The electrogenicity of the rat sodium–bicarbonate cotransporter NBCe1 requires interactions among transmembrane segments of the transporter

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The electrogenic Na^+–HCO_3^- cotransporter (NBCe1) plays a central role in intracellular pH (pH_i) regulation as well as HCO_3^- secretion by pancreatic ducts and HCO_3^- reabsorption by renal proximal tubules. To understand the structural requirements for the electrogenicity of NBCe1, we constructed chimeras of NBCe1-A and the electroneutral NBCn1-B, and used two-electrode voltage clamp to measure electrogenic transporter current in *Xenopus* oocytes exposed to 5% CO_2–26 mM HCO_3^- (pH 7.40). The chimera consisting of NBCe1-A (i.e. NBCe1-A 'background') with the cytoplasmic N-terminal domain (Nt) of NBCn1-B had a reversal potential of −156.3 mV (compared with a membrane potential V_m of −43.1 mV in a HCO_3^--free solution) and a slope conductance of 3.0 μS (compared with 12.5 μS for NBCe1-A). Also electrogenic were chimeras with an NBCe1-A background but with NBCn1-B contributing the extracellular loop (L) between transmembrane segment (TM) 5 and 6 (−140.9 mV/11.1 μS), the cytoplasmic C-terminal domain (Ct; −123.8 mV/9.7 μS) or Nt + L + Ct (−120.9 mV/3.7 μS). Reciprocal chimeras (with an NBCn1 background but with NBCe1 contributing Nt, L, Ct or Nt + L + Ct) produced no measurable electrogenic transporter currents in the presence of CO_2–HCO_3^-. pH_i recovered from an acid load, but without the negative shift of V_m that is characteristic of electrogenic Na^+–HCO_3^- cotransporters. Thus, these chimeras were electroneutral, as were two others consisting of NBCe1(Nt–L)/NBCn1(TM6–Ct) and NBCn1(Nt–L)/NBCe1(TM6–Ct). We propose that the electrogenicity of NBCe1 requires interactions between TM1–5 and TM6–13.

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Cells regulate their internal ionic environment via membrane proteins that mediate the transmembrane movement of ions. The *SLC4A* gene family, which has 10 members, consists mainly of transporters that are specialized to move HCO_3^- (or in some cases, CO_3^{2-}) across plasma membranes (for review see Romero *et al.* 2004). For many *SLC4* family members, major physiological roles include the regulation of intracellular pH (pH_i) and/or the transepithelial movement of HCO_3^-. Regarding pH_i regulation, some *SLC4* members mediate the efflux of HCO_3^- (or CO_3^{2-}) and thus act to lower pH_i (acid loaders), whereas others mediate the influx of HCO_3^- (or CO_3^{2-}) and thus act to raise pH_i (acid extruders). The balance between the activities of acid extruders and loaders, as well as metabolic production and consumption of H^+ ions, determines the steady-state pH_i (Boron, 1989). For example, the electrogenic Na^+–HCO_3^- cotransporter NBCe1 (*SLC4A4*) (Bevensee *et al.* 1995) – operating with a Na^+:HCO_3^- stoichiometry of 1 : 2 in cells such as astrocytes – is an acid extruder. This HCO_3^--mediated acid extrusion helps to avoid pH_i in astrocytes falling below normal levels during cerebral acidification (Giffard *et al.* 2000).

In HCO_3^--transporting epithelia, at least one member of the *SLC4A* gene family is usually present in the basolateral membranes, mediating a crucial step in the transepithelial movement of HCO_3^-. For example, NBCe1, apparently operating with a 1 : 2 stoichiometry, mediates HCO3$^-$ uptake across the basolateral membrane of the pancreatic duct for secretion into the lumen (Marino *et al.* 1999). In the renal proximal tubule, which reclaims more than 80% of filtered HCO_3^-, NBCe1 mediates the efflux of HCO_3^- across the basolateral membrane for delivery to the blood. An essential requirement for this HCO_3^- exit is that NBCe1 in the proximal tubule operates with an apparent Na^+:HCO_3^- stoichiometry of 1 : 3, which

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Table 1. Summary of regions swapped between NBCe1-A and NBCn1-B

Region	NBCe1-A	NBCn1-B
Nt	MSTEN...ALNIQ (424)	MEADG...ALSLQ (611)
Loop	ADYYP...CNFVP (83)	GEIYA...GPYVP (76)
Ct	KGMDY...RHTSC (92)	KLMDL...AETSL (95)
Front (start–Loop)	MSTEN...CNFVP (646)	MEADG...GPYVP (826)
Back (TM6–end)	DITLM...RHTSC (389)	DVLFW...AETSL (392)

Each cell contains the single-letter codes of the first five and last five amino acids of the fragment; the total number of amino acids is in parentheses.

makes the reversal potential for the transporter more positive than the basolateral membrane potential of the proximal tubule cell (Soleimani *et al.* 1987; Boron & Boulpaep, 1989). By moving Na^+ out of the cell, NBCe1 in the proximal tubule may be unique in moving Na^+ against the Na^+ electrochemical gradient without utilizing ATP directly.

In spite of the importance of the electrogenicity of NBCe1 for its physiological function, the protein domains responsible for electrogenic ion translocation are not known. Sequence analysis reveals a high level of amino acid identity (> 50%) among the five known Na^+–HCO_3^- transporters of the *SLC4* family (Romero *et al.* 2004), two of which are electrogenic (NBCe1/*SLC4A4* and NBCe2/*SLC4A5*) (Burnham *et al.* 1997; Romero *et al.* 1997; Sassani *et al.* 2002; Virkki *et al.* 2002) and three of which are electroneutral (NBCn1/*SLC4A7*, NDCBE/*SLC4A8* and NCBE/*SLC4A10*) (Pushkin *et al.* 1999; Choi *et al.* 2000; Wang *et al.* 2000; Grichtchenko *et al.* 2001). It is not surprising that the identity level is generally higher in the transmembrane domains of the proteins than in the cytoplasmic N- and C-terminal domains and the large, third extracellular domain between transmembrane segment (TM) 5 and TM6. This relationship is consistent with the hypothesis that the transmembrane domains mediate the actual transport event, but that differences in these less-conserved regions somehow modulate the transmembrane domains and thereby control the stoichiometry of ion transport. Several studies have shed light on the structure–function relationships of NBCe1. A large-scale mutagenesis study targeted acidic and basic amino acids near or outside transmembrane segments, and identified several that block transport (Abuladze *et al.* 2005). However, it remains unclear whether those residues directly participate in ion translocation or determine stoichiometry.

In the present study, we systematically constructed a series of chimeric transporters between NBCe1 and the electroneutral Na^+–HCO_3^- cotransporter NBCn1 (for a sequence comparison of the two transporters, see Choi *et al.* 2000). We swapped transmembrane and non-transmembrane domains of NBCe1 and NBCn1, expressed them in *Xenopus* oocytes, and analysed the HCO_3^--dependent currents mediated by chimeras in two-electrode voltage clamp. We found that neither the cytoplasmic N-terminal domain (Nt), the large, third extracellular loop (L) nor the cytoplasmic C-terminal domain (Ct) affects electrogenicity. However, both the transmembrane domain before the third extracellular loop (TM1–5) and the transmembrane domain after the third extracellular loop (TM6 to the last TM) are necessary for electrogenicity.

