Glucoma, cataracts, and proximal renal tubular acidosis are diseases caused by point mutations in the human electronegic Na\(^+\) bicarbonate cotransporter (NBCe1/SLC4A4) (1, 2). One such mutation, R298S, is located in the cytoplasmic N-terminal domain of NBCe1 and has only moderate (75%) function. As SLC transporters have high similarity in their membrane and N-terminal primary sequences, we homology-modeled NBCe1 onto the crystal structure coordinates of Band 3 (AE1) (3). Arg-298 is predicted to be located in a solvent-inaccessible subsurface pocket and to associate with Glu-91 and Glu-295 via H-bonding and charge-charge interactions. We perturbed these putative interactions between Glu-91 and Arg-298 by site-directed mutagenesis and used expression in *Xenopus* oocyte to test our structural model. Mutagenesis of either residue resulted in reduced transport function. Function was “repaired” by charge reversal (E91R/R298E), implying that these two residues are interchangeable and interdependent. These results contrast the current understanding of the AE1 N terminus as protein-binding sites and propose that hkNBCe1 (and other SLC4) cytoplasmic N termini play roles in controlling HCO\(_3^-\) permeation.

Regulating and maintaining acid-base homeostasis is critical for normal cell, tissue, and systemic function. Transporters in several transporter families are involved in this multilevel pH regulation: Slc4 (bicarbonate transporters), Slc94 (Na\(^+\)-H\(^+\) exchangers), Slc16 (H\(^+\)/monocarboxylate cotransporters), and Slc26 (anion and bicarbonate transporters). Na\(^+\)-H\(^+\) exchangers (NHEs,\(^2\) Slc9 proteins) play important pH regulatory roles in many cells and tissues. Nevertheless, in many cells, HCO\(_3^-\) transporters carry even more acid-base equivalents than NHEs and are often more active in CO\(_2\)/HCO\(_3^-\) environments (normal cellular and tissue buffering system).

The importance of HCO\(_3^-\) transporters has been further highlighted by the existence of severe pathogenic mutations (reviewed in Ref. 4). Igarashi *et al.* (1) described the first patients with mutations in the NBCe1 (SLC4A4) gene and protein, R298S and R510H. Since then, several additional patients have been identified with recessive NBCe1 mutations (for review, see Ref. 5). These patients with mutations in the NBCe1 coding sequence have permanent proximal renal tubular acidosis (pRTA type II, i.e. pH\(_{\text{blood}}\) \(\sim\) 7.05, [HCO\(_3^-\)] \(\sim\) 3–11 mM; normal blood pH \(=\) 7.35–7.45, [HCO\(_3^-\)] \(\sim\) 23–25 mM) with early onset, bilateral glaucoma, bilateral cataracts, and band keratopathy, yet without obvious intestinal or pancreatic defects (1).

In the renal proximal tubule, the major apical step of bicarbonate absorption is acid secretion to the forming urine by the NHE3, Na\(^+\)-H\(^+\) exchanger (6). The basolateral step of proximal tubule bicarbonate absorption appears to rely exclusively on NBCe1 function (Na\(^+\)/HCO\(_3^-\) cotransport). For example, the NHE3 knock-out mice have only a slight metabolic acidosis (blood pH \(\sim\) 7.27 and [HCO\(_3^-\)] \(\sim\) 21 mM) (7, 8), indicating that NHE3 is not the rate-limiting step in transepithelial bicarbonate absorption. However, both humans with NBCe1 mutations (1, 9–13) and the NBCe1 knock-out mice (14) have significant metabolic acidosis (humans, see above; NBCe1 \(-/-\) mice, blood pH 6.86, [HCO\(_3^-\)] \(\sim\) 5.3 mM). Taken together, these data indicate that the basolateral exit of HCO\(_3^-\) via NBCe1 rather than apical H\(^+\) secretion via NHE3 is the dominant and rate-limiting step in kidney bicarbonate absorption. This loss-of-function/reduced-function phenotype also indicates that NBCe1 is the only HCO\(_3^-\) absorption pathway in the renal proximal tubule and that NBCe1 plays a key role for maintaining ocular pressure and corneal clarity.

Loss of NBCe1 function may result from (a) aberrant protein processing or folding (12, 13, 15), (b) protein truncation (10), or (c) misfolding of the NBCe1 protein (1, 9, 16). For example, S427L in transmembrane span 1 is a biophysical (functional) mutation resulting in unidirectional transport at 10% of wild-type (9), whereas L522P in transmembrane span 5 is a protein trafficking problem (12). R298S-NBCe1 was originally reported as having \(\sim\)50% wild-type function (1), i.e. also a biophysical mutation, but was more recently reported as a protein trafficking-
ing problem (1, 9, 16). A transmembrane topography of the human NBCe1-B has been proposed based on glycosylation studies (17). None of the proposed structural models dispute the Arg-298 location, i.e. residing in the center of the cytoplasmic N terminus of NBCe1 and not obviously associated with the transmembrane domain. How then does this placement translate to malfunction of the R298S-NBCe1 protein? Does this imply that HCO₃⁻ permeation and/or affinity is associated with the cytoplasmic N terminus? Transmembrane domains of membrane proteins are generally thought to control ion permeability across membranes. However, knowing the sequence and predicted structural location (based on linear sequence) is not always the best predictor of structure.

