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Review

Modular structure of sodium-coupled bicarbonate transporters

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Summary

Mammalian genomes contain 10 *SLC4* genes that, between them, encode three Cl–HCO_3 exchangers, five Na^+-coupled HCO_3 transporters (NCBTs), one reported borate transporter, and what is reported to be a fourth Cl–HCO_3 exchanger. The NCBTs are expressed throughout the body and play important roles in maintaining intracellular and whole-body pH, as well as contributing to transepithelial transport processes. The importance of NCBTs is underscored by the genetic association of dysfunctional NCBT genes with blindness, deafness, epilepsy, hypertension and metal retardation. Key to understanding the action and regulation of NCBTs is an appreciation of the diversity of NCBT gene products. The transmembrane domains of human NCBT paralogs are 50–84% identical to each other at the amino acid level, and are capable of a diverse range of actions, including electrogenic Na/HCO_3 cotransport (i.e. NBCe1 and NBCe2) and electroneutral Na/HCO_3 cotransport (i.e. NBCn1 and NBCn2), as well as Na^+-dependent Cl–HCO_3 exchange (i.e. NDCBE). Furthermore, by the use of alternative promoters and alternative-splicing events, individual *SLC4* genes have the potential to generate multiple splice variants (as many as 16 in the case of *NBCn1*), each of which could have unique temporal and spatial patterns of distribution, unitary transporter activity (i.e. flux mediated by one molecule), array of protein-binding partners, and complement of regulatory stimuli. In the first section of this review, we summarize our present knowledge of the function and distribution of mammalian NCBTs and their multiple variants. In the second section of this review we consider the molecular consequences of NCBT variation.

Key words: NBC, acid–base, splice variants.

Intracellular pH regulation

The regulation of intracellular pH (pH_i) depends on the balance between transporters that have the effect of loading the cell with acid and those that have the effect of extruding acid from the cell (reviewed by Roos and Boron, 1981). The acid loaders (Fig. 1A) include Cl–HCO_3 exchangers – members of the SLC4 and SLC26 families (reviewed by Alper, 2006; Casey and Cordat, 2009; Dorwart et al., 2008) – electrogenic Na$^+$-coupled bicarbonate transporters (electrogenic NCBTs; members of the SLC4 family) operating with a Na^+:HCO_3^- stoichiometry of 1:3, and channels that permit the passive influx of H^+ or efflux of HCO_3^-. The acid extruders (Fig. 1B) include V- and P-type H-pumps (reviewed by Cipriano et al., 2008; Kuhlbrandt, 2004), Na–H exchangers (SLC9 family) (reviewed by Orlowski and Grinstein, 2004), and both electrogenic and electroneutral NCBTs (members of the SLC4 family) operating with a Na^+:HCO_3^- stoichiometry of less than 1:3. In this review, we focus on the structural features of NCBTs, particularly those that affect transporter function.

The five mammalian NCBTS

Sodium-coupled bicarbonate transport activity was first described in vertebrates by Boron and Boulpaep in their study of the mechanism of HCO_3^- reabsorption in the salamander proximal tubule (Boron and Boulpaep, 1983). They demonstrated that the NCBT mechanism at the basolateral membrane is electrogenic, independent of Cl^-, and sensitive to the stilbene-derivate SITS. Fourteen years later, the expression cloning of this transporter – now known as NBCe1 – was reported (Romero et al., 1997). The predicted peptide sequence of NBCe1 was noted as being closely related to that of the stilbene-sensitive, Cl–HCO_3 anion exchangers AE1–3 (Romero et al., 1997). By comparison with the putative topology of the red cell anion exchanger AE1, variants of NBCe1 are predicted to have a large cytoplasmic N-terminus (Nt) with 424–468 amino acid residues, a transmembrane domain (TMD) with 521 residues and as many as 14 transmembrane (TM) segments, and a cytoplasmic C-terminus (Ct) with 90–105 residues (Fig. 2). The subsequent cloning of mammalian NBCe1 orthologs (Abuladze et al., 1998; Bevensee et al., 2000; Burnham et al., 1997; Choi et al., 1999; Thevenod et al., 1999) was followed in short succession by the cloning and characterization of four further paralogs (NBCe2, NBCn1, NBCn2 and NDCBE) with demonstrated NCBT activity (Fig. 3). NBCe1 forms dimers, within which each monomer appears to be capable of NCBT activity (Kao et al., 2008). Below, we individually discuss the distribution and known physiological roles of these five NCBTs and their variants.

NBCe1

Molecular function

NBCe1 is one of two electrogenic NCBT paralogs in mammals (Fig. 3). In the kidney NBCe1 operates with an apparent Na^+:HCO_3^- stoichiometry of 1:3 (Soleimani et al., 1987), in which mode the transporter mediates net HCO_3^- efflux across the basolateral membrane of proximal tubule epithelia. [In principle, each of the NCBTs could carry CO_3^{2-} in addition to (or instead of) HCO_3^-. Thus, NBCe1-A could achieve an apparent 1:3 stoichiometry by transporting 1 Na^+, 1 HCO_3^- and 1 CO_3^{2-}. This