Methods

Construction of chimeras

The 'renal' splice variant of rat NBCe1 (NBCe1-A, GenBank accession number NM_053424) and NBCn1 (NBCe1-B, GenBank accession number NM_058211) were used for constructing chimeric proteins. Table 1 summarizes the regions that we swapped. To swap regions of interest, we introduced restriction endonuclease sites (generating silent mutations that did not affect amino acid composition) at both ends of the corresponding regions of both transporters, using the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). Details are available upon request. PCR was carried out using *Pfu* DNA polymerase with high proofreading capability. Chimeric constructs were sequenced by the Biomedical Facility at Emory University or the Keck Center at Yale University. The constructs were inserted in the oocyte expression vector pGH19 (Choi *et al.* 1999), which contains the 5′ and 3′ untranslated regions of the *Xenopus* β-globin gene. For wild-type NBCe1 and chimeras containing the N-terminal domain of NBCe1, we used the pTLN2 vector (Romero *et al.* 1998) because of incompatibility of the restriction enzyme sites. pTLN2 is virtually identical to pGH19 except for the polycloning sites.

Transporter expression in *Xenopus* oocytes

The DNAs encoding chimeric transporters were linearized with Not I or Nhe I for constructs in pGH19 and *in vitro* transcribed with T7 RNA polymerase using the mMessage/mMachine transcription kit (Ambion, Austin, TX, USA). For constructs in pTLN2, Mlu I

was used for linearization and SP6 RNA polymerase for transcription. Oocytes (stages V and VI) were prepared from *Xenopus laevis*. A piece of ovary containing oocytes was excised, rinsed five times with Ca^{2+}-free ND96 solution containing (mM): NaCl 96, KCl 2, $MgCl_2$ 1 and Hepes 5 (pH 7.5), for 20 min each. The tissue was then agitated in sterile-filtered Ca^{2+}-free ND96 with collagenase (Type 1 A, Sigma-Aldrich, St Louis, MO, USA) for 40 min. Defolliculated oocytes were washed with Ca^{2+}-free ND96, sorted and stored at 18°C in OR3 media (50% Leibovitz L-15 media in 5 mM Hepes, pH 7.5) supplemented with 5 U ml^{-1} penicillin-streptomycin. Oocytes were injected with 50 nl of either RNA (0.4 $\mu g\,\mu l^{-1}$). Injected oocytes were maintained for 3–7 days at 18°C.

Two-electrode voltage clamp

Two-electrode voltage clamp was performed using the oocyte clamp OC-725C (Warner, Hamden, CT, USA). The electrodes were filled with 3 M KCl with a resistance of less than 1.5 MΩ. Oocytes were clamped at a holding potential of −60 mV and subjected to staircase voltage commands stepping from −140 to +60 mV (20 mV increment) for 100 ms. Measurements of the electrogenic current were made at 1 min after switching bath solutions from modified amphibian ND96 solution containing (mM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 2 and Hepes 5 (pH 7.4), to one buffered with 5% CO_2–26 mM HCO_3^- (pH 7.4; NaCl replaced by $NaHCO_3$). Measurements of the ionic currents associated with NBCn1 were done in ND96, as previously described (Cooper *et al.* 2005). In Na^+-free solutions, Na^+ was replaced by $NMDG^+$. The voltage signal was sampled by the Digidata 1322 interface (Axon Instruments, Union City, CA, USA) connected to an IBM-compatible PC. Data were acquired and analysed using pClamp 8 (Axon Instruments).

Measurements of pH_i in oocytes

We impaled oocytes with two electrodes, one sensitive to pH and the other a conventional voltage electrode, as previously described (Siebens & Boron, 1987; Romero *et al.* 1997; Lu *et al.* 2006). The pH electrode was made with borosilicate fibre capillaries, silanized, and filled with the proton ionophore 1 cocktail B (Sigma-Aldrich), and back filled with a phosphate buffer at pH 7.0. The voltage electrode was filled with 3 M KCl and had a resistance of 0.7–2.0 MΩ. Electrodes were connected to a high-impedance electrometer FD-223 (WPI, Sarasota, FL, USA). The voltage signal was subtracted from the voltage from the pH electrode, which yields a voltage due solely to pH. The data were sampled by an A/D converter interfaced to a computer. Measurements of pH_i were first done in the nominally HCO_3^--free ND96 solution (pH 7.5) and then in a solution equilibrated with 1.5% CO_2–10 mM HCO_3^- (pH 7.5). NaCl was replaced by $NaHCO_3$. The osmolality was adjusted to 196–200 mosmol kg^{-1}. All experiments were performed at room temperature (∼22°C).

Statistical analysis

Data were reported as means ± S.E.M. Levels of significance were assessed using the unpaired, two-tailed Student *t* test. $P < 0.05$ was considered significant. Rates of pH_i change were fitted by a line using a least-squares method.

Results

Electrogenic HCO_3^- currents mediated by NBCe1

In analysing the electrogenicity of chimeric transporters, our experimental approach was to express the transporters in *Xenopus* oocytes and determine steady-state current–voltage (I–V) relationships with the oocyte first exposed to the CO_2–HCO_3^--free ND96 solution and then to the solution buffered with 5% CO_2–26 mM HCO_3^- (pH 7.4). Figure 1*A* shows average data for water-injected control oocytes. In the ND96 solution, they had a mean reversal potential (E_{ND96}) around −41 mV, low background currents (I_{ND96}) and a low slope conductance (G_{ND96}). After the switch to CO_2–HCO_3^-, the reversal potential (E_{HCO3}), currents (I_{HCO3}) and slope conductance (G_{HCO3}) were not substantially different from those in the presence of ND96 solution. The difference current $I_{HCO3} - I_{ND96}$ (red triangles) nearly lies on the voltage axis. Table 2 summarizes the data from control oocytes as well as oocytes (discussed below) expressing NBCe1-A, NBCn1-B and various other chimeras.

Figure 1*B* shows average data for an oocyte expressing NBCe1-A. I_{ND96} values (at positive voltages) as well as G_{ND96} values were somewhat higher than in H_2O-injected control oocytes, consistent with the report by Sciortino & Romero (1999). I_{HCO3} values at positive voltages as well as G_{HCO3} values were much larger than for H_2O-injected control oocytes, with a substantial negative shift of E_{HCO3}. These changes are due to the cotransport activity of NBCe1-A as it moves net negative charge (i.e. 1 Na^+ and the equivalent of 2 HCO_3^- ions) into the cell. The difference $I_{HCO3} - I_{ND96}$ (red symbols) is a good estimate of the electrogenic cotransport current (I_{NBC}), particularly for the larger outward currents (Lu *et al.* 2006). The slope conductance of I_{NBC} (G_{NBC}) was substantially greater than G_{ND96}, and the reversal potential of I_{NBC} (E_{NBC}) was about −129.3 mV so that I_{NBC} was outwardly directed at all voltages more positive than this value.

