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NBCe1 as a Model Carrier for Understanding the Structure-Function Properties of Na⁺-Coupled SLC4 Transporters in Health and Disease

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Abstract

SLC4 transporters are membrane proteins that in general mediate the coupled transport of bicarbonate (carbonate) and share amino acid sequence homology. These proteins differ as to whether they also transport Na⁺ and/or Cl⁻, in addition to their charge transport stoichiometry, membrane targeting, substrate affinities, developmental expression, regulatory motifs, and protein-protein interactions. These differences account in part for the fact that functionally, SLC4 transporters have various physiological roles in mammals including transepithelial bicarbonate transport, intracellular pH regulation, transport of Na⁺ and/or Cl⁻, and possibly water. Bicarbonate transport is not unique to the SLC4 family since the structurally unrelated SLC26 family has at least three proteins that mediate Cl⁻-HCO₃⁻ exchange. The present review focuses on the first of the sodium-dependent SLC4 transporters that was identified whose structure has been most extensively studied: the electrogenic Na⁺-base cotransporter NBCe1. Mutations in NBCe1 cause proximal renal tubular acidosis (pRTA) with neurologic and ophthalmologic extrarenal manifestations. Recent studies have characterized important structure-function properties of the transporter and how they are perturbed as a result of mutations that cause pRTA. It has become increasingly apparent that the structure of NBCe1 differs in several key features from the SLC4 Cl⁻-HCO₃⁻ exchanger AE1 whose structural properties have been well-studied. In this review, the structure-function properties and regulation of NBCe1 will be highlighted and its role in health and disease will be reviewed in detail.

Keywords

SLC4A4; carbonate; bicarbonate; renal tubular acidosis; transport; acid-base; pH

Overview of the SLC4 Family

The majority of SLC4 genes encode base transporting proteins that differ in their Na⁺, -Cl⁻, and electrogenic properties Fig.1A-D, [61, 91]. 3 homologous transporters, AE1, AE2, and AE3 (*SLC4A1*, -2, -3 respectively) function as exchangers mediating the electroneutral exchange of Cl⁻ and bicarbonate. A fourth protein encoded by the *SLC4A9* gene (originally named AE4) was also initially reported to mediate anion exchange however its amino acid

sequence more closely resembles Na⁺-coupled SLC4 transporters and its function is controversial. NDCBE (encoded by *SLC4A8*) is an ion exchanger like AE1, AE2, and AE3 yet differs in that it transports Na⁺ and base in exchange for Cl⁻ electroneutrally. Two transporters, NBCn1 (*SLC4A7* gene) and NBCn2 (*SLC4A10* gene) are electroneutral Na⁺-base cotransporters. NBCe1 (*SLC4A4* gene) and NBCe2 (*SLC4A5* gene) are the two members of the family that mediate electrogenic Na⁺-base transport. *SLC4A11* does not appear to transport HCO₃⁻ or CO₃²⁻ however its function remains controversial having been assigned various transport modes including electrogenic Na⁺-BO₄⁻ cotransport, ion channel activity (Na⁺, OH⁻/H⁺), Na⁺-coupled OH⁻/H⁺ transport, and water channel flux [87, 90, 123]. Of the Na⁺-coupled SLC4 transporters, NBCe1 is the best understood structurally and is the focus of this review. A dendrogram of the SLC4 transporters is shown in Fig.2.

NBCe1 variants and tissue expression

There are 5 variants of NBCe1 (NBCe1-A-E) encoded by the *SLC4A4* gene [3] (human chromosome 4q21) that have been reported in mammals, which differ in their extreme N- and C-termini but have an identical transmembrane region (Fig.3; [61, 91]). Three of the variants have been functionally characterized, all of which mediate electrogenic Na⁺-base cotransport, but differ in their tissue expression, intrinsic activity, and modulation by regulatory factors.

NBCe1-A is highly expressed on the basolateral membrane of kidney in S1 and S2 proximal tubules where it mediates the absorption of bicarbonate [2, 78, 104, 111]. In addition to the proximal tubule, NBCe1-A is also expressed at the protein level in the eye [14, 121] and salivary gland [18]. In turbinate mucosa and nasal polyps, NBCe1-A mRNA is detectable in nasal submucosal glands [64]. A second more widely expressed NBCe1 variant, NBCe1-B, is identical to NBCe1-A except for its extreme N-terminus wherein 85 aa replaces the 41 aa in NBCe1-A (Fig.3). NBCe1-B was originally cloned from pancreas and contributes to transepithelial pancreatic secretion [2, 39, 53, 54, 77]. NBCe1-B has been localized at the transcript and/or protein level (that should also have detected NBCe1-C/-E in some instances) in various tissues including brain, eye, heart, intestine, gall bladder, lung, skeletal muscle, submucosal glands, and nasal mucosa [1, 2, 14, 25, 29, 35, 58, 59, 64, 76, 83, 94, 121, 141]. A third variant originally cloned from rat brain [76, 80], NBCe1-C, has a unique C-terminus with a type I PDZ motif (Fig.3). NBCe1-D and NBCe1-E transcripts more recently isolated from mouse reproductive tissues have the identical sequence as NBCe1-A and NBCe1-B respectively, and differ in that they lack a nine amino-acid cassette in the cytosolic N-terminus (Fig.3; [72]). The latter transcripts have not been demonstrated to be expressed at the protein level nor has their function been reported.

Familial isolated proximal renal tubular acidosis

Hereditary proximal renal tubular acidosis (pRTA) represents a group of diseases that impair proximal tubular bicarbonate absorption [45]. The defect in bicarbonate absorption can be isolated (Table 1) or accompanied by additional proximal tubule transport defects (Table 2). Proximal tubular bicarbonate absorption is an indirect process mediated by the coupling of apical NHE3 (to a lesser extent the apical H⁺-ATPase) and basolateral NBCe1-A transport

(Fig.4A; [16, 44]. The rate of bicarbonate absorption is enhanced by membrane bound CAIV and cytoplasmic CAII that catalyze the hydration/dehydration of CO₂ in the lumen and cytoplasm respectively. Currently, mutations in NBCe1 are the only known cause of hereditary pRTA in the absence of other proximal tubule transport defects [52]. Fortuitously, the diagnosis is straightforward because of the unique spectrum of extra-renal manifestations that include short stature, ocular findings (cataracts, band keratopathy, glaucoma), neurological abnormalities (mental retardation, basal ganglia calcifications, migraine headaches), and tooth (enamel) defects that do not occur in other diseases that cause pRTA (Table 1).

Following the original report by Igarashi et al. [52] a total of eight missense mutations (R298S, S427L, T485S, G486R, R510H, L522P, A799V, and R881C), 2 nonsense mutations (Q29X, W516X), and 2 frameshift deletions (2311 delA, and a C-terminal tail 65bp-del) have been reported (Table 3) [61]. The NBCe1-A-Q29X mutation was originally thought to be variant specific [8, 51] but could theoretically involve NBCe1-D [72] assuming the latter variant is expressed in humans. Interestingly, the patient reported with the NBCe1-A Q29X mutation did not have cataracts or band keratopathy as do patients with mutations common to all variants suggesting that NBCe1-A *per se* is not involved in these ocular abnormalities. The phenotype in mice with congenital loss/truncated NBCe1 is more severe than in humans and results in decreased survival, volume depletion and colonic obstruction [36, 63]. Ocular and neurologic manifestations have not been reported in mice suggesting that these abnormalities require a longer period of time to become manifest.

