membrane domain of human NBCe1-A (Fig. 11B). The topology of the SLC4 HCO$_3^-$ transporters is not well defined in this region, presumably because of flexibility and conformational sensitivity to ionic strength (18, 30). Furthermore, studies on human AE1 suggest that the topology of the molecule may depend on the maturation status of the protein (27) and may even be influenced by the very experimental modifications made to the protein for the purposes of elucidating its native topology (30).

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Evidence provided by a mouse monoclonal anti-AE1 antibody, BRIC 132, which recognizes its epitope—residues 813–824 of AE1 (52)—in permeabilized but not intact red blood cells, suggests that R808 of AE1 (R881 of NBCe1-A) may reside near the cytoplasmic side of the membrane (53). Furthermore, on the basis of the NMR solution structure of a synthetic peptide corresponding to residues 796–841 of AE1, Askin et al. (4) concluded that R808 of AE1 is located in a helical region proximal to the cytoplasmic loop that forms the BRIC 132 epitope. In addition, the authors propose that an ion pair exists between R808 and D821 of AE1 (D894 of NBCe1-A), which may help to stabilize the cytoplasmic loop structure. Assuming that AE1 and NBCe1-A have similar topologies in this region, the BRIC 132 and the NMR data, taken together, suggest that R881 of NBCe1-A is cytoplasmically disposed in a helical region close to the membrane (Fig. 11B). It is intriguing to note that cysteines introduced to the BRIC 132 epitope can be accessible to chemical modification from the extracellular side of the membrane when expressed in a kidney cell line (56), which is consistent with the notion that the R881C mutation induces a structural perturbation in NBCe1-A.

A recent mutational analysis of NBCe1-A demonstrated that the double mutant R881D/K883D retains function yet exhibits impaired trafficking when transiently expressed in HEK293 cells (1). It is also noteworthy that NBCe2, the electrogenic NBC in liver, has a cysteine at what would be position 882 in AE1 (57), which may help to stabilize the cytoplasmic loop. In conclusion, we report here that the missense mutation R881C in NBCe1-A (20), which causes pRTA. All of these mutations—as well as R298S in the cytosolic NH2 terminus of NBCe1-A (20)—result in reduced trafficking of protein to the cell surface (25, 32, 36, 49). At the seven residues, it is largely unknown whether replacing arginine with lysine, which also has a positively charged side chain, would affect SLC4 trafficking or activity. However, it is interesting to note that the mutant R589K in AE1 is reported to have impaired trafficking in HEK cells (36).

In the absence of a suitable crystal structure for any SLC4 family member, we can only speculate on the extent of the structural alteration in R881C and how the mutation results in the mistrafficking of the protein. It is unlikely that R881C is grossly misfolded, inasmuch as this mutant had normal activity (when normalized for expression levels) as expressed in oocytes. The mutation most likely causes local misfolding of the region surrounding the mutation—perhaps by destabilizing an R881-D894 ion pair—resulting in the protein being retained by chaperones for eventual delivery to the ER-associated degradation system in kidney cells. A similar explanation was suggested to explain the mistrafficking observed with the R589H and S613F mutations of AE1, which cause a form of dominant dRTA (49) and which, in polarized MDCKI cells, cause ER retention.

In conclusion, we report here that the missense mutation R881C in NBCe1-A does not affect the activity of individual protein molecules. Rather, the molecular basis of the disease is likely to be due entirely to ER retention of the mutant protein. As a consequence, the proximal tubules of affected patients have a vastly reduced ability to reabsorb HCO3- from the glomerular filtrate into the blood, resulting in severe pRTA.

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