Figure 1*C* shows average data for an oocyte expressing NBCn1-B. I_{ND96} values (at positive voltages) as well as G_{ND96} values were higher than in H_2O-injected

Table 2. Reversal potentials and slope conductances of oocytes expressing chimeric transporters

Chimera name	Figure	*n*	ND96		HCO_3–CO_2		Cotransport current	
			E_{ND96}	G_{ND96} (μS)	E_{HCO3}	G_{HCO3} (μS)	E_{NBC}	G_{NBC} (μS)
water	Fig. 1*A*	5	−41.0 ± 5.6	1.1 ± 0.1	−47.4 ± 6.6	0.8 ± 0.1	−27.7 ± 2.8	−0.4 ± 0.1
NBCe1-A	Fig. 1*B*	7	−36.5 ± 1.2	7.7 ± 0.9	−91.5 ± 2.8	24.0 ± 1.4	−129.3 ± 4.5*	12.5 ± 0.9**
NBCn1-B	Fig. 1*C*	7	−33.4 ± 5.1	5.9 ± 0.3	−26.2 ± 0.5	4.7 ± 0.2	−23.7 ± 1.8	−1.3 ± 0.3
n1(Nt)/e1	Fig. 3	7	−43.2 ± 2.4	3.2 ± 0.4	−98.9 ± 4.8	6.0 ± 0.6	−156.3 ± 9.2*	3.0 ± 0.4**
n1(L)/e1	—	6	−37.9 ± 2.2	4.9 ± 0.5	−100.3 ± 2.5	14.3 ± 0.9	−140.9 ± 6.4*	11.1 ± 0.7**
n1(Ct)/e1	—	7	−43.2 ± 2.1	3.0 ± 2.1	−113.1 ± 1.9	9.8 ± 1.1	−123.8 ± 1.6*	9.7 ± 1.2**
n1(NtLCt)/e1	Fig. 4	5	−38.0 ± 2.5	1.9 ± 0.1	−94.0 ± 3.9	5.7 ± 0.6	−120.9 ± 3.3*	3.7 ± 0.6**
e1(Nt)/n1	Fig. 5	7	−27.2 ± 0.4	6.1 ± 0.3	−27.9 ± 0.4	5.2 ± 0.3	−24.9 ± 2.0	−0.9 ± 0.9
e1(L)/n1	—	7	−30.9 ± 1.1	6.3 ± 0.5	−33.0 ± 1.1	4.9 ± 0.3	−22.6 ± 1.2	−1.3 ± 0.2
e1(Ct)/n1	—	6	−30.4 ± 1.3	4.1 ± 0.3	−33.6 ± 3.0	3.1 ± 0.5	−23.5 ± 3.3	−0.9 ± 0.3
e1(NtLCt)/n1	Fig. 6	7	−33.3 ± 0.9	5.6 ± 1.1	−37.8 ± 1.0	3.9 ± 0.9	−23.2 ± 0.8	−1.7 ± 0.3
n1(1–821)/e1	Fig. 7	8	−30.5 ± 2.2	1.3 ± 0.2	−32.3 ± 2.5	0.9 ± 0.2	−26.4 ± 1.6	−0.4 ± 0.1
n1(822–1218)/e1	Fig. 8	7	−30.3 ± 0.6	7.7 ± 1.0	−32.5 ± 0.8	6.4 ± 0.9	−20.8 ± 1.2	−1.3 ± 0.2

Conductance (*G*) was computed from current–voltage (*I–V*) values crossing the zero currents. * and **Significant compared to water-injected controls ($P < 0.05$).

oocytes, and E_{ND96} was relatively positive, reflecting the background Na^+ current apparently carried by NBCn1 (Choi *et al.* 2000; Cooper *et al.* 2005). However, I_{HCO3} (at positive voltages) was no higher than I_{ND96}, and E_{HCO3} was not appreciably different from E_{ND96}. In other words, NBCn1 did not produce HCO_3^--dependent currents. Nevertheless, compared to water-injected controls, oocytes expressing NBCn1-B had a greater G_{ND96} (5.9 *versus* 1.1 μS). Previous work showed that this additional ionic conductance is mostly due to a Na^+ current but does not require HCO_3^-. This Na^+ current is mediated by NBCn1 expressed both in *Xenopus* oocytes and human embryonic kidney (HEK) cells, indicating that the conductance is probably intrinsic to the transporter (Choi *et al.* 2000; Cooper *et al.* 2005).

Design of chimeric transporters

Rat NBCe1-A and NBCn1-B share overall ∼55% amino acid identity (more than 65% in the transmembrane domains), and have similar hydropathy plots (Choi *et al.* 2000). We presumed that this similarity would facilitate the construction of chimeric proteins between the two transporters without major problems in retaining protein conformation. Although both NBCe1 and NBCn1 exist as multiple splice variants, we are aware of no marked functional differences among variants of NBCn1. In the absence of protein binding partners such as IRBIT (Shirakabe *et al.* 2006), NBCe1-A, with its unique Nt, is substantially more active than either NBCe1-B or NBCe1-C (McAlear *et al.* 2006). Therefore, we made our constructs with rat NBCe1-A, which is expressed

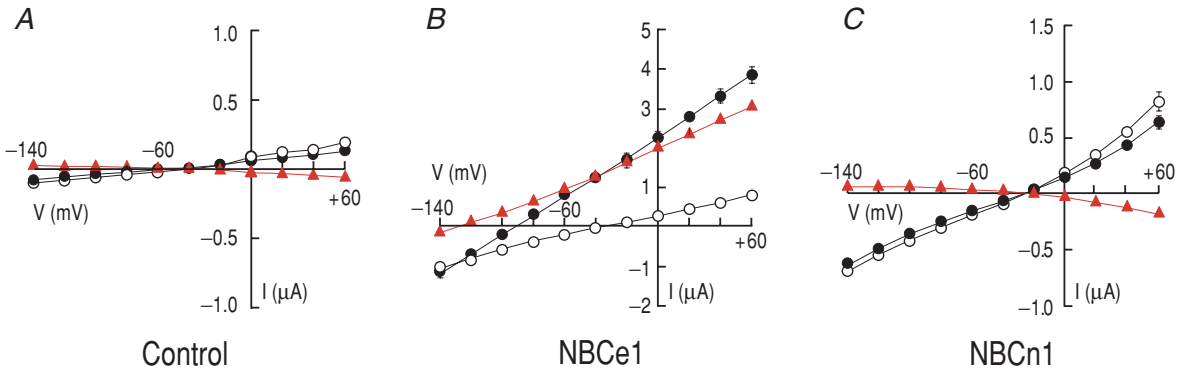

Figure 1. Mean steady-state current–voltage (*I–V*) relationships in control oocytes and oocytes expressing wild-type NBCe1 or NBCn1