The disease causing mechanism of the known nonsense/frameshift mutations is due to absence of the full-length NBCe1 protein, whereas missense mutations affect the intrinsic function of the transporter and/or impair plasma membrane processing/targeting (Table 3). Unlike the majority of the findings in patients with NBCe1 mutations, migraine headaches have been reported that are mutation specific. Specifically, headaches have been described in patients with R510H, L522P, R881C, 2311 delA, and 65bp-del mutations [117]. Heterozygous family members of a patient with a 65 base-pair C-terminal deletion and the L522P mutation have also been reported to have headaches. It has been hypothesized that headaches in homozygotes may be due to abnormal NMDA-mediated neuronal hyperactivity resulting from ER retained misfolded NBCe1-B in brain astrocytes, whereas in heterozygotes, ER retained mutant-wild type NBCe1-B hetero-oligomer formation may be involved [117, 137].

Unlike AE1 mutations where both autosomal recessive and dominant inheritance has been reported [12], there are no known cases with NBCe1 mutations where only one allele is mutated. Whether individuals heterozygous for NBCe1 mutations have subtle defects in proximal tubule bicarbonate transport and/or mild ocular, brain, growth, and pancreatic enzyme abnormalities is unknown. In addition, there are no reports of naturally occurring gain of function NBCe1 mutations in patients although residues have been identified whose substitution modestly increases the base transport function [4]. Whether various NBCe1 SNPs have functional effects has recently been addressed. Yamazaki et al. examined the NBCe1 SNPs E122G, S356Y, K558R, and N640I and reported that the base transport function of K558R was decreased 41-47% [136]. The impact of these SNPs on more subtle

changes in proximal tubule bicarbonate transport and extrarenal cellular function is unknown.

In addition to NBCe1 mutations, familial isolated pRTA with a dominant inheritance has also been described in patients with short stature, and decreased bone density (Table 2) [19, 56, 68]. Mutations in proximal tubule proteins involved in H⁺/base transport including CA II, CA IV, CA XIV, NBCe1, NHE3, NHE8, NHERF1 and NHERF2, and PAT1(CFEX) were not detected [56]. Intron and promoter region mutations in the genes encoding these proteins have not yet been excluded in these patients.

Extrarenal manifestations in patients with NBCe1 mutations and pRTA

In addressing the pathophysiology of the extrarenal manifestations in patients with NBCe1 mutations, there is currently insufficient data to attribute a specific phenotype to defective NBCe1 function at the cellular level *per se* versus the effect of chronic systemic acidemia that all patients have prior to treatment. Moreover, we currently lack an understanding of the role of each of the NBCe1 variants in several of the tissues that are affected in patients; specifically the role of the local loss of NBCe1 function in brain (NBCe1-B, NBCe1-C), eye (NBCe1-A, NBCe1-B, and teeth ameloblasts (NBCe1-B)). Finally, several of the studies that have examined the tissue protein expression of NBCe1 have used antibodies that could theoretically pick up multiple variants i.e. NBCe1-B/-C/-E, and therefore the tissue expression of these variants is an area that requires further study. H⁺/base transport models of some of the cells where NBCe1 proteins are expressed are depicted in Fig.4A-D.

Growth

The decreased growth that is seen in patients with NBCe1 mutations also occurs in other pediatric diseases where metabolic acidosis is present from birth [12, 45]. Although not well recognized, fetal blood is normally more acidic than the maternal circulation and therefore patients with NBCe1 mutations might be exposed to an even more severe metabolic acidosis prior to birth [115]. This consideration has not been previously addressed and could also contribute to growth retardation seen in other causes of metabolic acidosis in early childhood.

Brain

Abnormal brain development leading to a low IQ has not been attributed to acid-base disturbances *per se*, making it more likely that this phenotype is due to the loss of NBCe1 function in specific cell types in the brain, particularly given the known expression of NBCe1 variants in the cortex and hippocampus [76]. NBCe1 transcripts are also expressed in the striatum that might account for the finding that patients with NBCe1 mutations have calcified basal ganglia [50] as do patients with CAII mutations [17].

Eye

The eye is an additional targeted organ in patients with NBCe1 mutations. Normal lid opening during blinking creates an intermittent loss of CO₂ resulting in acute alkalization of the anterior corneal tear coat (Fig.4B). The increased corneal pH would normally be

ameliorated by modulation of endothelial cell NBCe1-B transport. Loss of NBCe1-B function is thought to cause an abnormal increase in corneal pH resulting in the precipitation of calcium phosphate (band keratopathy) in the central cornea (region exposed during lid opening). In addition to the cornea, electrogenic Na^+ -base transport has previously been detected in the toad lens [127] likely mediated by NBCe1-B [14] that is expressed in the lens epithelium and potentially accounts for the poorly understood finding that these patients have cataracts implying that lens transparency is pH-dependent. NBCe1-B is also expressed in the pigmented ciliary body [14] where Woloson et al. has identified an electrogenic Na^+ -base transport process in rabbit [128]. Exactly how the loss of NBCe1-B activity leads to glaucoma is currently unknown.

Teeth

The abnormal tooth enamel in patients [32] has been attributed to loss of local ameloblast NBCe1-B function leading to abnormal pH regulation of the extracellular matrix during enamel formation (Fig.4C) [63]. Whether the low systemic pH is also playing an important role is not known. In a recent study, mandibular E11.5 explants from NBCe1^{-/-} mice maintained in host kidney capsules of normal for 70 days, resulted in teeth with enamel and dentin with morphological and mineralization properties similar to cultured NBCe1^{+/+} mandibles suggesting that the cause of the enamel phenotype is primarily to be due to abnormalities in systemic pH [126].

Gastrointestinal Tract

In the pancreas although NBCe1-B is expressed on the ductal cell basolateral cell membrane and mediates base influx (Fig.4D) [39, 53, 54, 77, 106], the loss of NBCe1-B function does not lead to any clinically apparent ductal abnormalities unlike in cystic fibrosis where transepithelial bicarbonate secretion is thought to be impaired [89]. Loss of NBCe1-B somehow leads to an abnormal increase in systemic amylase and lipase in patients suggesting a pH dependency of the secretion and or change in permeability of acinar cells producing these enzymes. Although in rats NBCe1-B is expressed in acinar cells, in humans acinar cell NBCe1-B has not been detected [106]. Although NBCe1-B is also expressed in the intestine, the loss of the transporter does not lead to a severe GI phenotype as it does in mice [36] suggesting that compensatory mechanisms play a more important role in the physiology of the GI tract in humans.