NBCe1 is a member of the HCO₃⁻ transporter gene family that includes Band 3 (AE1/SLC4A1). All SLC4 members have >35% sequence identity, particularly in predicted membrane spans. Although crystals were recently obtained for this region of NBCe1, only gross topology rather than amino acid assignment was reported (18). We hypothesized that we could gain insights into NBCe1 structure and function by mapping its amino acid sequence onto the AE1 N-terminal structure (3). This structural prediction indicated that Arg-298, a conserved residue in SLC4 proteins, is located in a solvent-inaccessible pocket. The model further predicted that Arg-298 has charge interactions with Glu-295 and Glu-91, both of which are also conserved in SLC4 HCO₃⁻ transporter proteins.

Are these sequence alignments coincidence, or is the Band 3 N-terminal structure a good predictor of NBCe1 N-terminal structure? In this study, we use point mutations to perturb the charge interaction between Glu-91 and Arg-298. Our results indicate that Arg-298, Glu-91, and their interaction are crucial for the NBCe1 N-terminal structure as well as the normal physiological function of NBCe1. Thus, this structural model and the following experiments elucidate, on the molecular level, “why” R298S causes a proximal RTA with bilateral cataracts (27). Using an anti-HA 1o antibody and horseradish peroxidase-conjugated 2o antibody with a chemiluminescent substrate, we were able to quantify surface expression of hkNBCe1 clones in a luminometer. The single-oocyte chemiluminescence technique utilizes enzyme amplification of chemiluminescence substrate to quantify a HA-tagged protein expressed at the cell surface (28, 29). This technique has a linear relationship between surface expression detected by single-oocyte chemiluminescence and functional activity of the K⁺ channel, ROMK (Kir 1.1), as reported by Yoo et al. (30).

**Experimental Procedures**

**NBCe1 N Terminus Structure Modeling**—A pair-wise alignment of sequences of human kidney NBCe1 (residues 62–371) and AE1 (residues 55–356) accession codes M27819 was prepared in the Swiss-PdbViewer (SPDBV (19)) or externally with SIM and the Blossum62 algorithm (20). These nearly identical alignments, when submitted together with the coordinates for AE1 (Protein Data Bank accession code 1HYN) to the Swiss-model server (21), did not yield an initial model due to failure of identifying appropriate loops. Despite a 36.5% overall sequence identity between these proteins, there is a region among NBCe1 (residues 113–174) and AE1 (residues 165–218) that does not show much sequence similarity, reducing the threading reliability in this area. Thus, multiple sequence alignments with the ClustalW algorithms (DNASTAR) were made to identify boundaries of conserved and variable sequence regions within and across homologous domains of the Slc4 protein families. The resulting manually optimized binary alignment between NBCe1 and AE1 served as input for Swiss model. The returned initial model, based on the four individual copies of the domain in HYN, was briefly minimized. Side chain conformations were subsequently optimized with SCWRL3 (22) and minimized to yield the present model structure (Fig. 2B). The NBCe1 model was then analyzed with the programs VADAR, Procheck, and WHAT IF (23–25) (Fig. 2C–E).

**Cloning and Mutations**—The human kidney NBCe1 (hkNBCe1) clone in a *Xenopus* expression plasmid was previously described (9). hkNBCe1 mutations were generated using QuickChange (Stratagene, La Jolla, CA) and sequenced for verification (W. M. Keck Biotechnology Resource Laboratory, New Haven, CT). Linearized cDNA was used to make capped cRNA with the SP6 mMessage mMachine kit (Ambion, Austin, TX) as described previously (26).

A hemagglutinin affinity tag “HA tag” was engineered into the extracellular loop of hkNBCe1 at the Ser-596 → Ser-610 region with a linker (SNDTTLAP-DYPYDVPDYAG-EYLPTMS) as that described in McAlear et al. (27). This HA tag insertion does not affect NBCe1 activity or sensitivity to stilbenes (27). Using an anti-HA 1o antibody and horseradish peroxidase-conjugated 2o antibody with a chemiluminescent substrate, we were able to quantify surface expression of hkNBCe1 clones in a luminometer. The single-oocyte chemiluminescence technique utilizes enzyme amplification of chemiluminescence substrate to quantify a HA-tagged protein expressed at the cell surface (28, 29). This technique has a linear relationship between surface expression detected by single-oocyte chemiluminescence and functional activity of the K⁺ channel, ROMK (Kir 1.1), as reported by Yoo et al. (30).