A, control oocytes injected with water ($n = 5$). *B,* oocytes expressing NBCe1 ($n = 7$). *C*, oocytes expressing NBCn1 ($n = 7$). Oocytes were clamped at −60 mV and voltage was stepped from −140 to +60 mV for 100 ms in 20 mV increments. The recordings were done in the pH 7.40 HCO_3^--free ND96 solution (○) or in a comparable solution buffered to pH 7.40 with 5% CO_2–26 mM HCO_3^- (●). The difference I_{HCO3}–I_{ND96} is an estimate of the electrogenic cotransport current (▲).

in the proximal tubules of the kidney (Romero *et al.* 1998), and rat NBCn1-B, which is predominantly present in the cardiovascular system (Cooper *et al.* 2006). One specific prediction is that replacing the Nt of NBCe1-A with that of NBCn1-B may be analogous to replacing the Nt of NBCe1-A with that of NBCe1-B, and thus render an electrogenic transporter less active. Chimeras might also be less active than the wild-type molecules because of 'mismatches' of various domains, which might lead to decreased surface expression, or to decreased activity of individual molecules. Here we use the shorthand terminology 'e1' for NBCe1-A and 'n1' for NBCn1-B. We made 10 chimeric constructs by swapping different regions of NBCe1-A with the corresponding ones of NBCn1-B (Fig. 2 and Table 1). In establishing boundaries of regions to be swapped, we followed the 13-TM topology model proposed for the Cl^-–HCO_3^- exchanger AE1 (Fujinaga *et al.* 1999; Kuma *et al.* 2002).

The cytoplasmic N and C termini and the third extracellular loop of NBCe1-A are not important for electrogenicity

Figure 3 summarizes the function of the chimeric transporter n1(Nt)/e1 (Fig. 3*A*), which has the cytoplasmic N-terminal domain of NBCn1-B, with the remainder of the molecule contributed by NBCe1 (i.e. NBCe1 'background'). In voltage-clamp experiments (e.g. Figure 3*B*), mean I_{ND96} (at positive voltages) and G_{ND96} (Table 2) were relatively low compared to NBCe1-A (Fig. 1*B*), but higher than for H_2O-injected controls (Fig. 1*A*). E_{ND96} for n1(Nt)/e1 was about −43 mV. In the presence of 5% CO_2–26 mM HCO_3^-, I_{HCO3} (at positive voltages) and G_{HCO3} were much higher than the corresponding values in the presence of ND96. Moreover, E_{HCO3} was markedly more negative than E_{ND96}. From the difference I_{HCO3}–I_{ND96}, we see that I_{NBC} (at positive voltages) and G_{NBC} were much greater than the corresponding values for the chimera in ND96, but much less than G_{NBC} for wild-type NBCe1-A. For the chimera, E_{NBC} was more negative than −150 mV. These results indicate that replacing the N-terminal domain of NBCe1-A with the corresponding region of NBCn1-B results in a transporter that is electrogenic. The relatively low G_{NBC} of the chimera, which presumably reflects mismatching the N terminus to the rest of the transporter, could be due to either reduced protein delivery to the cell surface or to reduced activity of individual transporters.

To assess the acid–base transport characteristics of n1(N)/e1, we used microelectrodes to monitor pH_i and membrane potential (V_m) of oocytes (Fig. 3*C*). After an initial CO_2-induced acidification in the solution containing 1.5% CO_2–10 mM HCO_3^-, the pH_i of oocytes expressing n1(Nt)/e1 recovered as a result of the influx of HCO_3^- (or CO_3^{2-}). Na^+ removal in the continued presence of CO_2–HCO_3^- reversed the pH_i recovery, indicating that the recovery process is Na^+-dependent. Application of CO_2–HCO_3^- also caused a hyperpolarization, indicating that the chimera mediates the flux of 1 Na^+ and at least 2 HCO_3^- (or equivalent) ions. Thus, the chimera n1(Nt)/e1 is an electrogenic Na^+–HCO_3^- cotransporter.

In this series of experiments, we analysed three other chimeras. The first, n1(L)/e1, had the third extracellular loop of NBCn1-B on a background of NBCe1-A (not shown). The second, n1(Ct)/e1, had the cytoplasmic C-terminal domain of NBCn1-B on a background of NBCe1-A (not shown). The third chimera, n1(NtLCt)/e1, had the N terminus, extracellular loop and C terminus from NBCn1-B on a background of NBCe1 (Fig. 4*A*). Voltage-clamp data for all three chimeras (summarized in Table 2) were similar to those shown for the triple chimera in Fig. 4*B*. G_{HCO3} was substantially higher than G_{ND96}, G_{NBC} was much higher than G_{ND96} in H_2O-injected oocytes (Fig. 1*A*), and E_{NBC} for n1(NtLCt)/e1 was more negative. In other words, all three had the functional characteristics of an electrogenic transporter. These data show that the major non-transmembrane domains (i.e. cytoplasmic N and C termini and third extracellular loop, which are less-well conserved than the transmembrane domains) of NBCe1-A are not critical for electrogenicity. In other words, the transmembrane domains of NBCe1-A are the only components required for electrogenic function.

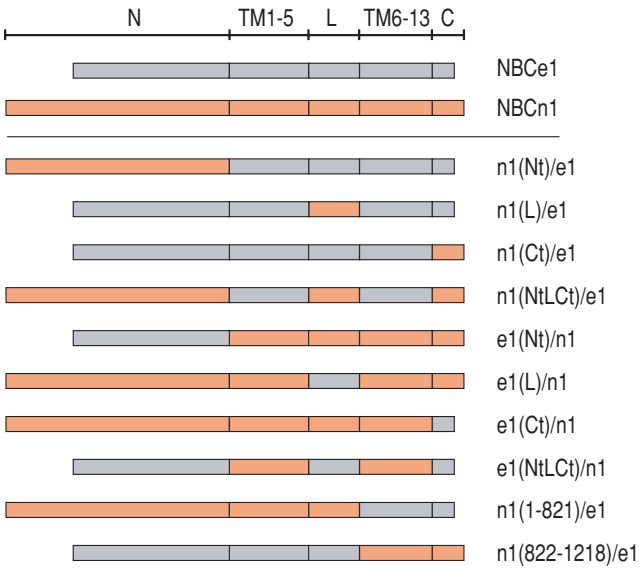

Figure 2. Cotransporters used in the present study
We generated chimeras between NBCe1-A (grey areas) and NBCn1-B (orange areas). N, N-terminal domain; TM1–5, transmembrane segments 1–5; L, third extracellular loop; TM6–13, transmembrane segments 6–13; C, C-terminal domain.