Structure-function properties of NBCe1

Of the 5 variants the structure of NBCe1-A has been most thoroughly studied providing a topologic framework not only for the remaining NBCe1 variants that share an identical transmembrane region, but potentially other Na^+ -coupled SLC4 bicarbonate transporters. NBCe1-A is a dimer as recently demonstrated in the native kidney *in situ* using fluorescence image moment analysis and spatial intensity distribution analysis (SpIDA) [109]. Intracellular pH and/or bicarbonate may modulate the dimerization of the N-terminal region [37]. Each monomer is a ~ 140-kDa glycoprotein protein containing 1035 amino acids (Fig. 5A) with the ability to independently transport ions [55]. The topologic structure of NBCe1 is conveniently divided into 3 regions: a long N-terminal cytoplasmic region, a large

transmembrane region, and a shorter C-terminal cytoplasmic tail. Using cysteine scanning mutagenesis, Zhu et al. analyzed the topologic properties of NBCe1-A showing that it has 14 transmembrane segments (TMs); the N-terminal transmembrane region has 8 TMs homologous to AE1, whereas TMs 10-14 fold significantly differently [148, 150] (Fig. 5A,B). The N-terminal region and C-terminal tail of NBCe1 proteins are located in the cytoplasm in all NBCe1 variants and all share a large glycosylated extracellular loop between transmembrane segment (TM) 5 and 6 [26] and a smaller extracellular loop between TMs 7 and 8. The larger loop is not normally accessible to enzymatic digestion suggesting that it is compactly folded within the protein [145]. NBCe1 lacks the two re-entrant loops that were previously reported in AE1 [144]. More recently, the topologic structure of AE1 has been modified based on homology with a prokaryotic CIC protein (Fig. 5C) [15, 134]. The extracellular surface of NBCe1-A is compactly folded and the transporter has certain topologic features that resemble the vGLUT and LeuT prokaryotic Na⁺-coupled transporters [125, 135, 148, 150].

Cytoplasmic N-terminal region

A preliminary low resolution crystal of the N-terminal region of NBCe1-A has been reported [38]. Alignment with AE1 showed similar predicted helical regions and hydrophobic residues that can potentially stabilize the N-terminal NBCe1-A dimer interface. The N-terminal cytoplasmic region contains two known NBCe1 mutations: The Q29X nonsense mutation is specific for NBCe1-A (and would be predicted to also truncate NBCe1-D), and the R298S mutation that is common to all NBCe1 variants (Table 3; Fig. 5A). The Q29X mutation was the first example of an NBCe1 mutation where potential mutant-specific therapy was demonstrated [8]. In this mutation, a wt-CAG sequence encoding glutamine replaces a UAG stop codon causing premature truncation [51]. Azimov et al. found that in HEK 293-H cells expressing the NBCe1-A-Q29X mutant, the aminoglycoside G418 could induce ribosomal read-through producing full-length functional NBCe1-A protein [8]. These studies also offered the opportunity to treat the eye abnormalities in patients using topical aminoglycosides thereby avoiding their systemic toxicity. Less toxic more potent aminoglycosides with a flexible N-1-AHB group ((S)-2-hydroxy-4-aminobutyl group at the N-1 position) [86] in addition to non-aminoglycoside compounds such Ataluren [93] that induce ribosomal read-through may also prove efficacious.

The N-terminal domain R298S pRTA mutation common to all NBCe1 variants appears to reside in a tightly folded region that may form a “HCO₃⁻ tunnel” in the wild-type transporter but whose structure is disrupted in the mutant [22, 49, 52, 117, 148]. Zhu et al. have hypothesized that the N-terminal and transmembrane regions normally interact and that in the mutant, the efficient delivery of base to the ion permeation pathway is perturbed [146]. Other groups have shown that the NBCe1-A-R298S and corresponding NBCe1-B-R342S mutant when expressed in MDCK cells have a trafficking defect. Specifically, rather than the typical polarized expression on the basolateral membrane, the arginine to serine mutation mistargets the transporter to both the apical and basolateral membranes [70, 116] suggesting that a targeting signal in the N-terminal domain is perturbed in the mutant.

Transmembrane Region (TM1-14)

TM1 contains 31 amino acids (longer than a standard TM) with an N-terminal cytosolic portion that has a helical conformation connecting the transmembrane portion with the cytoplasmic portion [146]. Structure-function studies of the wild-type transporter have demonstrated that the extracellular C-terminal end of TM1 forms part of the ion permeation pathway where Thr⁴⁴² (which is located within a ⁴²¹ITFGGLLG⁴²⁸ motif (NBCe1-A numbering) found in most SLC4 transporters) appears to be part of an extracellular gate involved in ion entry [147]. TM1 contains several key residues including Ala⁴²⁸, Ala⁴³⁵ in addition to Thr⁴⁴² that are functionally important and appear to line the ion permeation pathway [147]. Misfolding of the transporter due to substituting Asp⁴¹⁶, Gln⁴²⁴, Tyr⁴³³, and Asn⁴³⁹ with cysteine causes intracellular retention.

pRTA is caused by a TM1 S427L pRTA mutation which decreases transporter function by 90% and at very negative membrane potentials also blocks the reversal of the direction of the transport current [32]. Ser⁴²⁷ has been recently localized in TM1 to space-confined region wherein the serine side chain hydrophobicity is involved in helix packing ionic / interactions between helices [146]. The S427L functional impairment is caused by conformational change in TM1 associated with a collapse/alterd configuration of the ion permeation pathway. An additional mechanism has been reported when the S427L mutant is expressed in MDCK cells, where the mutant transporter is mistargeted to the apical membrane [70].

The T485S and R486R mutations are adjacent substitutions that independently cause pRTA [49, 116, 117, 148, 149]. Unlike all other known NBCe1 mutations, the T485S mutation would not be predicted to cause pRTA because of the structural and chemical similarity between serine and threonine. Zhu et al. hypothesized that Thr⁴⁸⁵ must therefore reside in a critical region within the transporter either in the aqueous ion permeation pathway or an ion interaction site [149]. Thr⁴⁸⁵ was subsequently shown to be localized to an aqueous confined region accessible to NEM labeling, and functional sensitivity to MTS reagents was substrate-dependant (due to intra- and extracellular facing conformational changes). These results provided the first evidence that Thr⁴⁸⁵ is located in an ion interaction site. In addition, the G486R mutation was shown to alter the orientation of Thr⁴⁸⁵ impairing its labeling with NEM [149].

The T485S mutation decreases the overall base transport function of NBCe1-A by ~ 50%. [49, 116, 117, 148, 149]. The results of whole cell patch clamp experiments indicated that the wild-type transporter functioned as an electrogenic Na⁺-CO₃²⁻ cotransporter with one anion interaction site [149]. The T485S mutation converted NBCe1-A from an electrogenic to an electroneutral transporter suggesting that the mutant transporter mediates Na⁺-HCO₃⁻ cotransport [149]. Moreover using NO₃⁻ as a surrogate CO₃²⁻ transport, unlike wt-NBCe1-A, the mutant transporter failed to mediate Na⁺-driven NO₃⁻ [149] suggesting that it favors bicarbonate as a substrate.

The R510H, L522P, and W516X pRTA mutations are located in TM4 that appears to function as a scaffolding helix essential for normal protein folding, and also has potential stop transfer and signal anchor sequences [31, 49, 52, 70, 116, 117, 137, 148]. The W516X

nonsense mutation results in a truncated and likely misfolded protein [73]. The R510H and L522P mutations induce misfolding of the mutant proteins and retention in the ER [31, 49, 52, 70, 116, 117, 137, 148]. Why R510H is retained in the ER is currently unclear however it has been hypothesized that the size of the side-chain and/or magnitude of the positive charge is required for ionic interaction between TM4 and neighboring TMs. Given that L522I and L522C are processed to the plasma membrane normally, [137, 148] the L522P substitution likely causes increased TM flexibility resulting in helix disruption, protein misfolding, and ER retention.