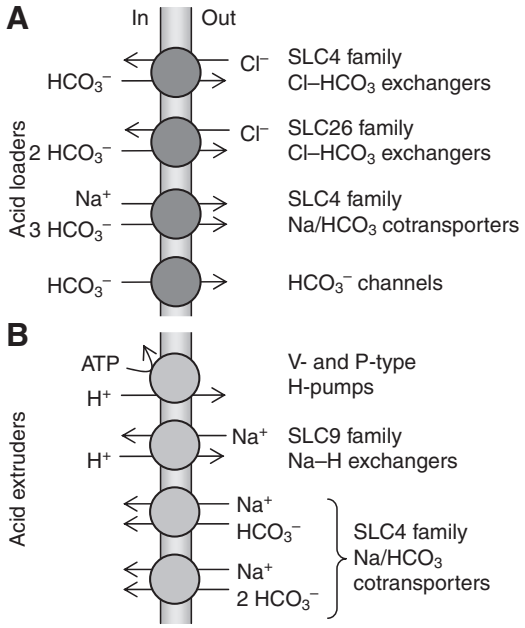

Fig. 1. Acid–base transporters. Acid–base homeostasis is achieved by the balanced action of acid-loading and acid-extruding mechanisms. (A) Acid loaders. The influx of acid equivalents across cell membranes is mediated by acid-loading transporters such as anion exchangers, electrogenic Na^+-coupled bicarbonate transporters and HCO_3 channels. (B) Acid extruders. The efflux of acid equivalents across cell membranes is mediated by acid-extruding transporters such as H-pumps, Na–H exchangers, and both electrogenic and electroneutral Na^+-coupled bicarbonate transporters.

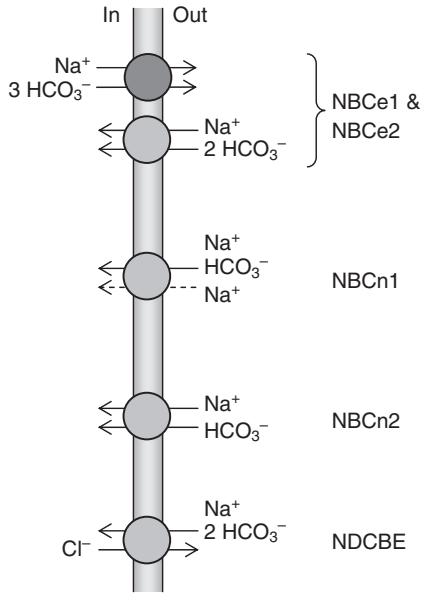

Fig. 3. Net transport activity of mammalian NCBTs. NBCe1 and NBCe2 are both electrogenic NCBTs capable of mediating Na^+:2 HCO_3^- influx or Na^+:3 HCO_3^- efflux in a tissue-specific manner. NBCn1, NBCn2 and NDCBE are all electroneutral NCBTs that mediate net HCO_3^- influx. NBCn1 is associated with a Na^+ conductance (dashed arrow). Na/HCO_3 influx mediated by NDCBE is coupled to Cl^- efflux.

and similar considerations are discussed elsewhere (Boron and Knakal, 1989; Boron, 1985; Pushkin and Kurtz, 2006).] In most other cell types – including astrocytes (Bevensee et al., 1997), pancreatic duct cells (Gross et al., 2001a) and ventricular myocytes (Aiello et al., 1998; Villa-Abrille et al., 2007) – NBCe1 operates with a 1:2 stoichiometry, in which mode the transporter mediates net HCO_3^- influx. NBCe1 also operates with a 1:2 stoichiometry when heterologously expressed in *Xenopus* oocytes (Heyer et al., 1999; Sciortino and Romero, 1999) and human embryonic kidney cells (Shao et al., 2008). This mechanistic flexibility (reviewed by Gross and Kurtz, 2002) is reported to be controlled by changes in

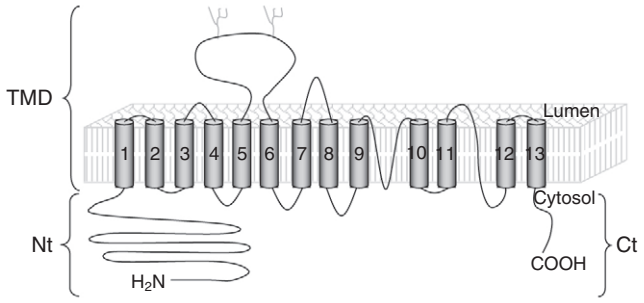

Fig. 2. Three-domain structure of a generic mammalian Na^+-coupled HCO_3 transporter (NCBT). By comparison with the predicted topology of the Cl–HCO_3 exchanger AE1 (Askin et al., 1998; Fujinaga et al., 1999; Zhu et al., 2003), NCBTs are predicted to have an extended N-terminus (Nt), a transmembrane domain including 12–14 transmembrane spans (TMD), and a shorter C-terminal domain (Ct). An extended extracellular loop connecting transmembrane spans 5 and 6 includes multiple *N*-glycosylation sites.

$[Ca^{2+}]$ (Muller-Berger et al., 2001) and may involve phosphorylation of the transporter (Gross et al., 2001b). Defective functional expression of NBCe1 is linked to proximal renal tubular acidosis (pRTA) (Igarashi et al., 1999), which can be associated with growth and mental retardation as well as ocular and dental defects (e.g. Dinour et al., 2004; Inatomi et al., 2004). Furthermore, NBCe1-null nice have a severe metabolic acidosis (Gawenis et al., 2007).