**Oocyte Experimental Solutions**—The CO₂/HCO₃⁻-free ND96 contained 96 mm NaCl, 2 mm KCl, 1 mm MgCl₂, 1.8 mm CaCl₂, and 5 mm HEPES. In CO₂⁻/HCO₃⁻-equilibrated solutions, 33 mm NaHCO₃ replaced 33 mm NaCl; all CO₂⁻/HCO₃⁻ solutions are 5% CO₂ (33 mm HCO₃⁻ (pH 7.5). In 0-Na⁺ solutions, choline replaced Na⁺. All the solutions used in the experiments were adjusted to pH 7.5 and 195–200 mosm.

**Oocyte Electrophysiology**—50 nL of water (control) or RNA solution (25 ng of hkNBCe1 or mutant cRNA) was injected into stage V/VI *Xenopus* oocytes. Voltage electrodes, made from fiber-capillary borosilicate and filled with 3 m KCl, had resistances of 1–10 megohms (31). Ion-selective electrodes were pulled similarly and silanized with bis-(dimethylamino)-dimethylsilane (Fluka Chemical Corp., Ronkonkoma, NY). pH electrode tips were filled with hydrogen ionophore 1 mixture B (Fluka) and back-filled with phosphate buffer (pH 7.0). Electrodes were connected to a high-impedance electrometer (WPI-FD223 for intracellular pH (pHᵢ) and Vᵢ (experiments), and digitized output data (filtered at 10Hz) were acquired by PCLAMP software sampling at 0.5 Hz. All ion-selective microelectrodes had slopes of −54 to −57 mV/decade ion concentration (or activity). pH electrodes were calibrated at pH 6.0 and 8.0. For voltage-clamp experiments, electrodes were filled with 3 m KCl/agar and 3 m KCl and had resistances of 0.2–0.5 megaohms. Oocytes were clamped at −80 mV, and current was constantly monitored and recorded at 10 Hz (Warner Inst. Co.,
Oocyte Clamp OC-725C. Voltage steps pulses (75 ms) were executed from −160 to +60 mV in 20 mV steps; the resulting I-V traces were filtered at 2 kHz (8 pole Bessel filter) and sampled at 10 kHz. Data were acquired and analyzed using Pulse and PulseFit (HEKA Instruments, Germany).

**Oocyte Surface Protein Expression**—Oocyte labeling was performed at 4 °C. Oocytes were fixed with 4% paraformaldehyde in ND96 for 15 min, washed and incubated in 1% bovine serum albumin-ND96 blocking solution for 30 min. Oocytes were labeled with a 1° antibody (1:200 dilution, monoclonal rat-α-HA 1° antibody (Roche Applied Science)) for 60 min, and then with a 2° antibody (1:2000 dilution, hors eradish peroxidase-conjugated goat-α-rat IgG (Jackson ImmunoResearch Laboratories)) for 30 min in 1% bovine serum albumin-ND96 blocking solution. Labeled oocytes were washed several times and incubated in ND96 for 10 min before exposure to 50 µl of extracellular HCO₃⁻ into the cell (via NBCe1), counteracting acidification by CO₂ hydration (Fig. 1B).

The bicarbonate transport is accompanied by a large positive (outward) current (Fig. 1, B and E, squares) due to the 1 Na⁺: nHCO₃⁻ stoichiometry of NBCe1 and the negative charge movement (32–34). No similar current can be observed in the water-injected oocytes (Fig. 1, A and E, circles). Sodium replacement with choline (hereafter referred as Na⁺ removal or “0 Na⁺”) in a HCO₃⁻ solution reverses the HCO₃⁻ transport direction (Fig. 1, B and D, 0Na⁺). That is, HCO₃⁻ is now moving out of the cell resulting in a fast acidification and an inward current for a hkBNCe1-expressing oocyte (9) (Fig. 1, B and D, 0Na⁺, and Fig. 1F), equivalent to renal NaHCO₃ absorption. Na⁺ removal in HCO₃⁻ creates no detectable pH change or current in the water-injected oocytes. One can use these parameters (current magnitude, I_max, and the rate of acidification...
HCO₃⁻ transport and elicited currents from WT- and mutant NBCe1

Calculations are as indicated under “Experimental Procedures” and as previously described (9). The value for Δβ₂₉₈₈₆₆ is β₂₉₈₈₆₆ − β₂₉₈₈₆₆ (water). These data were collected using the three-electrode experiments (see “Experimental Procedures”) to voltage-clamped oocytes at −60 mV while also measuring pHᵢ, Iᵢ, membrane current. For pHᵢ and ΔpHᵢ values, there are actually four significant digits, although three are shown for readability. Italicized columns are the average value for each parameter. Note that values should be compared with both WT-NBCe1 as well as the water-control because the water-control versus the WT-NBCe1 percentage increases and decreases depending on the parameter.