TM5 plays an important role in anion interaction and selectivity [139]. Within TM5, Asp⁵⁵⁵ appears to prevent various anions from being transported by NBCe1-A nonspecifically. In addition to functioning as an electrogenic Na⁺-base transporter, when glutamate is substituted at Asp⁵⁵⁵, NBCe1 also mediates the transport of Cl⁻, NO₃⁻, and SCN⁻. Residues in TM5 also interact with the well-known stilbene inhibitor 4, 4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS). DIDS binds from the extracellular surface of the transporter to a KKMIK motif in TM5 [74]. Whether DIDS sterically blocks ionic interaction/permeation through the transporter is currently unknown. DIDS may also prevent substrate induced conformational changes required to mediate ion transport. The apparent affinity between DIDS and NBCe1 is increased at more positive membrane voltages; a phenomenon attributed to voltage dependent changes in transporter conformation [74, 131]. DIDS also inhibits transporter function from the intracellular surface at an undetermined binding site [46]. The mechanism and site of inhibition of other known NBCe1 inhibitors including tenidap [33], benzamil [33], and S0859 [21] has not been studied.

NBCe1 has two extracellular loops that appear to play a functional/structural role. Extracellular loop 3, EL3, located between TM5 and TM6 is the largest (56 amino acids) loop in NBCe1 and other Na⁺-driven SLC4 transporters, and contains 4 cysteine residues [145] and is glycosylated [26]. The 4 cysteines are intramolecular disulfided and form a highly ordered topologically domain [145]. As in the Cys-loop ligand-gated ion channel superfamily, it has been hypothesized that this region in EL3 may bind a ligand(s) that regulates the function of the transporter. The next extracellular loop, EL4, located between TM7 and TM8 can interact with membrane bound CAIV [5] and CAIX [88]. EL4 has also been reported to be involved in the electrogenic properties of NBCe1 [23]. Whether EL4 interacts with residues in the ion permeation pathway thereby modulating the electrogenic properties of NBCe1 remains to be determined.

McAlear et al. proposed that NBCe1-A-TM8 as had previously been shown in AE1 [118] might play an important role in ion permeation [79]. Using cysteine scanning mutagenesis of TM8, pCMBS accessibility was modulated by the presence of substrate ions and the stilbene inhibitor 4,4-dinitro-stilbene-2, 2'-disulfonate (DNDS). Leu⁷⁵⁰ was reported to play an important role in ion permeation.

Patients with generalized proximal tubule transport impairment (Fanconi's syndrome) and less often with isolated proximal renal tubular acidosis have associated hypokalemia [103]. Several factors including enhanced HCO₃⁻ delivery to the collecting duct and an increased aldosterone level due to volume depletion result in enhanced principal cell K⁺ secretion. In

2001 Deda et al. reported a patient with the phenotype of patients with NBCe1 mutations who had extra-renal K^+ loss due to diarrhea and vomiting causing severe acute hypokalemia [30]. It was subsequently shown that the patient had an A799V pRTA mutation that significantly decreased the function of mutant NBCe1-A [49, 117, 148]. Parker et al. showed that the mutant transporter had an associated HCO_3^- -independent cation leak conductance [92] and hypothesized that given the expression of NBCe1 in skeletal muscles (sarcolemma and possibly t-tubules), during severe acute hypokalemia a patient with the A799V mutation would manifest exacerbated muscle weakness.

The TM12 R881C pRTA mutation represents another example where the membrane expression in mammalian cells and *Xenopus* oocytes differs [49, 117, 120, 148]. When expressed in mammalian cells the R881C substitution induces ER retention suggesting that the transporter is misfolded and that TM12 is involved in helix packing [150]. In *Xenopus* oocytes the plasma membrane expression of the mutant transporter is decreased [49, 120]. Moreover, the reported functional abnormality of the mutant transporter appears to be entirely due to abnormal plasma membrane expression [120].

Using a TM swapping approach, Chen et al. concluded that TM12 and TM6 form a “functional unit” that plays a role in plasma membrane expression [24]. In these experiments a chimera was formed by replacing TM6 and what was referred to as “TM12” with corresponding TMs from electroneutral NBCn1, and membrane processing was significantly decreased. Given that according to the current topological model rather than TM12 alone, residues from TM12, intracellular loop 6 (IL6), and TM13 were also swapped, and the finding that mixed chimeras *per se* can potentially fold improperly resulting in ER retention [34] the interpretation of the data is likely premature. Moreover, residues in TM12 are known to play a role in helix packing [150].

Not only does TM12 but the entire C-terminal transmembrane region from TM10-14 (Ala⁸⁰⁰ and Lys⁹⁶⁷), plays an important role helix packing and protein folding [150]. The NBCe1 structure is stabilized by 18 residues clustered on the surface of that form intramolecular hydrogen bonds. The loops connecting TMs11-14 are not aqueous exposed and are tightly folded in the protein. Met⁸⁵⁸ bracketed by Pro⁸⁵⁷ and Pro⁸⁵⁸ is the residue where TM11 and 12 are abruptly bent into the lipid bilayer. TM12 and 13 are connected by a cryptic intracellular loop 6 (IL6) which is also tightly folded and likely interacts with the cytoplasmic region. TM12-Lys⁹²⁴ acts as a counter ion and contributes to helix packing. Extracellular loop 7 (EL7; Thr⁹²⁶-Ala⁹²⁹) is likely folded in the transmembrane region since it is minimally exposed to the aqueous media [150].

Cytoplasmic C-terminal tail

Asp⁹⁶⁰ marks the intracellular lipid/aqueous interface of TM14. A kink is introduced at Pro⁹⁶³ exposing the C-terminal cytoplasmic tail to the cytoplasm [150]. There is a close association between the C-terminal cytoplasmic tail of each monomer in the NBCe1 dimer grouping of two stretches of strongly charged amino acids that may form a regulatory motif [150]. Evidence suggests that the C-terminal tail plays a role in membrane processing and targeting. Specifically, a pRTA 65 bp-del frame shift mutation truncates the C-terminal tail and is retained in the ER in mammalian cells (although not in *Xenopus* oocytes) [117]. In

addition, a C-terminal tail ¹⁰¹⁰QQPFLSP¹⁰¹⁵ motif has been identified that functions as a basolateral targeting signal in MDCK cells [71].