NBCe1 variants

The *SLC4A4* gene that encodes NBCe1 can be transcribed to include sequence encoding either of two alternative Nt (under the control of two distinct promoters) (Abuladze et al., 2000) and either of two alternative Ct (by alternative splicing of transcripts) (Bevensee et al., 2000). Although in theory this modularity could allow for the production of four possible mRNA transcripts, so far only three splice variants (NBCe1-A, -B and -C; Fig. 4) have been identified from cDNA.

NBCe1-A (Burnham et al., 1997) – sometimes known as kNBC1 – is the shortest of the three NBCe1 forms and its transcripts are predominantly expressed in the kidney (Abuladze et al., 1998). NBCe1-A is located in the basolateral membrane of proximal tubule epithelia (Maunsbach et al., 2000), where it plays a vital role in the reabsorption of HCO_3^- (i.e. movement from lumen to blood) previously filtered in the glomerulus, thereby preventing whole-body acidosis.

NBCe1-B (Abuladze et al., 1998; Choi et al., 1999) – sometimes known as pNBC1 – is predominantly expressed in the pancreas (Abuladze et al., 1998). NBCe1-B is identical to NBCe1-A save for a longer and different Nt (85 amino acids, blue, replace 41 amino acids, maroon; see Fig. 4). In pancreatic

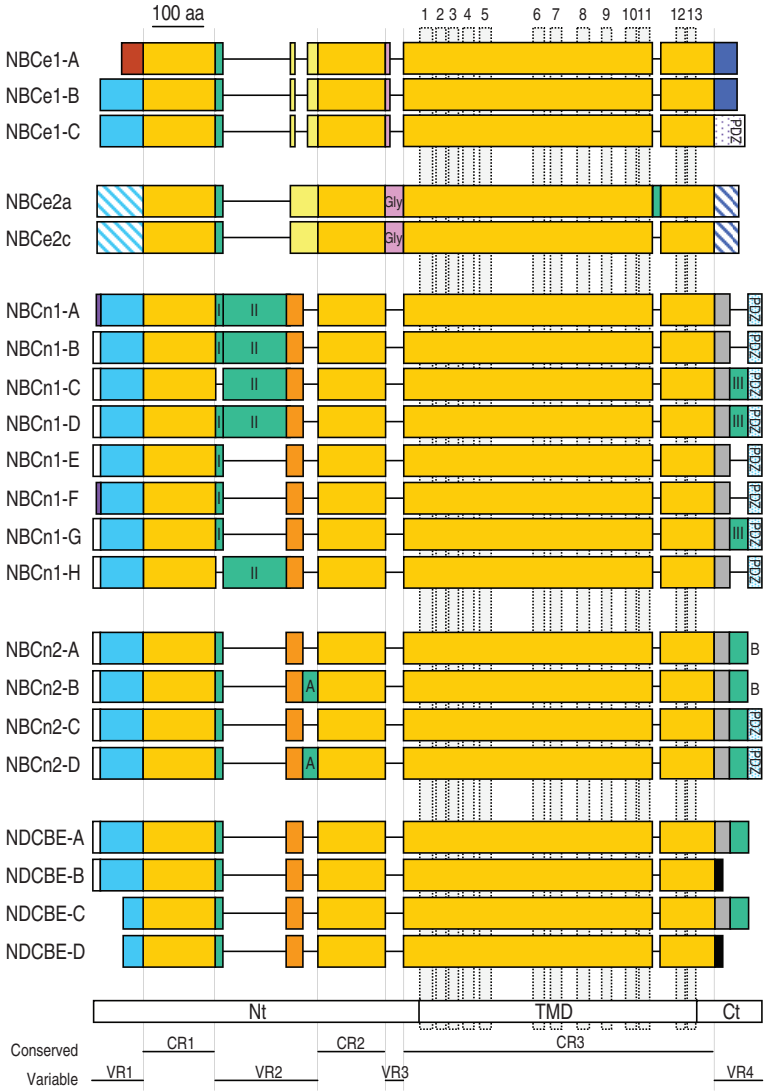

Fig. 4. Alignment of protein modules comprising NCBT variants. NCBT variants are aligned to scale from Nt to Ct. Numbered gray columns mark the putative positions of the transmembrane helices represented in Fig. 2. Conservation of color in a vertical direction on the figure represents conservation of sequence among NCBTs. Internal splice cassettes are represented as green boxes and labeled with their name. PDZ-binding motifs (PDZ) and glycine-rich regions (Gly) are also marked. A horizontal line connecting bars represents the absence of homologous sequence in a variant. The position of conserved regions (CRs) and variable regions (VRs) discussed in the text are indicated by lines at the bottom of the diagram. Unless otherwise stated, protein sequences are human. Alignment is based on a manually adjusted version of a sequence alignment that was originally generated by ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) using GenBank protein accession numbers for NBCe1-A (NP_003750), NBCe1-B (AAD42020), NBCe1-C (ABQ43327), NBCe2a (AAK26741), NBCe2c (AAK97072), NBCn1-A (Q9Y6M7), rat NBCn1-B (AAD46389), NBCn1-C (ACH61962), NBCn1-D (ACH61960), NBCn1-E (ACH61961), NBCn1-F (AAG16773), NBCn1-G (ACB47400), NBCn1-H (ACH61958), NBCn2-A (NP_071341), NBCn2-B (AAQ83632), rat NBCn2-C (AAS89263), rat NBCn2-D (derived *in silico* from rat NBCn2-C), NDCBE-A (AAY79176), NDCBE-B (NP_004849), NDCBE-C (ABJ09587) and NDCBE-D (ABJ91577).

acinar cells NBCe1-B protein is present in abundance at the basolateral membrane (Roussa et al., 2004; Satoh et al., 2003; Thevenod et al., 1999), where it would mediate HCO_3^- uptake and thereby contribute to HCO_3^- secretion into the pancreatic ducts (Abuladze et al., 1998), helping to keep pancreatic enzymes inactive within the ducts and to neutralize acidic gastric chyme in the duodenum.