| Units | Water | hkBNCe1 | R298S | E91R | R298E | E91R-R298E |
|-------|-------|---------|-------|------|-------|------------|
| dpHᵢ/dt | (x10⁻³ pH unit/s) | CO₂/HCO₃⁻ | −299.33 | 167.16 | 21.29 | −297.86 | 54.00 | 7 178 | −373.67 | 42.94 | 9 224 | −286.89 | 36.11 | 9 172 | −233.57 | 28.57 | 7 140 |
| dpHᵢ/dt | 0Na⁺-CO₂⁻ | 17.83 | 5.42 | 6 | −225.47 | 24.10 | 19 | −131.57 | 10.38 | 7 | 58 | −56.22 | 8.00 | 9 | 25 | −129.89 | 23.50 | 9 | 58 | −138.00 | 10.51 | 7 | 61 |
| dpHᵢ/dt | ND96 wash | 140.17 | 24.37 | 6 | 173 | 80.89 | 30.18 | 19 | 164.86 | 23.72 | 7 | 204 | 202.00 | 18.62 | 9 | 250 | 138.78 | 22.59 | 7 | 129 | 134.86 | 14.12 | 17 | 66 |
| tₐ (mV) | −60 mV | nA | Basal | −17.36 | 39.27 | 6 | 24 | −72.63 | 11.01 | 39 | −36.51 | 8.73 | 7 | 50 | −37.01 | 13.58 | 9 | 50 | −48.56 | 24.45 | 9 | 66 | −82.11 | 19.86 | 7 | 112 |
| nA | CO₂/HCO₃⁻ | 1.63 | 10.68 | 6 | 0 | 764.37 | 50.97 | 19 | 568.85 | 43.23 | 7 | 74 | 38.66 | 5.80 | 5 | 9 | 543.44 | 64.15 | 9 | 71 | 753.73 | 63.27 | 9 | 77 |
| nA | 0-Na⁺-CO₂⁻ | −10.58 | 9.56 | 6 | 2 | −688.99 | 66.14 | 19 | −506.42 | 60.94 | 7 | 74 | −36.04 | 7.72 | 5 | 9 | −495.42 | 69.52 | 9 | 72 | −666.42 | 40.04 | 7 | 97 |
| nA | ND96 wash | −49.80 | 39.00 | 5 | 24 | −208.29 | 25.61 | 19 | −158.34 | 11.64 | 7 | 76 | −42.32 | 14.21 | 9 | 20 | −487.02 | 303.23 | 9 | 234 | −223.22 | 61.11 | 7 | 107 |

For the R298S pathophysiology, we recreated this point mutation in wild-type hkBNCe1 (WT) and expressed it in Xenopus oocytes. Results show that R298S is a moderately functioning mutation with decreased HCO₃⁻ affinity/capacity (Fig. 1, C–F, and Table 1). The CO₂/HCO₃⁻-elicited outward current and the R298S-Na⁺-dependent currents are significantly lower than those observed in WT. The R298S acidification rate is 78% faster than that of WT in CO₂/HCO₃⁻ (indicating slower transport), and the acidification due to Na⁺ removal (renal absorption) is significantly slower than that of WT (Fig. 1D; 71 versus −130 × 10⁻² pH units/s). Comparing the rate before (NaHCO₃ influx) and after (NaHCO₃ efflux) Na⁺ removal in HCO₃⁻ solution, the dpHᵢ/dt of R298S is decreased rather than increased as in WT, showing that HCO₃⁻ transport function is deficient in this human mutant. The β₂ of R298S is also much less than that of WT (Table 1). The differences in the current magnitude between WT and R298S are voltage-dependent, yet the reversal potential (at 0 current) remains unchanged (Fig. 1E). The NBCe1 I-V responses further illustrate that R298S is a moderate mutation with lower apparent HCO₃⁻ transport capacity and Na⁺ affinity than WT.

To elucidate the role of R298S in NBCe1 transport, we initiated structure-function studies. Our rationale was that sequence alignments of highly conserved residues of well-characterized Slc4 protein sequences from divergent species could reveal candidate residues of transport importance (Fig. 2).

Members of the Slc4 family share 48.7% sequence identity through predicted membrane-spanning regions, although the animal Slc4 family includes functionally distinct transporters (35): (a) anion exchangers, (b) Na⁺/HCO₃⁻ cotransporters, and (c) Na⁺-driven Cl⁻/HCO₃⁻ exchangers. Interestingly, even higher identities among Slc4 members are found within their N termini, particularly within spans of the folded N-terminal cytoplasmic domains (57.2% on average; NBCe1 has 67% identity among orthologs).

The structural model indicates that Arg-298 is located in a secondary-structure element (Fig. 2), suggesting structural differences between NBCe1 and AE1.