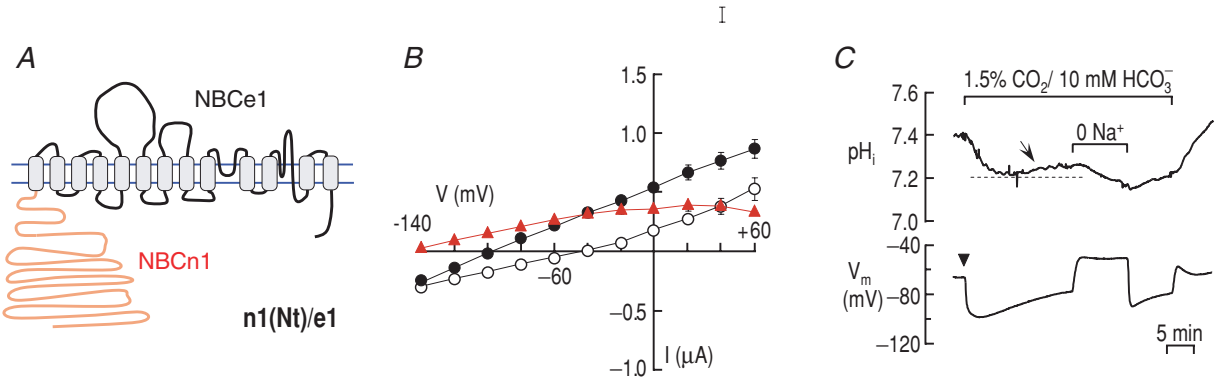

Figure 3. Replacing the N-terminal domain of NBCe1-A with the N-terminal domain of NBCn1-B
A, chimeric transporter n1(Nt)/e1. *B*, (*I–V*) relationships obtained from oocytes expressing n1(Nt)/e1 ($n = 7$) in the absence (○) and presence (●) of 5% CO_2–26 mM HCO_3^- (pH 7.4). The difference I_{HCO3}–I_{ND96} is an estimate of the electrogenic cotransport current (▲). *C*, representative recordings of pH_i and membrane potential (V_m) in an oocyte expressing n1(Nt)/e1. The pH_i recovery from a CO_2-induced acidification (arrow) and the hyperpolarization upon application of 1.5%CO_2–10 mM HCO_3^- (arrowhead) are hallmarks of electrogenic Na^+–HCO_3^- transport. One of four experiments.

Replacing the major non-transmembrane domains of NBCn1-B with the homologous domains of NBCe1-A does not induce electrogenic function

To further confirm the above findings, we constructed the four opposite chimeras in which the major non-transmembrane domains of NBCe1-A replaced the homologous domains of NBCn1-B. The first, e1(Nt)/n1, had the cytoplasmic N terminus of NBCe1-A on a background of NBCn1-B (Fig. 5*A*). The second and third, e1(L)/n1 and e1(Ct)/n1, respectively, had the third extracellular loop or cytoplasmic C terminus of NBCe1-A on a background of NBCn1-B (not shown). Finally, e1(NtLCt)/n1 had the N and C termini as well as the third extracellular loop of NBCe1-A on a background of NBCn1-B (Fig. 6*A*). Voltage-clamp data for all four chimeras (summarized in Table 2) were similar to data shown in Figs 5*B* and 6*B*. I_{ND96} (at positive voltages) and G_{ND96} values were higher than for H_2O-injected oocytes. E_{ND96} values were around −30 mV. The chimeras share these characteristics with wild-type NBCn1-B, which has a background conductance. I_{HCO3} (at positive voltages) and G_{HCO3} for the chimeras were somewhat lower than corresponding values in the presence of ND96; this is also a characteristic of wild-type NBCn1-B (Fig. 1*C*). The *I–V* relationship of the triple chimera e1(NtLCt)n1 exhibited slight outward rectification (Fig. 6*B*). Clearly, none of these four chimeras exhibited the currents characteristic of electrogenic cotransport. Conversely, the presence of the background conductance in all four chimeras is consistent with previous conclusions that the ionic conductance is likely to be a property of the transmembrane domains of NBCn1-B.

In the experiments on pH_i recovery, the initial CO_2-induced acidification was followed by a robust pH_i recovery in oocytes expressing e1(Nt)/n1 (Fig. 5*C*). Na^+ removal in the continued presence of HCO_3^- reversed the pH_i recovery. Note that the pH_i recovery was not accompanied by the hyperpolarization characteristic of electrogenic cotransport. Moreover, Na^+ removal caused a substantial hyperpolarization, reflecting the background Na^+ conductance of wild-type NBCn1-B.

Taken together, the data in this section demonstrate that the major non-transmembrane domains (i.e. cytoplasmic N and C termini and third extracellular loop) of NBCe1-A do not confer electrogenicity on chimeras in which the transmembrane domains come from NBCn1-B. Conversely, the transmembrane domains of NBCn1-B

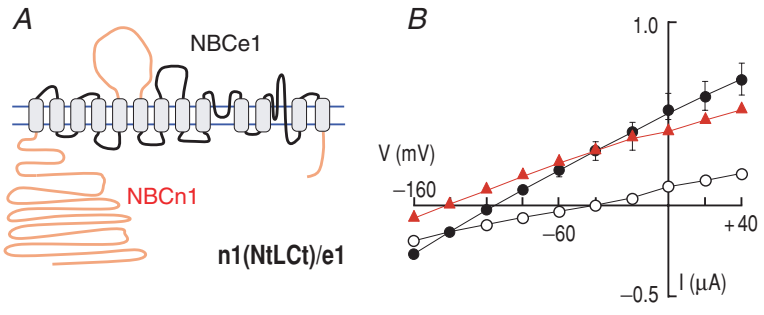

Figure 4. Replacing the major non-transmembrane domains of NBCe1-A with the corresponding domains of NBCn1-B
A, chimeric transporter n1(NtLCt)/e1. *B*, (*I–V*) relationships obtained from oocytes expressing n1(NtLCt)/e1 in the absence (○) and presence (●) of HCO_3^-–CO_2 ($n = 5$).

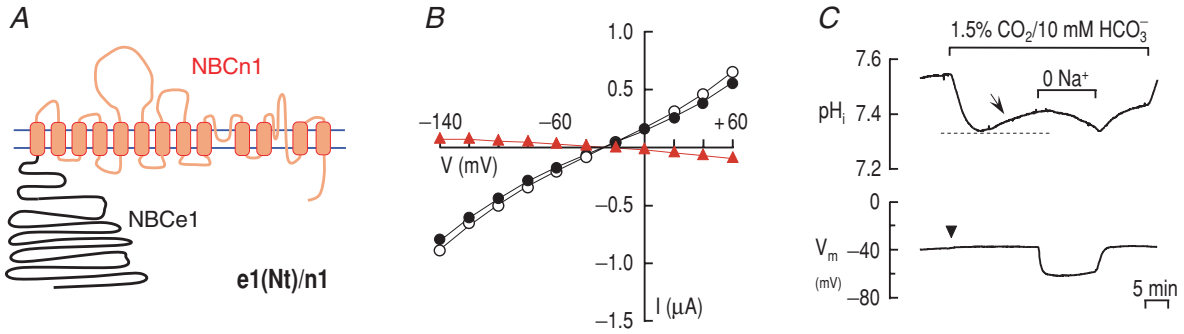

Figure 5. Replacing the N-terminal domain of NBCn1-B with the N-terminal domain of NBCe1-A
A, chimeric transporter e1(Nt)/n1. *B*, (*I–V*) relationships obtained from oocytes expressing e1(Nt)/n1 in the absence (○) and presence (●) of HCO_3^-–CO_2 ($n = 7$). *C*, representative recordings of pH_i and V_m in an oocyte expressing e1(Nt)/n1. The pH_i recovery from a CO_2-induced acidification (arrow) without affecting membrane potentials (arrowhead) are hallmarks of electroneutral Na^+–HCO_3^- transport. A substantial hyperpolarization caused by Na^+ removal is due to an associated Na^+ conductance. One of four experiments.

are the only components required for the background conductance.