NBCe1-C (Bevensee et al., 2000) is the longest of the three transporter variants and is identical to NBCe1-B save for a longer and different Ct (61 amino acids, dark blue dots, replace 46 amino acids, solid dark blue; see Fig. 4). NBCe1-C is predominantly expressed in the brain (Bevensee et al., 2000), particularly in glial cells (Majumdar et al., 2008) where – as proposed for electrogenic NCBT activity in leech glia (Deitmer 1992; Rose and Dietmer, 1994) – NBCe1 activity could help to counter changes in extracellular pH resulting from neuronal firing.

NBCe2

Molecular function

NBCe2 – sometimes known as NBC4 – is the second of two mammalian electrogenic NCBT paralogs (Fig. 3). As with NBCe1, the stoichiometry of NBCe2 is cell-type specific. For example, NBCe2 operates with an apparent Na^+:HCO_3^- stoichiometry of 1:3 when heterologously expressed in the mPCT renal cell line (Sassani et al., 2002), but a 1:2 stoichiometry when heterologously expressed in *Xenopus* oocytes (Virkki et al., 2002).

NBCe2 protein is predominantly detected in the liver (Pushkin et al., 2000a), especially in the sinusoidal (i.e. basolateral) membrane of hepatocytes (Abuladze et al., 2004), where electrogenic Na/HCO_3 cotransport (Fitz et al., 1991b; Renner et al., 1989) is a major contributor to Na^+ and HCO_3^- influx under basal conditions (Fitz et al., 1989; Fitz et al., 1991a; Fitz et al., 1991b). This influx influences such hepatic functions as urea synthesis and gluconeogenesis (Kashiwagura et al., 1984). Apical NBCe2 immunoreactivity is reported in the membranes of bile duct cholangiocytes (Abuladze et al., 2004), renal collecting duct intercalated cells (Damkier et al., 2007), and uroepithelial cells in the renal pelvis (Abuladze et al., 2004). Apically expressed NBCe2 in choroid plexus epithelia (Praetorius and Nielson, 2004) operates with a 1:3 stoichiometry (Millar and Brown, 2008), secreting HCO_3^- into the cerebrospinal fluid. Polymorphisms in the *SLC4A5* gene locus have been genetically linked to hypertension (Barkley et al., 2004; Hunt et al., 2006), although none of the associated genetic changes alter the predicted NBCe2 peptide sequence.

NBCe2 variants

NBCe2 is encoded by the *SLC4A5* gene. Of the six NBCe2 splice variants reported (Pushkin et al., 2000b; Sassani et al., 2002; Xu et al., 2003), only NBCe2a–d sequences are confirmed by the human genome database and of these four transcripts only NBCe2a and NBCe2c (see Fig. 4) include a full complement of TM segments. The predicted sequence of NBCe2a differs from that of NBCe2c in having a unique 16 amino acid extension (green in Fig. 4, NBCe2a) between TM segments 11 and 12. NBCe2c is the only variant confirmed to have electrogenic NCBT activity (Sassani et al., 2002; Virkki et al., 2002).

NBCn1

Molecular function

NBCn1 (Fig. 3) was the first of three mammalian electroneutral NBC paralogs to be characterized at the molecular level (Choi et al., 2000; Pushkin et al., 1999). NBCn1 mediates the apparent 1:1 cotransport of Na^+ and HCO_3^- and has an associated conductance, about 50% of which is carried by Na^+ (Choi et al., 2000). NBCn1 is widely expressed in mammalian organs, with especially high levels of transcript expression in muscular tissue (Choi et al., 2000; Cooper et al., 2006; Damkier et al., 2006; Pushkin et al., 1999). Elsewhere in the body, NBCn1-mediated HCO_3^- transport may (1) influence neuronal activity (Cooper et al., 2005) by setting the resting pH_i of neurons and contributing towards recovery of pH_i after neuronal firing, (2) contribute to secretion of cerebrospinal fluid by mediating HCO_3^- influx across the basolateral membrane of the choroid plexus epithelium (Praetorius et al., 2004), (3) promote osteoclast survival by contributing to an anti-apoptotic alkaline pH_i (Bouyer et al., 2007), and (4) neutralize the protons formed during the conversion of NH_4^+ to NH_3 by mediating HCO_3^- influx across the basolateral membrane of mTAL epithelia (Vorum et al., 2000). NBCn1 knockout mice are auditorily and visually impaired (Bok et al., 2003).

NBCn1 variants

NBCn1 is encoded by the *SLC4A7* gene, which can be transcribed to include sequence encoding either of two alternative Nt (purple *vs* white in Fig. 4, NBCn1) – presumably transcribed from distinct promoters – and these transcripts can be processed to include perhaps any combination of three internal splice cassettes (neighboring cassettes I and II in the Nt and cassette III in the Ct; green in Fig. 4, NBCn1). Although in theory 16 possible mRNA transcripts could be produced, so far only eight of these variants (NBCn1-A to -H; represented in Fig. 4) have been amplified from cDNA. The distribution and individual character of these variants is still under investigation (Damkier et al., 2006).