High sequence similarity of these two transporters and the other family members facilitated a straightforward structure prediction (Fig. 2, B–E). Corresponding sequence and structural elements are found for AE1 and NBCe1 in this domain, thus confirming the identity of the entire fold. The lowest identity is observed for one larger loop region (NBCe1 residues 114–170; Fig. 2, B and D, light blue region), suggesting structural differences around the hairpin loop that binds ankyrin in Band 3. These differences are likely responsible for the lack of ankyrin binding by NBCe1. The domain fold is comprised of a central sheet surrounded by multiple helices. The spatially and somewhat separate helix-loop-helix motif represents a dimerization domain (Fig. 2B), similar to Band 3 in NBCe1 appears to dimerize (18). We represent NBCe1 as a monomer since we lack ultimate proof of the dimeric nature of NBCe1.

The structural model indicates that Arg-298 is located in a largely solvent-inaccessible, polar subsurface pocket (Fig. 2C) and that Arg-298 is surrounded by multiple other charged and polar residues. Foremost, Arg-298 likely forms H-bonds with either Glu-295 or Glu-91 (approximate residue distance 3–3.5 Å) (Fig. 2, C and E). NBCe1 residues Glu-295 and Glu-91 are equivalent to human AE1 residues Glu-283 and Glu-85, respectively. Other residues of this “pocket” are Thr-108 at the top.
the consensus level of the conserved residues in the gene family. The
exchanger; AB040457), NDCBE (Na\(^+\) bicarbonate cotransporter 1 and 2; AF124441 and AF254802), hpNBCe1 (human pancreas form
bicarbonate cotransporter 1; AF047468), ceNBC (Canorhabditis elegans Na\(^+\) bicarbonate cotransporter; AF004926), nNBCe1 (Ambystoma tigrinum Na\(^+\) bicarbonate cotransporter 1; AF010912), hNBCe1 (rat kidney epithelial Na\(^+\) bicarbonate cotransporter 1 and 2; AF114134), rbNBCe1 (rat brain epithelial Na\(^+\) bicarbonate cotransporter 1 and 2; AF124441 and AF254802), hpNBCe1 (human pancreas form
electrogenic Na\(^+\) bicarbonate cotransporter 1; AF083793), NBCe1-dace (Osteoblast dace electronegic Na\(^+\) bicarbonate cotransporter 1; AB0053467), NBCe1-trout (rainbow trout Na\(^+\) bicarbonate cotransporter 1; AF434166), NBC3 (human Na\(^+\) bicarbonate cotransporter 3; AF069512), NBC4c (human Na\(^+\) bicarbonate cotransporter 4; AF293337), NBC1-D (rat electroneutral Na\(^+\) bicarbonate cotransporter 1-D, NM_058211), NBCe (Na\(^+\)-Cl\(^-\) /bicarbonate exchanger; AB040457), NDCBE (Na\(^+\)-Cl\(^-\) /bicarbonate exchanger; AY151155).

To test our putative structure for the NBCe1 N terminus, we created point mutations in hkBNCe1 to perturb the putative charge interactions among three residues (Arg-298, Glu-91, and Glu-295) (Fig. 3). Our NBCe1 model (Fig. 2, B and E) predicted that the mutations would cause charge repulsion, thereby opening the N-terminal structure and altering NBCe1 function. We began by evaluating the effect of R298E on the hkBNCe1 transport function by altering the charge of the disease-mutation from a neutrally charged (Ser) to a negatively charged residue (37). The HCO\(_3^\)-evoked currents and Na\(^+\)-dependent currents in R298E are ~71% of WT (Fig. 3, A and E, and Table 1). CO\(_2\) acidifies R298E oocytes 72% faster than WT, whereas Na\(^+\) removal in HCO\(_3^\)-acidified WT 74% faster than R298E (Fig. 3, A and D). These results represent an impaired NBCe1 transport function resulting from the R298E mutation. All of these results are consistent with R298E-expressing oocytes having a buffering power (\(\beta_i\)) three times smaller than that of WT. Nevertheless, the I-V relationships for R298E (Fig. 3, E and F) are similar to the R298S disease mutation (Fig. 1, E and F). The CO\(_2\)/HCO\(_3^\)- reversal potentials (−80 mV) are similar for R298S-, R298E-, and WT-hkBNCe1 (Figs. 1E and 3E), indicating no fundamental change in stoichiometry of ion transport.