Replacing either the front or back half of NBCe1-A with the homologous region of NBCn1-B abolishes electrogenic Transport

Front half n1. Our data described above show that the major non-transmembrane domains of NBCe1-A, despite their unique amino acid composition, are not necessary for, nor do they by themselves confer, electrogenicity of Na^+–HCO_3^- cotransport. Thus, the better-conserved transmembrane domains must determine whether cotransport is electrogenic. Based on the work of others on AE1 (for review see Alper *et al.* 2001), it is reasonable to divide the transmembrane domains of NBCe1-A into two regions: TM1–5 and TM6–13. Separating the two regions is the third extracellular loop that, as demonstrated by the above data, has no significant role in electrogenicity of NBCe1-A or the background conductance of NBCn1-B. To examine the functional role of these two transmembrane regions, we made two final chimeras. The first, n1(1–821)/e1, had the composition of NBCn1-B from the N terminus to the end of the third extracellular loop ('front half'), and the composition of NBCe1-A from TM6 to the C terminus ('back half', Fig. 7*A*). Voltage-clamp data for oocytes expressing this chimera (summarized in Table 2) show that G_{ND96} was very low, suggesting that the chimera does not have the background conductance characteristic of NBCn1-B (Fig. 7*B*). Moreover, G_{HCO3} was slightly lower than G_{ND96} and E_{HCO3} was not shifted to negative values, suggesting that the chimera does not have the electrogenic cotransport characteristic of NBCe1-A.

In the experiments on pH_i (Fig. 7*C*), oocytes expressing n1(1–821)/e1 recovered from a CO_2-induced acid load at about half the rate ($4.8 \pm 0.5 \times 10^{-5}$ pH units s^{-1}, $n = 5$) of those expressing the n1(Nt)/e1 chimera as shown in Fig. 3*C* ($10.3 \pm 3.6 \times 10^{-5}$ pH units s^{-1}, $n = 4$). The difference between these two values is statistically insignificant ($P > 0.05$) Immunocytochemistry (see Supplemental Material) indicates that the surface expression of the n1(1–821)/e1 chimera is less than that of the wild-type NBCe1-A. The slower pH_i recovery of n1(1–821)/e1 (Fig. 7*C*) compared with that of n1(Nt)/e1 (Fig. 3*C*) could be due to lower surface expression or to lower activity of individual molecules. Because both n1(1–821)/e1 and n1(Nt)/e1 lack the Nt of NBCe1-A, the part of the molecule responsible for the relative slowness of n1(1–821)/e1 must be TM1–5 (i.e. a mismatch between the front and back halves of the chimeric cotransporter).

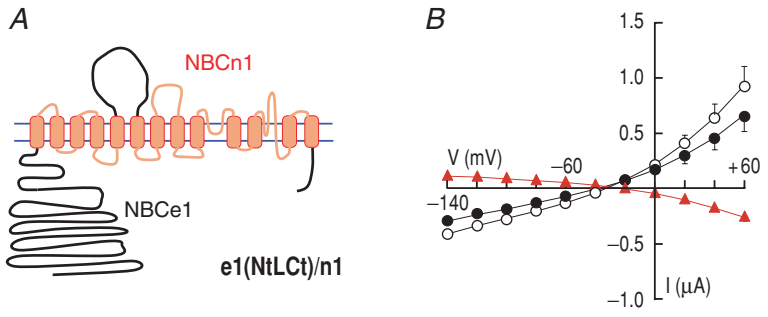

Figure 6. Replacing the major non-transmembrane domains of NBCn1-B with the corresponding domains of NBCe1-A
A, chimeric transporter e1(NtLCt)/n1. *B*, (*I–V*) relationships obtained from oocytes expressing e1(NtLCt)/n1 in the absence (○) and presence (●) of HCO_3^-–CO_2 ($n = 7$).

Nevertheless, oocytes expressing n1(1–821)/e1 clearly exhibited a more rapid pH_i recovery than water-injected controls ($-0.6 \pm 0.5 \times 10^{-5}$ pH units s^{-1}, $n = 5$), and the difference is highly significant ($P = 0.001$, unpaired two-tailed t test). Thus, n1(1–821)/e1 engages in significant acid–base transport. Because even very slow pH_i recovery rates produce large changes in V_m (Romero *et al.* 1997) (i.e. V_m is a very sensitive indicator of electrogenicity), we are in a position to judge the electrogenicity of the n1(1–821)/e1 chimera. However, application of CO_2–HCO_3^- did not cause the large, abrupt hyperpolarization characteristic of NBCe1-A or even of the n1(Nt)/e1 chimera shown in Fig. 3*C* (change in V_m, -33.3 ± 2.8 mV, $n = 4$), but instead led to a slow, small depolarization (change in V_m, $+14.7 \pm 1.7$ mV, $n = 5$) that we often observed in H_2O-injected, control oocytes. Moreover, Na^+ removal caused neither the large abrupt depolarization characteristic of NBCe1-A or even of the n1(Nt)/e1 chimera ($+38.3 \pm 10.0$ mV, $n = 4$), nor the large hyperpolarization (reflecting the background Na^+ conductance) of the n1(822–1218)/e1 chimera (-29.1 ± 4.9, $n = 3$), but only a small hyperpolarization (-6.4 ± 0.7, $n = 5$) similar to the observation in H_2O-injected, control oocytes (-2.9 ± 1.6, $n = 5$). These data show that the chimera n1(1–821)/e1, although active in terms of pH_i changes, has neither electrogenic cotransport nor a background Na^+ conductance.

Back half n1. The absence of electrogenic cotransport in n1(1–821)/e1 shows that TM1–5 of NBCe1 is essential for electrogenicity. To test whether TM6–13 is also involved, we then made the reciprocal chimera n1(822–1218)/e1, which had the composition of NBCe1-A from the N terminus to the end of the third extracellular loop ('front half'), and the composition of NBCn1-B from TM6 to the C terminus ('back half', Fig. 8*A*). Voltage-clamp data for oocytes expressing n1(822–1218)/e1 (summarized in Table 2) show that G_{ND96} was relatively high, suggesting that the chimera has the background conductance characteristic of NBCn1-B (Fig. 8*B*). On the other hand, G_{HCO3} was slightly lower than G_{ND96} and E_{HCO3} was not shifted towards more negative values, suggesting that this chimera lacks the electrogenic cotransport characteristic of NBCe1-A.