NBCn2

Molecular function

NBCn2 (Fig. 3) is the second of three mammalian, electroneutral NCBT paralogs and – under physiological conditions – mediates the apparent 1:1 net cotransport of Na^+ and HCO_3^-, accompanied by a 1:1 exchange of Cl^- (Parker et al., 2008b). Thus – unlike NDCBE – NBCn2 does not couple the transport of Na^+ and HCO_3^- across the cell membrane to the net transport of Cl^- in the opposite direction. Similar to the GABA transporter GAT1 (Loo et al., 2000), the inclusion of Cl^- in transport cycles may be facultative, as – in the absence of extracellular Cl^- – NBCn2 seems capable of Na^+-driven Cl–HCO_3 exchange (Giffard et al., 2003; Parker et al., 2008b; Wang et al., 2000).

NBCn2 is expressed throughout the brain (Chen et al., 2008b; Hübner et al., 2004; Parker et al., 2008b; Wang et al., 2000) and choroid plexus (Praetorius et al., 2004). Consistent with a role for NBCn2 in cerebrospinal fluid secretion, NBCn2-knockout mice have a reduced brain ventricle size (Jacobs et al., 2008). NBCn2 – because of its contribution to pH_i regulation – also appears to be important for modulating the firing rate of CA3 pyramidal neurons (Jacobs et al., 2008). Repetitive firing leads to a fall in pH_i, from which the neuron subsequently recovers, likely due in part to NBCn2-mediated HCO_3^- uptake. Imposing a large intracellular acid load reduces neuronal excitability. NBCn2 knockout mice exhibit a slower pH_i recovery from such acid loads, and thus a slower recovery of neuronal excitability (Jacobs et al., 2008). Indeed, NBCn2-null mice have an enhanced survival rate from epileptic seizure (Jacobs et al., 2008). Unexpectedly, however, a case of epilepsy has been genetically linked to a disrupted human *SLC4A10* gene (Gurnett et al., 2008).

NBCn2 variants

NBCn2 is encoded by the *SLC4A10* gene which is capable of producing four splice variants (NBCn2-A to -D; represented in Fig. 4) that arise from the optional inclusion of two internal splice cassettes. The 90 nucleotides of cassette A encode 30 amino acids in the Nt, represented by the green box labeled 'A' in Fig. 4 for NBCn2-B and NBCn2-D (hereafter NBCn2-B/D). The 39 nucleotides of cassette B include sequence encoding the last three, unique amino acids of NBCn2-A/B, followed by a termination codon. Thus, transcripts lacking cassette B (NBCn2-C/D) encode a transporter with a longer and different Ct (21 unique residues replacing three).

NDCBE

Molecular function

The Na^+-driven Cl–bicarbonate exchanger NDCBE (Fig. 3) is the third of the three known mammalian electroneutral NCBT paralogs, and the sole obligate Na^+-driven Cl–HCO_3 exchanger (Grichtchenko et al., 2001). Thus – unlike NBCn1 and NBCn2 – NDCBE couples the transport of Na^+ and $2\,HCO_3^-$ (or perhaps $1\,CO_3^{2-}$) across the cell membrane to the net transport of Cl^- in the opposite direction. The magnitude of the net Cl^- efflux mediated by NDCBE is dwarfed by a larger component of futile Cl–Cl exchange that requires both Na^+ and HCO_3^- (Grichtchenko et al., 2001).

NDCBE variants

NDCBE protein – encoded by the *SLC4A8* gene – is expressed throughout the central nervous system (Chen et al., 2008a). *SLC4A8* produces at least four protein variants (NDCBE-A to -D; represented in Fig. 4), resulting from the choice of a full-length or truncated Nt and a choice of two Ct (Parker et al., 2008a). Determining differences in distribution among the variants requires tools still in development.

Structural elements OF NCBTS

NCBTs can be considered to be composed of variable and conserved regions as shown in the black-and-white lower panel in Fig. 4. Subtle variations in the conserved regions (gold boxes labeled 'CR' in Fig. 4) are likely responsible for the functional differences among NCBTs, while the more obviously variable regions (non-gold boxes labeled 'VR' in Fig. 4) – often brought about by the optional inclusion of splice cassettes – seem to play a regulatory role. In this section we consider these conserved and

variable regions – linearly scanning the molecule from the Nt to the Ct – and summarize our present knowledge of the consequences of sequence variation among the five NCBTs.

Nt variable region 1 (Nt-VR1)

Nt-VR1 has at least three, non-overlapping subdomains of interest.

Putative SH3-binding domain

A short exon encoding 16 amino acids (white boxes in Fig. 4) commences the sequences of certain variants of NBCn1, all known variants of NBCn2, and NDCBE-A/B. The encoded protein is not necessary for basal NCBT activity as evidenced by (1) the absence of this exon in NBCe1 and NBCe2 transcripts, (2) the lack of a functional difference between NDCBE-A/B *vs* NDCBE-C/D (Parker et al., 2008a), and (3) the lack of functional effect upon removal of this sequence from NBCn2 (Parker et al., 2007a).