Interestingly, E91R-hkBNCe1 exhibits very severe defects in ion transport function (Fig. 3B and Fig. S1B). The CO\(_2\)/HCO\(_3^\)-evoked current in E91R appears gradually and reaches a plateau only slowly, instead of maximizing quickly fol-
hkNBCe1 Cytoplasmic N Terminus Controlling HCO$_3^-$ Permeation

The expression of the NBCe1 transporter protein in the oocytes was quantified by detecting an HA tag engineered into the extracellular loop of hkNBCe1 and the NBCe1 mutants. Basal luminescence of oocyte surfaces was determined using oocytes expressing the untagged WT-hkNBCe1 transporter. The luminescence values of the HA-tagged mutants were not significantly different from that of the HA-tagged WT-hkNBCe1, demonstrating that there was no difference in surface expression of NBCe1 protein between the WT and mutants. These data confirm that the E91R-hkNBCe1 protein is appropriately trafficked and expressed at the oocyte plasma membrane (Fig. 4A). These results also verified that the extracellular HA tag did not alter the HCO$_3^-$-elicited current (Fig. 4B) or the 0Na$^+$/HCO$_3^-$-elicited currents (Fig. 4C) when compared with untagged NBCe1 proteins (Figs. 1, E and F, and 3, E and F).

The CO$_2$/HCO$_3^-$-induced acidifications (Figs. 3, C and D) and currents (Fig. 5, A and B) of E91R/R298E are not significantly different from WT (Table 1). The Na$^+$-dependent current (Fig. 5B) and Na$^+$ removal-elicited acidifications (Fig. 3D) are similar for E91R/R298E and WT. Wt has a slightly higher transport) due to solution change for E91R (Fig. 3, B and D) is also significantly different from water-injected controls (Fig. 1, A and D).

The surface expression of the NBCe1 transporter protein in the oocytes was quantified by detecting an HA tag engineered into the extracellular loop of hkNBCe1 and the NBCe1 mutants. Basal luminescence of oocyte surfaces was determined using oocytes expressing the untagged WT-hkNBCe1 transporter. The luminescence values of the HA-tagged mutants were not significantly different from that of the HA-tagged WT-hkNBCe1, demonstrating that there was no difference in surface expression of NBCe1 protein between the WT and mutants. These data confirm that the E91R-hkNBCe1 protein is appropriately trafficked and expressed at the oocyte plasma membrane (Fig. 4A). These results also verified that the extracellular HA tag did not alter the HCO$_3^-$-elicited current (Fig. 4B) or the 0Na$^+$/HCO$_3^-$-elicited currents (Fig. 4C) when compared with untagged NBCe1 proteins (Figs. 1, E and F, and 3, E and F).

DISCUSSION

We hypothesized that we might gain additional insights of NBCe1 structure and function by mapping the hkNBCe1, SLC4A4, amino acid sequence onto the AE1 crystal structure (Protein Data Bank (PDB) number 1HYN) (3). From this NBCe1 structural model, Arg-298 of NBCe1 is predicted to hydrogen-bond with Glu-91 and Glu-295. R298S is a human NBCe1 mutation resulting in renal and eye disease (see “Results”). Interestingly, Arg-298 is a conserved residue in the animal Sle4 protein family and is predicted to lie in a “solvent-inaccessible pocket.” To test whether our structural model of NBCe1 was valid, we mutated these putative interacting resi-
**hkNBCe1 Cytoplasmic N Terminus Controlling HCO\(_3^-\) Permeation**

**FIGURE 4. NBCe1 surface expression in oocytes.** A, the normalized luminescence value (mean \(\pm\) S. E.) of oocytes expressing HA-tagged hkNBCe1 mutants. The surface expression of the transporter protein on the oocytes was labeled by a monoclonal rat-\(\alpha\)-HA 11° antibody and a goat-\(\alpha\)-rat horse-radish peroxidase-IgG 2° antibody and measured with a luminometer after adding chemiluminescent substrate. The luminescence values of the clones were normalized to the value of the WT-hkNBCe1 with HA tag. The oocytes were from three different donor frogs and sample sizes of each clone are shown in the bars. The asterisk indicates a luminescence value for that clone that is statistically different (\(p < 0.01\)) from that of HA-tagged WT-hkNBCe1. B and C, the current-voltage relationship of hkNBCe1 mutants in CO\(_2\)/HCO\(_3^-\) (B) and in Na\(^+\)−CO\(_2\)/HCO\(_3^-\) solutions (C).

The modeling and functional data of the E91R-nbcCe1 mutant imply that charge alterations (particularly the introduction of additional cationic side chains) are disruptive to the pocket structure around Arg-298. To further probe the role of residue charge at this putative solvent-inaccessible pocket, we examined the effect of charge reversal through a double mutation, E91R/R298E-hkNBCe1 (Fig. 3, C–F and Fig. S1C). If structure and charge interaction rather than exact sequence are important, we predicted that a clone that simultaneously reverses the charge of Glu-91 and Arg-298 (E91R/R298E) would have transporter function that resembles those of wild-type NBCe1.