Comparison of NBCe1 and AE1

Until the topologic structure of NBCe1 was demonstrated by cysteine scanning mutagenesis studies, it was assumed that NBCe1 and other SLC4 proteins adopted a membrane topology similar to AE1. The differences in the topologic structure of the two proteins likely reflects their functional properties in keeping with the well-characterized differences in the atomic structure of prokaryotic ion exchangers and Na⁺-coupled substrate transporters. Recent mutagenesis and cryoEM studies have shown that the AE1 fold resembles a prokaryotic CIC protein (Fig.5C) [15, 134] whereas the LeuT and vSGLT prokaryotic Na⁺-coupled substrate transporters [125, 135] and NBCe1-A share certain features. NBCe1-A and AE1 have structural differences which likely play an important role in their unique functional properties and ion preferences. In NBCe1-A the first transmembrane segment, TM1, plays an important role in forming part of the ion translocation pathway and interacts tightly with the cytoplasmic domain [147]. AE1-TM1 has not been shown to be involved in ion permeation and the AE1 N-terminal cytoplasmic region does not appear to form a substrate access tunnel [110]. The large extracellular loop (EL3) in NBCe1-A and AE1 differ in that in NBCe1-A, EL3 is intra-disulfided and resistant to enzymatic digestion suggesting it is tightly folded and may play an important role in ligand binding [145]. The C-terminal transmembrane region of NBCe1-A is also tightly folded and lacks reentrant loops that were previously reported in AE1 [144, 150]. In AE1, TM13 and 14 are involved in ion translocation whereas in NBCe1-A, these TMs do not have a similar role [143, 150].

Modulation of NBCe1 Function

Intrinsic structural features

The differential regulation of the function of NBCe1 variants is due in part to intrinsic differences in the structure of their N-terminal domains and their specific interaction with various regulatory factors. McAlear et al. first demonstrated that the unique N-terminus of NBCe1-A differs from other variants in that it has as an autostimulatory domain (ASD) that enhances the function of the transporter through a currently unknown mechanism [80]. Whether the identical N-terminus in the NBCe1-D variant functions similarly has not been studied. Zhu et al. recently reported evidence for interaction between the NBCe1-A N-terminal and transmembrane regions [146]. Whether this interaction is unique to NBCe1-A and/or plays a role in modulating its function via the ASD or a separate mechanism requires further study.

In contrast to the ASD in NBCe1-A, the N-terminus of NBCe1-B and NBCe1-C (and presumably the -E variant) functions as an autoinhibitory domain (AID) [66, 80]. The autoinhibition by AID is stabilized by recruitment of the WNK/SPAK pathway [47]. IRBIT which binds to the NBCe1-B N-terminus at residues 37-65 (a positively charged motif) stimulates the transporter by recruiting protein phosphatase 1 (PP1) that blocks the WNK/SPAK inhibition [47, 66, 138]. PIP₂ which can also activate the transporter may bind to the same motif as IRBIT [47]. PIP₂ mediated activation of NBCe1-B and -C appears to involve

a staurosporine-sensitive kinase [119]. Of interest, PIP₂ also activates NBCe1-A (and potentially NBCe1-D) however the mechanism remains undetermined [129].

Magnesium

Mg²⁺ inhibits the function of NBCe1-B [132, 133] and potentially NBCe1-C/-E. Whether Mg²⁺ and IRBIT compete for a common binding site or act through independent pathways is currently unknown. Mg²⁺ has also been shown to cause NBCe1-A rundown in *Xenopus* oocytes, through a mechanism that has been hypothesized to involve a Mg²⁺-dependent phosphatase (5'-lipid phosphatase) that dephosphorylates PIP₂ to PIP [129]. Wu et al. hypothesized that the inhibition of NBCe1 function by an elevation of intracellular Mg²⁺ may reduce cellular dysfunction during ischemia [129]. Finally, the Hsp70-like stress 70 protein chaperone STCH interacts with the residues amino acids 96–440 distal to the IRBIT interaction site in NBCe1-B inducing a significant increase in plasma membrane expression [10]. Whether STCH also increases the plasma membrane expression of NBCe1-A/-C/-D/-E is currently unknown. Bae et al. have proposed that STCH stimulation of NBCe1 plasma membrane expression could represent a regulatory pathway that prevents cellular dysfunction during acidemia [10]. The utility of STCH as a therapeutic tool to increase plasma membrane expression remains to be determined.

Phosphorylation state

The phosphorylation state of NBCe1 can affect membrane expression, charge transport stoichiometry, and ion flux through the transporter. In oocytes and proximal tubule cells, ANG II has a biphasic effect on NBCe1 mediated transport [27, 28, 48, 95, 142]. ANG II-induced inhibition of NBCe1-A via the AT_{1B} receptor is mediated by Ca²⁺-insensitive PKCε leading to decreased NBCe1-A surface expression [94, 95]. In *Xenopus* oocytes expressing NBCe1-A, Ca²⁺ also alters the charge transport stoichiometry shifting it from 1:2 to 1:3, possibly via PKC phosphorylation [85]. PKA dependent phosphorylation of NBCe1-A-Ser⁹⁸² was also reported to shift the charge transport stoichiometry from 1:3 to 1:2 [43]. Thr⁴⁹ in the unique amino-terminus of NBCe1-B modulates the cAMP-induced increase in cotransporter current without altering its stoichiometry [41]. Accordingly, cAMP has also been found to increase intestinal NBCe1-B mediated transport in part via a change in membrane expression [9, 141]. Both conventional PKCs (PKCαβγ) and a novel PKCδ also participate in constitutive and stimulated (carbachol) endocytosis of NBCe1-A and NBCe1-B [96] providing a potential mechanism for modulating NBCe1-B transport in salivary duct, ileum, and colon [11, 96, 141]. In addition to PKCs, ATP has been hypothesized to phosphorylate NBCe1-A via an unidentified protein kinase thereby increasing the cotransporter current [85].

Carbonic anhydrase

Gross et al. proposed that NBCe1 and carbonic anhydrase II (CAII) form a transport metabolon based on the results of experiments using isothermal titration calorimetry, which showed that CAII binds to NBCe1-A at a C-terminal D⁹⁸⁶NDD⁹⁸⁹ motif [40]. Pushkin et al. and Becker et al. provided additional experimental support for interaction between NBCe1 and CAII [13, 99]. CAIV and CAIX were subsequently shown to bind to the extracellular

surface of NBCe1-B at EL4 [5, 88]. Other groups however were unable to show a functional interaction with CAII [75, 97, 130]. In comparing these studies, one needs to be cognizant of the techniques/preparations used in addition to the technical differences among the various assays employed. Regardless of whether a metabolon between CA proteins and NBCe1 exists *in vivo*, there does not appear to be a clinically important interaction between the proteins in that loss of CAII function results in a more mild phenotype than loss of NBCe1 function. Specifically, patients with loss of function mutations in CAII, and mice with targeted disruption of CAII do not have severe pRTA [69, 112]. In addition, patients with CAIV mutations have retinitis pigmentosa (RP17) and pRTA has not been reported [100]. Schueler recently reexamined this question and showed that CAI, CAII and CAIII stimulate NBCe1-A transport in *Xenopus* oocytes [107] and attributed the CA stimulation of NBCe1-A function to carbonic anhydrase enzymatic activity rather than intramolecular proton shuttling.

Systemic and hormonal factors

Both NaCl and NaHCO₃ loading have been shown to reduce the expression of proximal tubule NHE3 and NBCe1-A providing a mechanism for ameliorating both volume overload and metabolic alkalosis during systemic bicarbonate loading [7]. Potassium depletion with concomitant metabolic alkalosis is associated with an increase in proximal tubule renal bicarbonate reabsorption [20, 101, 102] that is potentially due to increased expression of NBCe1-A [6].