In addition to the start exon that encodes the 16 amino acids in most known NBCn1 variants, NBCn1 has a similar alternative start exon that encodes 11 amino acids (purple box in Fig. 4), which likely represents a product transcribed from an alternative promoter (Liu et al., 2008). Uniquely among these extreme Nt sequences, NBCn2 exon 1 includes a putative SH3 domain-binding sequence (M.D.P., unpublished) 'PxxPxR' (Knudsen et al., 1995) that may mediate interactions with protein partners (Kay et al., 2000).

Autostimulatory domain (ASD)

NBCe1-A has a unique 43 amino acid Nt sequence (maroon box in Fig. 4) that confers an increase in unitary transporter activity. In giant patches, the electrical current mediated by NBCe1-A lacking the ASD is 64% smaller than that mediated by the full-length cotransporter (McAlear et al., 2006). The mechanism of this stimulation has yet to be determined.

Autoinhibitory domain (AID) and IRBIT-binding domain

NBCe1-B/C (McAlear et al., 2006) and the three electroneutral NCBTs (Parker et al., 2007b) all include an AID (within the blue boxes in Fig. 4). Undefined determinants within the AID have an inhibitory effect on the unitary transporter activity, such that the current mediated by NBCe1-C is 230% greater in constructs lacking the AID (McAlear et al., 2006). Another NCBT, NDCBE-A, has a functional expression (defined here as the product of unitary transporter activity and surface expression of the cotransporter) that is indistinguishable from the NDCBE-C variant, which lacks a major portion of VR1 (Parker et al., 2008a). If these NBCe1 and NDCBE data are taken together, then we can conclude that the inhibitory determinants of the AID are located between the equivalent of residues 55 and 92 of NDCBE (i.e. the blue box of NDCBE-C/D). An AID has not been functionally defined in the Nt sequence of mammalian NBCe2, although an AID-like sequence is clearly identifiable in the predicted NBCe2 orthologs of zebrafish, frogs and chickens (M.D.P., unpublished).

Overlapping or neighboring the AID are determinants that are responsible for binding a protein partner that is known for its association with the IP_3 receptor (Shirakabe et al., 2006). Named IRBIT – for IP3R-binding protein released with inositol 1,4,5-trisphosphate – this soluble protein has a molecular mass of ~60 kDa (reviewed by Devogelaere et al., 2008). IRBIT increases the unitary transporter activity of NBCe1-B, as evidenced by the magnitude of the current (Shirakabe et al., 2006). IRBIT also increases the functional expression of NCBTs in two different systems, as evidenced in both cases by the rate of pH_i recovery from an acid load: (1) NBCe1-B in pancreatic duct cells (Yang et al., 2008), and (2) NDCBE-B, NBCn2-B and NBCn1-B heterologously expressed in *Xenopus* oocytes (Parker et al., 2007b). In light of the proximity – and perhaps the overlap – of the AID and the IRBIT-binding determinants (Fig. 4), it seems possible that IRBIT mediates its effect on NCBTs by sequestering the AID.

Two other interesting targets of IRBIT are NHE3 (Na–H exchanger 3) and CFTR (cystic fibrosis transmembrane conductance regulator). The combination of IRBIT and stimuli that elevate cytosolic [Ca^{2+}] leads to an increase in the surface presentation of NHE3 (He et al., 2008). In the case of CFTR, IRBIT decreases the mean closed time of the channels (Yang et al., 2008).

The inclusion of the AID and IRBIT-binding domain in certain splice variants of NCBTs allows for upregulation of HCO_3^- transport in response to physiological cues. IRBIT-mediated stimulation of other acid–base transporters such as NHE3 (He et al., 2008) and CFTR (Yang et al., 2008) allows the coordinated regulation of HCO_3^- reabsorption and secretory pathways.

Nt conserved region 1 (Nt-CR1)

Whereas NCBTs retain substantial HCO_3^- transport activity after removal of Nt-VR1, the Nt-CR1 includes sequence indispensable for functional expression of these transporters. For example, N-terminal truncation of NBCe1-C by 213 amino acids – removing most of Nt-CR1 – results in a loss of function despite normal surface presentation of the transporter (McAlear et al., 2006). The necessity of the Nt for NBCe1 function (Espiritu et al., 2006; McAlear et al., 2006) contrasts with the sufficiency of the TMD for the function of the Cl–HCO_3 exchanger AE1 (Groves and Tanner, 1992).

Mutation of a single residue – E91R – in the Nt-CR1 of the NBCe1-A construct causes intracellular retention of an N-terminally GFP-tagged construct in MDCK cells (Li et al., 2005). The E91R mutant of NBCe1-A is reported to have an additional defect in unitary transporter activity (Chang et al., 2008).

According to current models derived from X-ray diffraction studies of the Nt of AE1 (Zhang et al., 2000) and NBCe1 (Gill and Boron, 2006b), the beginning of the structured region of the Nt occurs within Nt-CR1. A homology model of the NBCe1-Nt, based on the crystal structure of the AE1 Nt, predicts that E91 forms a salt bridge with R298 (Chang et al., 2008), which is located in what we refer to as the 'Nt-conserved region 2' (see below). These residues form part of a chain of polar residues through the core of the Nt – including R86 and E92 in Nt-CR1 – that have been speculated to be part of an ion-translocation pathway (Chang et al., 2008).