The data presented here show that the double mutant (E91R/R298E) can restore E91R to full “WT-like” function (Fig. 5, A and B). Thus, either the double mutant reverses the structural effect of E91R on wild-type function or R298E quenches the effects of E91R on the transport function (Figs. 1, 3, and 4 and Fig. S1) by restoring the native structure (Fig. 2, B–E). Although all tested mutants imply a role for this domain and particularly this pocket in the transmembrane ion transport, this rescue mutation presents the strongest evidence and validation for the close proximity between Glu-91 and Arg-298, i.e. correctness of this model, and for a direct residue- and location-specific correlation of the structural and functional changes. A similar residue-charge interaction was reported recently between a pair of arginine and glutamate residues in a *Torpedo* acetylcholine receptor structure model (38). Acetylcholine-evoked single-channel currents were measured from receptors with R209E or E45R mutation and the corresponding double mutation. The charge-reversal double mutation yielded surface expression and rescued wild-type acetylcholine-function abolished by single mutations.

Glu-91 and its sequential neighbor Glu-92 are part of the highly conserved motif (WRETARWIKFEE; amino acids 336–347 in hkNBCe1) (Fig. 2A) proposed to determine pH sensitivity of murine anion exchanger AE2/SLC4A2 (39 – 41). Intriguingly, Glu-92 similarly is suggested by the model to be part of a second unusual feature of this fold. Glu-91 is located on the opposite side of the \(\beta\)-sheet from Glu-91. It is involved in a network of interacting residues as it hydrogen-bonds to arginine 86 (Arg-86) and potentially Lys-227 (Fig. 2). Arg-86 in turn interacts with Glu-272. Residues Arg-86, Glu-92, Lys-227, and Glu-272 are highly conserved in the Slc4 bicarbonate transporters (Fig. 2A), and these functionally important residues share number, charge, and residue type, nearly duplicating the properties of the pocket as for Glu-91, Glu-295, and Arg-298.
Together, these residues create an unusual continuous chain of interconnected polar residues and a steady path of high polarity through the core of this domain from the membrane oriented C-terminal side (Fig. 2, D and E) to the interior.

It is intriguing to speculate on the function of this feature. This pathway may attenuate ion transport (or even serve as an ion transport pathway). Interestingly, when the same group of residues is mutated in AE2 (R341A, W342A, E346A, and E347A), pH sensitivity of wild-type anion transport is abolished (39–41). A mutagenesis study of murine AE2 residues identified a histidine residue, corresponding to His-105 in NBCe1 amino acid sequence, important for regulation of Cl− transport (40). Histidine and lysine are hydrophilic, positively charged basic amino acids highly likely to be a potential pH sensor(s) for NBCe1. These residues have been characterized as pH sensors in many studies: acid-sensing ion channels (42), tandem pore domain acid-sensitive K+ channel (TASK-3) (43), Na+/H+ antiporter (44), and ROMK1 channels (45). Glutamate, negatively charged and acidic amino acid, was also identified as the pH sensor in other investigations of uncoupling protein (46), TRPV5 channel (47), and CIC-2G Cl− channel (48).

Finally, it is noteworthy that the Slc4 gene family spans eukaryotes from humans to yeast to plants. In plants and yeast, Slc4 proteins have not been shown to transport HCO3− but ratherborate (49). The Arabidopsis and mammalian boron transporter (BOR1/Slc4a11) (50) members lack 391 cytoplasmic N-terminal sequence found in mammalian NBCe1 or other animal Slc4 members, and these boron transporters do not transport HCO3−. In addition, human pancreatic NBCi isoform (pNBCi/NBCe1-B) has an N-terminal variation with a lower bicarbonate transport capacity (17), which is disinhibited by an inositol 1,4,5-trisphosphate receptor binding protein (IRBIT) (51). These results corroborate the suggestion of a critical role of NBCe1 N terminus in HCO3− transport. The structure modeling points us to candidate residues for mutation analysis that eventually gave rise to a severe functional mutation, E91R (Fig. 2).

The effect of the R298S-hkNBCe1 mutation is unclear in the literature. R298S has been reported reducing wild-type function (1) and as a protein trafficking problem (1, 9, 16). This latter report uses Xenopus oocytes as we have in this study. Horita et al. (16) implied oocyte surface expression by coincident fluorescence of a NBCe1-A N-terminal antibody (intracellular epitope) and wheat germ agglutinin as a general marker of plasma membrane (extracellular). The data presented in Fig. 4A use an extracellular tag of the hkBCE1 molecule, i.e., a direct assessment of the NBCe1 proteins at the plasma membrane. Contrary to the previous Xenopus oocyte report (1, 9, 16), the data in Fig. 4 also explicitly show that R298S-hkBCE1 affects NBCe1 function and not NBCe1 protein processing.

This report provides a structure model and biophysical role for the NBCe1 N terminus based in part on a human NBCe1 disease mutation (R298S), summarized in Fig. 5. R298S-hkBCE1 affects NBCe1 function and not NBCe1 protein processing (Fig. 4). Further, we detect the very unusual polarity of multiple core residues in the N-terminal domain, suggesting that this chain of connected residues may create and ion transport pathway, thus providing a possible explanation for its ion transport role and putative pH sensitivity. This solvent-inaccessible pocket appears conserved in all HCO3−-transporting Slc4 proteins. Thus, this work brings to light a new structural domain critical for HCO3− transport in the Slc4 proteins.