The chimera mediated a robust pH_i recovery from a CO_2-induced acid load, but without the abrupt hyperpolarization characteristic of NBCe1-A (Fig. 8*C*). The removal of Na^+ eliminated the pH_i recovery and evoked a large hyperpolarization. These observations confirm that the chimera is an electroneutral Na^+–HCO_3^- cotransporter, complete with the background Na^+ conductance.

In conclusion, our chimera studies indicate that the electrogenicity of NBCe1-A simultaneously requires both TM1–5 and TM6–13. On the other hand, the background Na^+ conductance of NBCn1-B requires only TM6–13.

Discussion

Overview

In this study, we provide the first molecular details of the structural requirements for electrogenic transport of Na^+–HCO_3^- cotransporters. By exchanging elements between NBCe1 and NBCn1, we determined that the relatively well-conserved transmembrane domains of NBCe1 are necessary and sufficient for electrogenic cotransport. That is, electrogenicity requires the simultaneous presence of TM1–5 and TM6–13 of NBCe1-A. However, electrogenicity is independent of the three, large, less-well conserved hydrophilic domains, namely, the cytoplasmic N terminus, the third extracellular loop and the cytoplasmic C terminus. We also found that the background Na^+ conductance of NBCn1-B requires

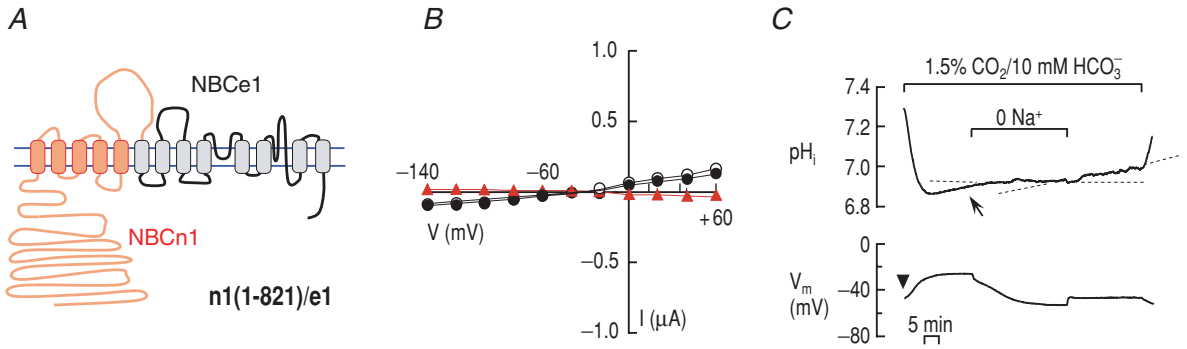

Figure 7. Replacing the front half (N-terminus, TM1–5 and loop) of NBCe1-A with the homologous parts of NBCn1-B
A, chimeric transporter n1(1–821)/e1. *B*, (*I–V*) relationships obtained from oocytes expressing n1(1–821)/e1 in the absence (○) and presence (●) of HCO_3^-–CO_2 ($n = 5$). *C*, representative recordings of pH_i and V_m in an oocyte expressing n1(1–821)/e1. The number in parenthesis (1–821) represents amino acids of NBCn1-B. The pH_i recovery from an acidification (arrow) without hyperpolarization (arrowhead) is shown. One of three experiments.

TM6–13 but not TM1–5 or any of the three large hydrophilic elements. These conclusions could be a valuable staring point for future studies aimed at determining the domains responsible for electrogenic *versus* electroneutral function as well as the background Na^+ conductance of NBCn1-B.

Structural domains and motifs

Electrogenic transport. Among the chimeras we constructed, those containing, and only those containing, both TM1–5 and TM6–13 of NBCe1 were electrogenic. Breaking the interaction between TM1–5 and TM6–13 of NBCe1-A (i.e. pairing an electroneutral TM1–5 with an electrogenic TM6–13, or vice versa) abolishes electrogenicity and yet maintains cotransport. In other words, both TM1–5 and TM6–13 of NBCe1-A have the machinery necessary for performing electroneutral transport, which is perhaps the basal state of NBCe1.

Electrogenic cotransport requires an interaction between at least one motif in TM1–5 and at least one motif in TM6–13. The identity of these motifs remains unclear. Up to now, only a handful of studies have focused on structure–function relationships of NBCe1. Abuladze *et al.* (2005) recently mutated a large number of acidic and basic amino acids in NBCe1 – in putative transmembrane segments and intra- or extracellular loops – to oppositely charged and/or neutral amino acids. Examining the effects on the Na^+-dependent recovery of pH_i from an acid load in HEK-293T cells, they found that many mutations caused substantial decreases in acid-extrusion rate, some resulting in severe functional defects. Whereas the report by Abuladze *et al.* (2005) provides useful information on residues that are important for transport activity, it was not intended to address the electrogenicity of Na^+–HCO_3^- transport and, indeed, the assays did not involve electrophysiological measurements. Moreover, the authors focused on amino acid residues that are conserved among members of the *SLC4A* family including Cl^-–HCO_3^- exchangers and electroneutral Na^+-coupled HCO_3^- transporters. Thus, it is unlikely that these conserved residues are critical for determining the electrogenicity of NBCe1.

Inferences from the AE subfamily. It is possible that some insight into the structural elements of Na^+–HCO_3^- transporters may be inferred from the AE-type Cl^-–HCO_3^- exchangers, which have been more thoroughly studied than the NBCs. Traditionally, AE was divided into two structurally and functionally distinct domains: the cytoplasmic N-terminal domain (43 kDa) that binds a variety of other proteins (Low, 1986) and the transmembrane domain (55 kDa) that mediates anion transport (Passow, 1986). Studies of AE1/AE2 chimeras reveal that the transmembrane domain determines the fundamental transport characteristics (i.e, Cl^-–HCO_3^- exchange and chloride–nitrate exchange), whereas the cytoplasmic N-terminal domain modulates the transport (i.e. pH dependence) (Alper *et al.* 2001). The membrane domain works as a 'sensor', whereas the cytoplasmic N-terminus is a 'modifier'. The study of AE2/AE3 chimeras supports this functional compartmentalization (Fujinaga *et al.* 2003). Muller-Berger *et al.* (1995*b*) have proposed that TM5, 8, 9, 10 and 13 of AE1 are functional segments for anion translocation. Several amino acids in these TMs have been proposed to serve as a proton coupling site (Jennings & Smith, 1992; Sekler *et al.* 1995), an anion selectivity filter (Zhu & Casey, 2004) or a pore-entry site (Muller-Berger *et al.* 1995*a*; Tang *et al.* 1998, 1999; Kuma *et al.* 2002; Jennings, 2005). These reports emphasized the structural importance of multiple amino acids in several TMs. It is possible that homologous amino acids in NBCs may also play similar roles in ion translocation (McAlear & Bevensee, 2006).