Norepinephrine, dopamine, and PTH, and potentially changes in blood pressure have also been reported to modulate the expression of NBCe1. In rats long-term infusion of noradrenaline increased the expression of the transporter [114]. Dopamine decreased the activity of NBCe1-A in rabbit and rat proximal tubules [60]. NBCe1 function is decreased by PTH in the rat possibly via a cAMP whereas in rabbit PTH has no effect [105]. NBCe1 protein expression is increased ~2 fold in the SHR rat in comparison to control WKY rats [113] however the exact mechanism is unknown.

In a rat model of acute renal transplant rejection, NBCe1-A is upregulated [122] whereas the calcineurin inhibitor FK506 decreases NBCe1-A expression [81]. NBCe1-A expression is upregulated in lithium induced distal renal tubular acidosis (dRTA) perhaps as compensatory mechanism [57]. In contrast, ureteral obstruction which induces hyperkalemic dRTA decreases NBCe1-A expression in the proximal tubule [124]. Hypothyroidism is associated with incomplete dRTA in humans, and in a rat hypothyroid model, was associated with decreased NBCe1 abundance [82]. During concomitant NH₄Cl loading and hypothyroidism, the expression of NBCe1 increased [82].

NBCe1 charge transport stoichiometry and substrate anion transport: carbonate versus bicarbonate transport

The electrochemical driving force (μ) across NBCe1 proteins dictates the direction of NBCe1 transport. Since in all cells the chemical gradient for Na⁺ and base (bicarbonate, carbonate) is inward (extracellular to cytoplasm), the charge transport stoichiometry of

NBCe1 and the membrane potential are the two major determinants of the overall electrochemical driving force [62]. Based on studies in other species, in the human kidney it is assumed NBC1-A has a charge transport stoichiometry of 1:3 (1 Na⁺:1 HCO₃⁻:1 CO₃²⁻) such that value of μ is positive. *In vivo* measurements in the rat proximal tubule reported a charge transport stoichiometry of 1:3 [140]. In the isolated perfused rabbit proximal tubule, the value varied from 1:2-1:2.7 depending on the composition of the solutions used [84, 108]. In *Necturus* proximal tubules *in vivo*, the charge transport stoichiometry decreases from 1:3 to 1:2 during an acute increase in the PCO₂ [98]. Gross et al. reported that the charge transport stoichiometry of NBCe1 is cell-type dependent [42]. Importantly, in heterologous expression systems where the signal/noise ratio is excellent and technical artifacts are minimal, the charge transport stoichiometry of human NBCe1-A in both human HEK293 cells and *Xenopus* oocytes is 1:2 rather than 1:3 [67, 149]. The question as to whether the charge transport stoichiometry can be modulated *in vivo* in humans as has been reported *in vitro* with altered phosphorylation status [43], intracellular Ca²⁺ [85] and respiratory acid-base parameters [98] is unknown.

In secretory epithelia such as pancreas, salivary glands, and intestine, and non-secretory cells such as astrocytes a charge transport stoichiometry of 1:2 is assumed given that NBCe1-B normally mediates cellular Na⁺-coupled base influx, and indeed in cultured pancreatic cells a value of 1:2 has been measured [39]. A charge transport stoichiometry of 1:2 is compatible with either 1 Na⁺:2 HCO₃⁻ transport (2 anion interaction sites) or Na⁺-CO₃²⁻ cotransport (1 anion interaction site). Using NO₃⁻ as a surrogate for CO₃⁻ transport Zhu et al. reported that human NBCe1-A expressed in HEK cells functioning with a 1:2 charge transport stoichiometry functions as Na⁺-CO₃²⁻ cotransporter [149]. Preliminary measurements of surface pH in the *Xenopus* oocyte expression system also suggests that rat NBCe1-A functions as a Na⁺-CO₃²⁻ cotransporter [65].

Although measurements will likely never be obtained in native human proximal tubule cells (leaving open the questions as to whether cell-specific factors can modulate the stoichiometry and the exact electrochemical driving force across NBCe1-A in the basolateral membrane in the human proximal tubule), the finding that human NBCe1-A has a charge transport stoichiometry of 1:2 in expression systems with excellent signal/noise, has prompted the re-evaluation of whether a 1:2 charge transport stoichiometry might be sufficient to drive Na⁺-CO₃²⁻ efflux in the human proximal tubule *in vivo*. Using data obtained in the rat proximal tubule since the necessary human data is unavailable, it has recently been shown that of NBCe1-A would have a positive value thereby driving cytoplasmic to peritubular Na⁺-CO₃²⁻ efflux; and that the transport process would be very sensitive to changes in small changes in μ [149]. These findings support the notion that that NBCe1 is actually a Na⁺-CO₃²⁻ cotransporter whose name should be changed to NCCe1-A (sodium carbonate cotransporter electrogenic 1-A).

Loss of NBCe1-A electrogenicity: Lessons learned in the context of the T485S mutation

It is instructive in this regard, to reconsider the loss of NBCe1 electrogenicity in the context of the T485S mutation in both the proximal tubule and in extra-renal secretory organs [149].

In the proximal tubule, independent of the initial charge transport stoichiometry (which is currently unknown), given the initial inwardly directed substrate ion gradients and the concomitant loss of the effect of the basolateral membrane potential as a driving force, electroneutral $\text{Na}^+\text{-HCO}_3^-$ influx mediated by the T485S mutant will significantly impair transepithelial bicarbonate absorption. Unlike NBCe1-A, NBCe1-B/-C normally mediate cellular base influx in the cells in which they are expressed. Assuming that the threonine to serine substitution also renders these variants electroneutral with an associated total base transport decrease of ~ 50%, in the steady state, a decrease in cellular base influx would be predicted to result from both the decrease in intrinsic base transport, and in addition, the decreased extracellular bicarbonate concentration (due to renal bicarbonate loss) that is available for transport. Future in vitro and in vivo studies are required to address studies are required. Given that many of these abnormalities are predicted to change dynamically, it would be useful to have a conditional transgenic mouse model system to study the effect of the T485S mutation on both the magnitude and direction of renal and extra-renal base transport as a function of time.

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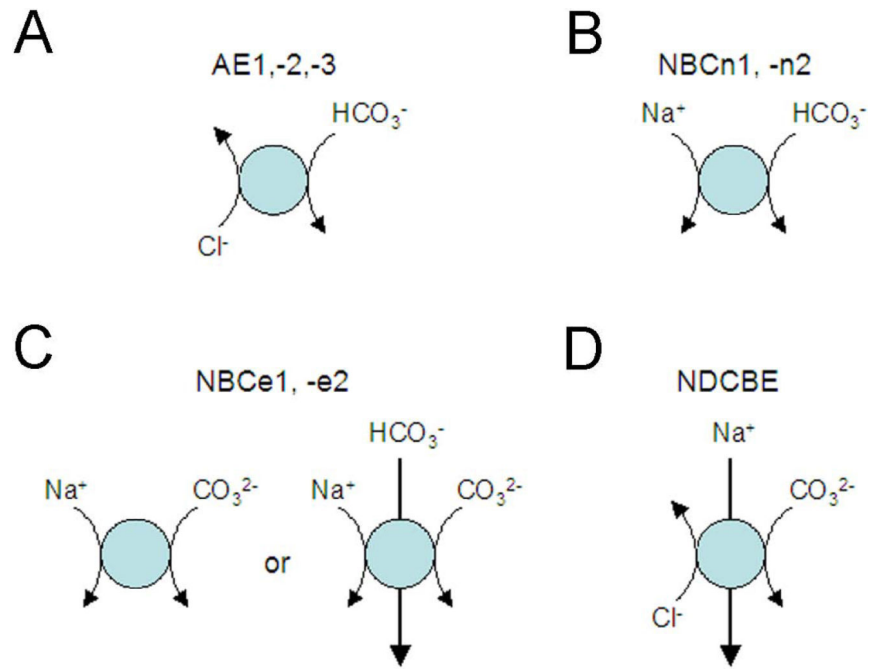


Figure 1.