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REFERENCES

1. Igarashi, T., Inatomi, I., Sekine, T., Cha, S. H., Kanai, Y., Kunimi, M., Tsukamoto, K., Satoh, H., Shimadzu, M., Tozawa, F., Mori, T., Shiobara, M., Seki, G., and Endou, H. (1999) Nat. Genet. 23, 264–266
2. Romero, M. F., and Smith, B. L. (2002) FASEB J. 16, a52–a53
3. Zhang, D., Kiyatkin, A., Bolin, J. T., and Low, P. S. (2000) Blood 96, 2925–2933
4. Alper, S. L. (2002) Annu. Rev. Physiol. 64, 899–923
5. Romero, M. F. (2005) Curr. Opin. Nephrol. Hypertens. 14, 495–501
6. Biemesderfer, D., Pizzonia, J., Abu-Alfa, A., Exner, M., Reilly, R., Igarashi, P., and Aronson, P. S. (1993) Am. J. Physiol. 265, F736–F742
7. Schultheis, P. J., Clarke, L. L., Meneton, P., Miller, M. L., Soleimani, M., Gavensis, L. R., Riddle, T. M., Duffy, J. I., Doetschman, T., Wang, T., Giebish, G., Endou, P. S., Lorenz, J. N., and Shull, G. E. (1998) Nat. Genet. 19, 282–285
8. Wang, T., Yang, C. L., Abbiati, T., Schultheis, P. J., Shull, G. E., Giebish, G., and Aronson, P. S. (1999) Am. J. Physiol. 277, F298–F302
9. Dinour, D., Chang, M.-H., Satoh, J.-I., Smith, B. L., Angle, N., Knecht, A., Serban, I., Holtzman, E. J., and Romero, M. F. (2004) J. Biol. Chem. 279, 52238–52246
10. Igarashi, T., Inatomi, I., Sekine, T., Seki, G., Shimadzu, M., Tozawa, F., Takeshima, Y., Takumi, T., Takahashi, T., Yoshikawa, Y., Nakamura, H., and Endou, H. (2001) J. Am. Soc. Nephrol. 12, 713–718
11. Igarashi, T., Inatomi, I., Sekine, T., Takeshima, Y., Yoshikawa, N., and Endou, H. (2000) J. Am. Soc. Nephrol. 11, A0573
12. Demirci, F. Y., Chang, M.-H., Mah, T. S., Romero, M. F., and Gorin, M. B. (2006) Mol. Vis. 12, 324–330
13. Toye, A. M., Parker, M. D., Daly, C. M., Lu, J., Virkki, L. V., Pelletier, M. F., and Boron, W. F. (2006) Am. J. Physiol. 291, C788–C801
14. Gavensis, L. R., Bradford, E. M., Prasad, V., Lorenz, J. N., Simpson, J. E., Clarke, L. L., Woo, A. L., Grisham, C., Sanford, L. P., Doetschman, T., Miller, M. L., and Shull, G. E. (2007) J. Biol. Chem. 282, 9042–9052
15. Inatomi, I., Horita, S., Braverman, N., Sekine, T., Yamada, H., Suzuki, Y., Kawahara, K., Moriyama, N., Kudo, A., Kawakami, H., Shimadzu, M., Endou, H., Fujita, T., Seki, G., and Igarashi, T. (2004) Pfluegers Arch. Eur. J. Physiol. 448, 438–444
16. Horita, S., Yamada, H., Inatomi, I., Moriyama, N., Sekine, T., Igarashi, T., Endo, Y., Daisukii, M., Ekin, M., Al-Gazali, L., Shimadzu, M., Seki, G., and Fujita, T. (2005) J. Am. Soc. Nephrol. 16, 2270–2278
17. Tatschsehve, S., Abuladze, N., Pushkin, A., Newman, D., Liu, W., Weeks, D., Sachs, G., and Kurtz, I. (2003) Biochemistry 42, 755–765
18. Gill, H. S., and Boron, W. F. (2006) Acta Crystallogr. F Struct. Biol. Crystal- liz. Comm. 62, 534–537
19. Guex, N., and Peitsch, M. C. (1997) Electrophoresis 18, 2714–2723
20. Huang, X., and Miller, W. (1991) Adv. Appl. Math. 12, 337–357
21. Schwede, T., Kopp, J., Guex, N., and Peitsch, M. C. (2003) Nucleic Acids Res. 31, 3381–3385
22. Canutescu, A. A., Shelenkov, A. A., and Dunbrack, R. L., Jr. (2003) Protein Sci. 12, 2001–2014
23. Willard, L., Ranjan, A., Zhang, H., Monzavi, H., Boyko, R. F., Sykes, B. D., and Wishart, D. S. (2003) Nucleic Acids Res. 31, 3316–3319