Nt variable region 2 (Nt-VR2)

The position of Nt-VR2 in NCBTs is coincident with a region that appears unstructured in the current models derived from X-ray diffraction studies of the Nt of AE1 (Zhang et al., 2000). In NCBTs, this region is extended compared with AE1. Moreover, in certain NCBT paralogs, the optional inclusion of protein cassettes (e.g. cassettes I and II of NBCn1) confers substantial variability in the length of Nt-VR2. Little is known about the function of these additional sequences short of the fact that their inclusion is unnecessary for NCBT activity. The inclusion of each of these cassettes, discussed individually below, appears to be regulated in a tissue-specific fashion.

Cassette I of NBCn1

Homologs of this 14 amino acid sequence (green boxes labeled 'I' in Fig. 4, NBCn1) are present in all NCBTs. This sequence

[originally named ‘cassette A’ of NBCn1 (Choi et al., 2000), but not equivalent to the ‘cassette A’ included in some NBCn2 variants] is lacking from NBCn1-C and NBCn1-H. The cassette appears to be always present in NBCn1 cDNAs amplified from certain organs (e.g. parotid gland), occasionally present in those from other organs (e.g. kidney), but exclusively absent from lung cDNA (Damkier et al., 2006; Odgaard et al., 2004).

Cassette II of NBCn1

Some rodent NBCn1 sequences include a 123 amino acid cassette II (green boxes labeled ‘II’ in Fig. 4, NBCn1) that abuts cassette I (Odgaard et al., 2004). In human variants, cassette II is 124 amino acids in length due to the inclusion of an additional, internal alanine residue. Inclusion of cassette II – encoded by a single exon not located in any other mammalian NCBT gene – is predominant in NBCn1 transcripts from heart (Cooper et al., 2006) and skeletal muscle (Pushkin et al., 1999) as well as from embryonic – but not adult – hippocampal neurons (Cooper et al., 2005). Cassette II includes several consensus binding sites for calcineurin (Parker and Boron, 2008), which might dephosphorylate NBCn1 and/or its associated protein-binding partners. According to a preliminary report (Cooper et al., 2006) the inclusion of cassette II is inhibitory to the functional expression of NBCn1 in *Xenopus* oocytes.

Cassette A of NBCn2

Cassette A is a 30 amino acid sequence unique to NBCn2 that inserts at a point 32 amino acids downstream from the end of cassette I, and therefore 32 amino acids downstream of what would be the point of insertion of cassette II in NBCn1. Although two groups have investigated the tissue distribution of cassette A (Giffard et al., 2003; Praetorius et al., 2004), the function of this cassette is unknown. An additional source of variation has been noted in rodent transcripts, which have an ambiguous 3′-splice boundary in the exon preceding cassette A, such that an extra alanine may be included in some transcripts (Giffard et al., 2003).

Nt conserved region 2 (Nt-CR2)

Residues important for functional expression

According to current models of the Nt of AE1 (Zhang et al., 2000) and NBCe1 (Gill and Boron, 2006b), the structured region of the Nt – which begins in Nt-CR1 – ends within Nt-CR2. Nt-CR2 includes important structural and regulatory features. For example, the naturally occurring mutation R298S in this region of human NBCe1-A is associated with pRTA (Igarashi et al., 1999), likely caused by a combination of aberrant protein trafficking [see R342S of NBCe1-B (Li et al., 2005)] and unitary transporter dysfunction (Chang et al., 2008). As noted above, R298 is part of a chain of polar residues though the Nt core structure that includes E295 – also in Nt-CR2 – and E91 in Nt-CR1 (Chang et al., 2008).

Dimerization arms

An assessment by size-exclusion chromatography shows that the Nt of NBCe1, NBCe2, NBCn1 and squid ‘NDCBE’ form dimers in solution (Gill and Boron, 2006a). [Note, squid ‘NDCBE’ is a Na^+-driven $Cl–HCO_3$ exchanger but is not the direct ortholog of mammalian NDCBE. The *SLC4A8* gene, which encodes mammalian NDCBE, is a vertebrate-specific *SLC4* paralog that emerged long after the divergence of vertebrates and invertebrates.] A preliminary structural analysis of NBCe1 Nt (Gill and Boron, 2006b) shows that the dimer interface is stabilized by interlocking arms, as is the case for the AE1 Nt (Zhang et al., 2000). Nevertheless, disrupting extracellular disulfide bridges in the TMD (discussed below) is sufficient to cause NBCe1-A to migrate as a monomer on a native gel (Kao et al., 2008).

Nt variable region 3 (Nt-VR3)

In NBCe1 and NBCe2, Nt-VR3 includes an extended stretch of glycine residues (pink boxes in Fig. 4). The exact length of the stretch varies between species, one of the longest being found in human NBCe2, where 25 residues in a 34 amino acid region are glycine. Presumably this adds flexibility between the Nt and TMD, although the exact nature and purpose of this region is yet to be determined. Nt-VR3 is absent from NBCn1, NBCn2 and NDCBE.

Nt conserved region 3 (Nt-CR3)

Residues important for functional expression

This small juxtamembrane stretch of Nt sequence contains two Asp residues separated by 10 amino acids. These separated Asp residues are retained among all NCBTs and are required for the appropriate trafficking of NBCe1 to the basolateral membrane of polarized epithelial cells (Li et al., 2008).