SLC4 HCO₃²⁻(CO₃⁻) transporters whose function is defined: A) AE1-3: Na⁺-independent Cl⁻-HCO₃⁻ exchangers; B) NBCn1 and NBCn2: electroneutral Na⁺-HCO₃⁻ transporters. It should be noted that under certain experimental conditions, NBCn2 can also mediate Na⁺-dependent Cl⁻-HCO₃⁻ exchange; C) NBCe1 and NBCe2: electrogenic Na⁺-CO₃²⁻(HCO₃⁻) transporters with either a 1:2 or 1:3 stoichiometry; D) NDCBE: Na⁺-driven Cl⁻-CO₃⁻ exchanger. Not depicted is AE4 whose function is not clearly characterized. The *SLC4A11* gene product does not transport HCO₃²⁻(CO₃⁻) nor is its function clearly defined.

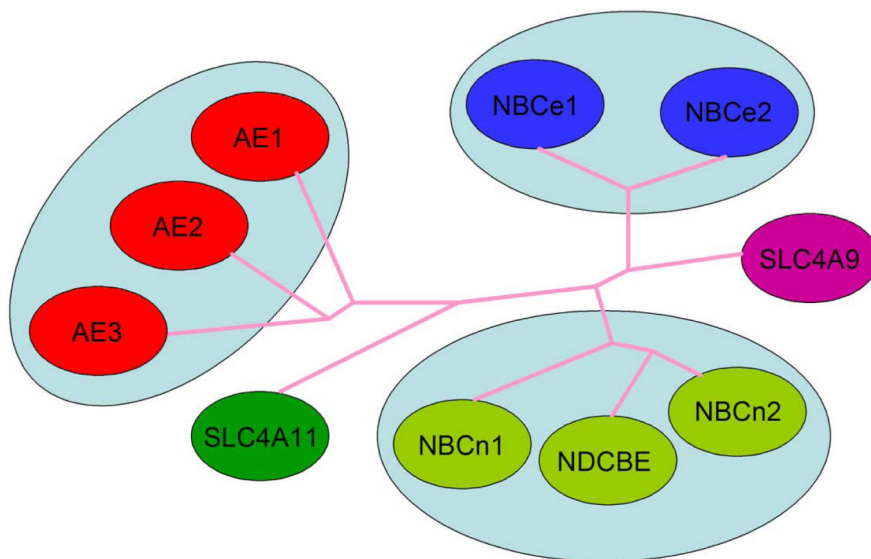


Figure 2. Dendrogram of SLC4 transporters. Depicted are the protein names of those transporters whose function is well-characterized. In general, proteins with similar function are clustered because function tends to follow structure. Since the function of the proteins encoded by the *SLC4A9* (“AE4”) and *SLC4A11* (“BTR1” and “NaBC1”) genes remains controversial, the gene name rather than the protein names (that in general refer to a specific function) are depicted.

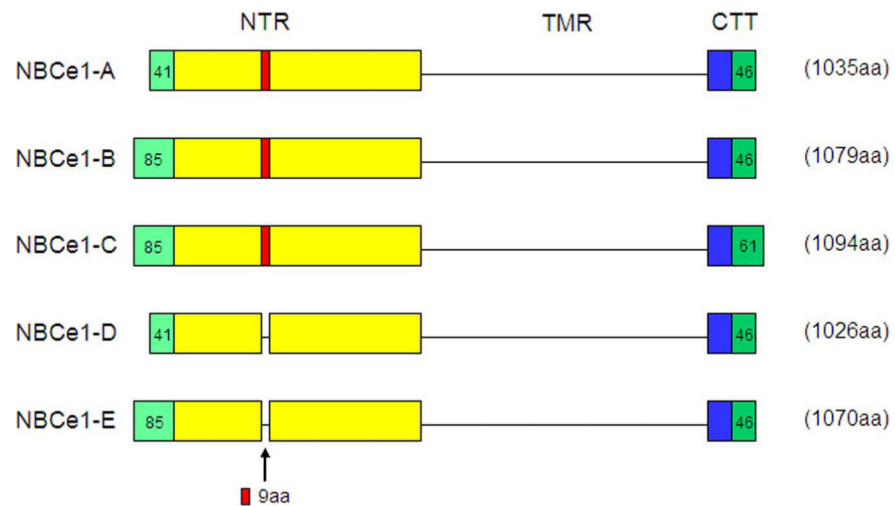


Figure 3.

NBCe1 variants: The N-terminal region (NTR), the common transmembrane region (TMR), and the C terminal tail (CTT) belonging to the 5 known mammalian NBCe1 variants are depicted diagrammatically (not to scale). All variants have an identical TMR but differ in their NTR and CTT. NBCe1-A and -D only differ in their NTR in that the -D variant lacks a stretch of 9 aa (RMFSNPDNG). The NBCe1-B and -E differ in that the latter lacks the same 9 aa cassette. NBCe1-B and -C differ in their CTT where the latter has a unique C-terminus with a type I PDZ motif. NBCe1-D/-E transcripts were detected in mouse reproductive tissues [72] and have not yet been demonstrated at the protein level.

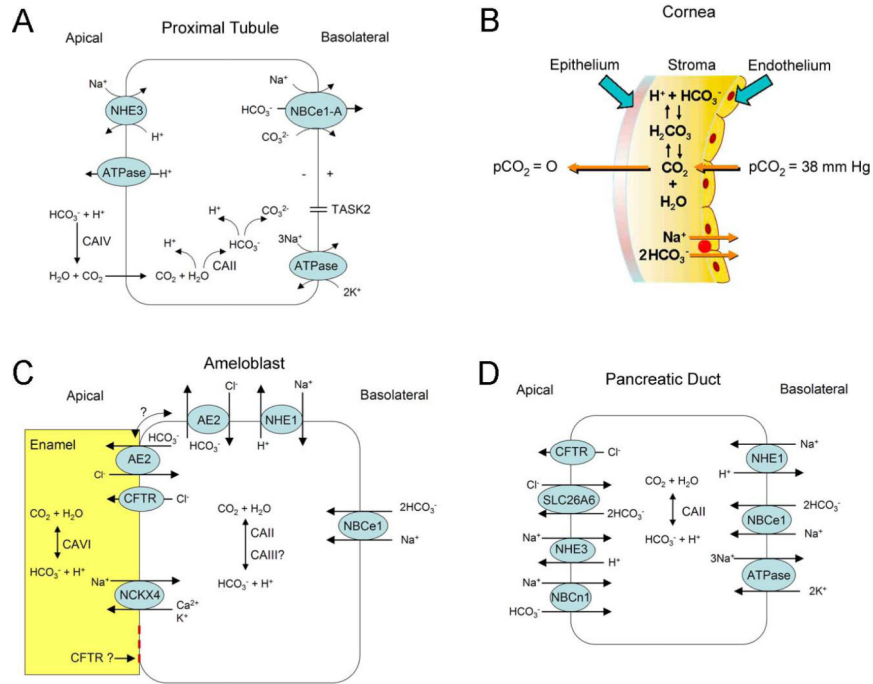


Figure 4. Transport models of various cell types expressing NBCe1-A. A) Proximal tubule cell; B) Corneal epithelial cell; C) Maturation stage ameloblast; D) Pancreatic duct cell. Note that in patients with NBCe1 a mutation, a pancreatic ductal phenotype has not been reported suggesting that pancreatic ductal cells likely have compensatory mechanisms mediating basolateral bicarbonate uptake. Patients typically have an increased blood lipase and amylase suggesting abnormal acinar involvement however unlike the rat, the human pancreatic acinus apparently does not express NBCe1-B [106].