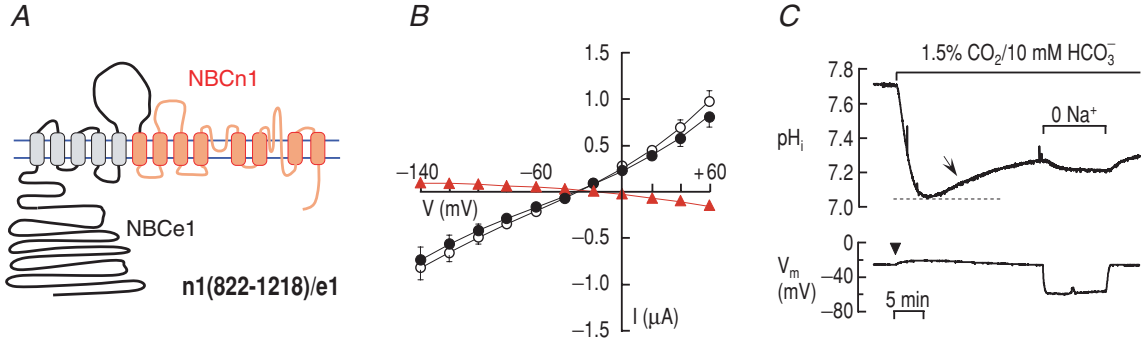

Figure 8. Replacing the second half (TM6-13 and C-terminus) of NBCe1-A with the homologous parts of NBCn1-B
A, chimeric transporter n1(822–1218)/e1. *B*, *I–V* relationships obtained from oocytes expressing n1(822–1218)/e1 in the absence (○) and presence (●) of HCO_3^-–CO_2 ($n = 7$). *C*, representative recordings of pH_i and V_m. The pH_i recovery from an acidification (arrow) without hyperpolarization (arrowhead) is shown. One of three experiments.

Natural NBCe1 mutations. At least eight naturally occurring mutations in human NBCe1 have been reported (Igarashi *et al.* 1999, 2002; Inatomi *et al.* 2004; Horita *et al.* 2005): Q29, R298S, S427L, R510H, T485S, A799V, R881C and a frameshift at codon 721. Q29 is a non-sense mutation, whereas the others, with the exception of the frameshift at 721, are missense. All of these mutations have no more than 50% of the wild-type activity, causing a severe proximal renal tubular acidosis with the arterial blood pH of below 7.10. Additional symptoms may include ocular abnormalities such as glaucoma, cataracts, band keratopathy and blindness. Many of these point mutations cause targeting defects of the transporters, whereas others appear to have normal function (Inatomi *et al.* 2004; Horita *et al.* 2005; Toye *et al.* 2006). Dinour *et al.* (2004) has recently reported that S427L mutation in TM1 alters voltage- and Na^+-dependent HCO_3^- movement, resulting in approximately 10% of HCO_3^- currents compared to wild-type renal NBCe1-A. The change in voltage- and Na^+-dependence in the S427L mutant may be due to the absence of the 'voltage-sensing' process of the transporter. Li *et al.* (2005) reported that S427L has an apical targeting process in polarized kidney cells. It is interesting to note that all electroneutral Na^+–HCO_3^- transporters have an alanine residue at the site corresponding to S427 in NBCe1-A.

The Na^+ conductance of NBCn1. Among the chimeras we constructed, only those containing TM6–13 of NBCn1 exhibited the Na^+ conductance typical of NBCn1 (Choi *et al.* 2000; Cooper *et al.* 2005). Thus, only TM6–13 is required for the Na^+ conductance. However, the identity of the critical motifs remains unclear.

Another electroneutral member of the *SLC4* family, AE1, can under some circumstances exhibit an ion conductance. AE1 may be responsible for a small, DIDS-sensitive anion conductance observed in human erythrocytes (Knauf *et al.* 1977; Kaplan *et al.* 1983). Although normally electroneutral in its natural environment, trout AE1 (tAE1) responds to cell swelling by exhibiting an anion conductance (for review see Motais *et al.* 1997) that contributes to regulated volume decrease. When this same tAE1 is expressed in *Xenopus* oocytes, it exhibits a Cl^- conductance even in the absence of cell swelling (Fievet *et al.* 1995). Studies with trout–mouse AE1 chimeras indicate that this anion conductance of tAE1 requires residues in the extracellular loop between TM5 and TM6, as well as residues in the remaining C-terminal portion of the molecule (i.e. the 'back half' of tAE1) (Fievet *et al.* 1995; Borgese *et al.* 2004). It is interesting to note that, when expressed in *Xenopus* oocytes, the human AE1 construct AE1Δ(6:7), which lacks transmembrane segments 6 and 7, also mediates a large anion conductance. In addition, oocytes expressing AE1Δ(6:7) exhibit a Na^+ conductance (Parker *et al.* 2006). As suggested by Parker *et al.* (2006), the TM6 and 7 module in human AE1 may be necessary to ensure that transport is electroneutral.

Thus, as is the case for AE1, data on NBCn1 point to the 'back half' of the molecule as being critical for ion conductance.

Preservation of cotransport activity

Our study was faciitated by the strong homology between NBCe1 and NBCn1, which permitted us to construct functional chimeras. Improper folding often results in failure of membrane proteins to target to the plasma membrane and instead to accumulate in the endoplasmic reticulum or Golgi. The C-terminal domains of NBCe1 and NBCn1 play an essential role in targeting and sorting of the protein. Using a series of deletion mutants with the C-terminal end of NBCe1-A, Li *et al.* (2004) have identified the sequence QQPFLS (amino acid residues 1010–1015) which contributes to the targeting of NBCe1-A to the basolateral membrane of the kidney epithelial cells. Nonetheless, the deletion of this motif does not abolish the targeting of the transporter to the plasma membrane in *Xenopus* oocytes. It is thus likely that QQPFLS would play a role in membrane polarity of the transporter. Our sequence analysis shows no significant amino acid homology in this motif among the *SLC4A* family. For NBCn1-B, the PDZ motif at the C-terminal domain is more important for targeting to the membrane (Loiselle *et al.* 2003). Deletion of the PDZ motif of the human homologue inhibited the transporter from being targeted to the plasma membrane in HEK cells and instead caused an intracellular accumulation of the transporter (Loiselle *et al.* 2003; Li *et al.* 2004). The information on the targeting role of the N-terminal domain is very rare. Despite this, our success in producing functionally active chimeras may be due at least in part to the presence of the native cytoplasmic N- and C-terminal domains of NBCe1 and NBCn1 in our chimeras.

Limitations

Strictly speaking, our results pertain only to NBCe1-A and NBCn1-B expressed in *Xenopus* oocytes, where NBCe1-A has a stoichiometry for Na^+:HCO_3^- of 1 : 2 (Heyer *et al.* 1999). It is impossible, from the present work, to draw conclusions about the structural requirements for the 1 : 3 stoichiometry that is believed to prevail in the renal proximal tubule.

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