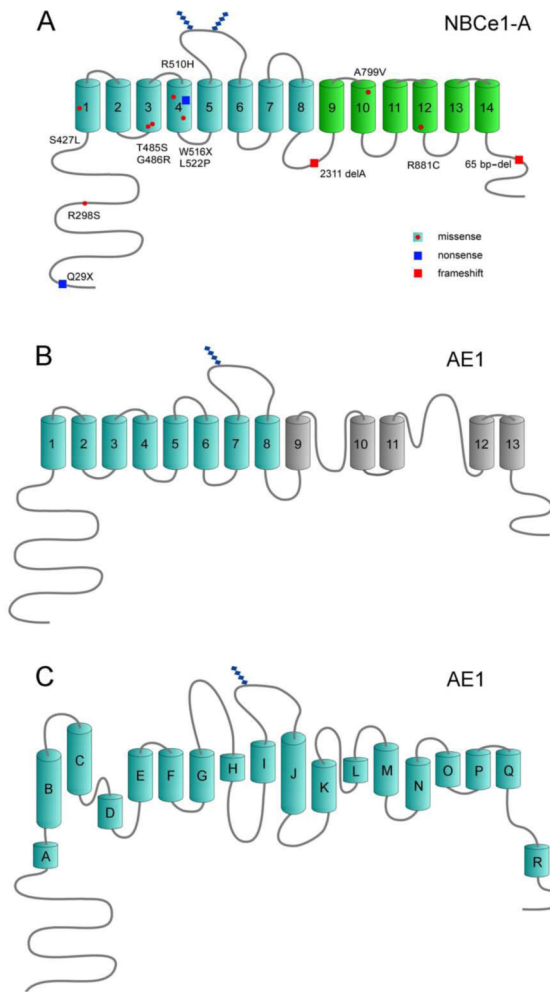


Figure 5.

A) Membrane topology of NBCe1-A [148, 150]. Known mutations throughout the transporter are depicted with the majority localized to the transmembrane region. All NBCe1 variants have a transmembrane region identical to NBCe1-A, which has been most thoroughly studied. B) Membrane topology of AE1 based on Zhu et al. [143, 150]; C) Membrane topology of AE1 based on recent homology with a prokaryotic CIC transporter [15, 134].

Table 1

Inherited Causes of Isolated pRTA with Associated Extrarenal Abnormalities

Gene	Protein	Inheritance	Renal Phenotype	Extra-Renal Phenotype
CA2	CAII	autosomal recessive	pRTA, dRTA, hypokalemia	osteopetrosis involving skull, axial skeleton, and long bones with widening of metaphyses; growth defect; intracerebral calcification
¹ SLC4A4	NBCe1	autosomal recessive	pRTA, hypokalemia	band keratopathy; glaucoma; cataracts; elevated serum amylase and lipase and enamel defects; intracerebral calcification; decreased IQ; growth defect
Unknown gene(s)	unknown	autosomal dominant	pRTA	colomboma; sub-aortic stenosis; decreased radial bone density; thinner iliac cortices; growth defect

¹Headaches occur in patients with the R510H, L522P, and R881C missense mutations, 2311delA, and a homozygous C-terminal 65 bp-del. In heterozygous patients with the 65 bp-del and the L522P mutations headaches also occur and have been attributed to a dominant-negative effect.

Table 2

Disorders Causing pRTA with Additional Proximal Tubule Transport Abnormalities

Gene	Protein	Inheritance	Disease
ALDOB	aldolase B	autosomal recessive	Hereditary fructose intolerance
ARSA	arylsulfatase A	autosomal recessive	Metachromatic leukodystrophy
ATP7B	Cu ⁺⁺ transporting ATPase β peptide	autosomal recessive	Wilson's disease
¹ CLCN5	2Cl ⁻ /H ⁺ exchanger	X-linked	Dent's disease 1
² Complex IV	cytochrome C oxidase	N/A	Cytochrome C oxidase deficiency
CTNS	cystinosis	autosomal recessive	Cystinosis
FAH	fumarylacetoacetase	autosomal recessive	Tyrosinemia type I
GALT	galactose-1-phosphate uridylyltransferase	autosomal recessive	Galactosemia
MMAB	methylmalonyl CoA mutase	autosomal recessive	Methylmalonic acidemia
OCRL1	PIP2 5-phosphatase	X-linked	Dent's disease 2
OCRL1	PIP2 5-phosphatase	X-linked	Lowe's syndrome
PC	pyruvate carboxylase	autosomal recessive	Pyruvate carboxylase deficiency
SLC2A2	GLUT2	autosomal recessive	Fanconi-Bickel syndrome

¹Dent's disease 1: 60% of patients have mutations in CLCN5 and 15% of patients have mutations in OCRL1. Mutations in OCRL1 also cause Lowe's syndrome.

²Potentially involves mutations in several mitochondrial or nuclear encoded genes.

Table 3

¹NBCe1-A Mutations Causing Proximal RTA

Mutation	Location	Classification	Effect of Mutation
Q29X	N-terminal region	nonsense	NBCe1-A protein truncation
R298S	N-terminal region	missense	- mistargeting: apical/basolateral membranes -abnormal interaction of the N- terminal region with the cytoplasmic region
S427L	TM1	missense	-mistargeting: predominant apical membrane -abnormal helix packing -decreased G_{HCO_3} -impaired I_{HCO_3} reversal at $-V_m$
T485S	TM3	missense	- altered ion interaction - electroneutral transport
G486R	TM3	missense	altered ion interaction
R510H	TM4	missense	ER retention
W516X	TM4	nonsense	truncation: all NBCe1 variants
L522P	TM4	missense	ER retention
2311 delA	IL4	frameshift	truncation: all NBCe1 variants
A799V	TM10	missense	- intracellular retention - decreased G_{HCO_3} - bicarbonate-independent G_{cation}
R881C	TM12	missense	ER retention (ER)
65 bp-del	C-terminal tail	frameshift	ER retention

G_{HCO_3} : bicarbonate conductance ; G_{cation} : cation conductance; I_{HCO_3} : bicarbonate-dependent current; $-V_m$: negative plasma membrane voltages

¹Numbered according to NBCe1-A amino acid sequence