Protein 4.1-binding site

A cluster of positively charged residues ‘KRK’ in the Nt of NCBTs is similar to an ‘RRR’ motif found at the equivalent position in the Nt of AE1. In AE1 this cluster – located between the two Asp residues mentioned above – binds to a cytoskeletal-stabilizing protein called protein 4.1 (Jons and Drenckhahn, 1992). It is likely no co-incidence then that protein 4.1B co-localizes with NBCe1 at the basolateral membrane of proximal tubule epithelia (Terada et al., 2004). This complex also includes the membrane-associated guanylate kinase homolog p55 (Terada et al., 2007), which likely mediates interactions between the transporter and the cytoskeleton.

TMD conserved region 3 (TMD-CR3)

Residues important for functional expression

As might be expected, most residues known to be important for unitary transporter activity and appropriate trafficking of NCBTs are located within the TMD. For example, seven naturally occurring, missense mutations within the TMD of human NBCe1 (S427L, T485S, G486R, R510H, L522P, A799V and R881C) are associated with pRTA (Demirci et al., 2006; Dinour et al., 2004; Horita et al., 2005; Igarashi et al., 1999; Suzuki et al., 2008). An extensive mutagenesis study has identified many other functionally important residues in the NBCe1 TMD (Abuladze et al., 2005).

Determinants of electrogenicity

The sequence within the TMD of NCBTs is well conserved, and thus we can conclude that subtle differences between these TMD sequences must be responsible for the most interesting variation among NCBT paralogs – their transport functions. For example, studies of chimeric constructs of NBCe1 and NBCn1 (Chen and Boron, 2008; Choi et al., 2007) have demonstrated that the electrogenicity of NBCe1 requires determinants in both the front half of the TMD (within TM1–5) and the back half of the TMD (within TM6–9). Between human NBCe1 and NBCn1, these regions are 56% (TM1–5) and 62% (TM6–9) identical.

Stilbene-binding site

A characteristic feature of most SLC4 family members is their sensitivity to inhibition by stilbene derivatives. Mutagenesis studies on AE1 (Kietz et al., 1991) and NBCe1-A (Lu and Boron, 2007) and cross-linking studies on AE1 (Okubo et al., 1994) indicate that the first lysine in a ‘KXXK’ motif at the extracellular end of TM5

is the most important determinant of stilbene sensitivity. All NCBTs retain this first lysine residue and all but NBCn1 (Choi et al., 2000) are highly stilbene sensitive (Grichtchenko et al., 2001; Virkki et al., 2002; Wang et al., 2000).

Variable glycosylation sites

The most obvious region of divergence among the TMDs of NCBTs is in the third extracellular loop (i.e. between TM5 and TM6). The number of potential *N*-glycosylation sites on this loop varies between two and four depending on the NCBT paralog (within a particular species). Conversely, the number of sites in any given NCBT can also vary among species (e.g. human NBCn1 has four, whereas rat NBCn1 has only three). NBCe1 (Choi et al., 2003), NBCn1 (Chen et al., 2007), NBCn2 (Chen et al., 2008b; Praetorius et al., 2004) and NDCBE (Chen et al., 2008a) are all *N*-glycosylated *in vivo*. Although NBCe1 has three potential *N*-glycosylation sites, only the most distal two sites are glycosylated with heterologous expression of NBCe1 in *Xenopus* oocytes (Choi et al., 2003). The disruption by mutagenesis of all glycosylation sites from the third extracellular loop can have disparate effects. Unglycosylatable NBCe1 appears to retain wild-type activity (Choi et al., 2003), whereas unglycosylatable NBCn2 is poorly expressed compared with the wild-type transporter (Chen et al., 2008b).

Conserved cysteine residues in the third extracellular loop

A second notable feature of the third extracellular loop of NCBTs is the spatial preservation of four cysteine residues. It has recently been suggested that all four Cys residues form disulfide bonds either within or between opposing NBCe1 monomers in a dimer. The inter-monomeric interactions mediated by the third Cys and fourth Cys residues on the loop play a key role in stabilizing NBCe1 dimers (Kao et al., 2008; Zhu et al., 2008).

Ct conserved region (Ct-CR3)

Ct-CR3 is a cytosolic juxtamembrane sequence contiguous with TMD-CR3. Ct-CR3 includes clusters of two to seven Lys residues of unascribed function, as well as a number of clusters of Asp residues. Clusters in which at least two out of four residues are Asp have been implicated in the activation of NCBTs *via* the binding of the soluble enzyme carbonic anhydrase II [CA II (Becker and Dietmer, 2007; Gross et al., 2002; Loiselle et al., 2004; Pushkin et al., 2004)]. However, neither injected CA II nor CA II fused to the Ct of NBCe1-A increases NBCe1-mediated current in *Xenopus* oocytes (Lu et al., 2006). Moreover, CA II does not bind to the purified Ct of NBCe1, NDCBE or AE1 (Piermarini et al., 2007). Finally, the prototype DADD motif originally proposed as the binding site for CA II on AE1 (Vince and Reithmeier, 2000) is similar to a DADE motif present in the Ct of a primitive SLC4-like protein from the bacterium *Nitrococcus mobilis* (Parker and Boron, 2007). When expressed in *Xenopus* oocytes, this protein transports Cl^- but not HCO_3^-.

Ct variable region (Ct-VR4)

Cassette III of NBCn1

Uniquely among NCBTs, NBCn1 achieves Ct variation *via* the optional inclusion of an internal 36 amino acid cassette III (green boxes labeled 'III' in Fig. 4) (Choi et al., 2000). This cassette is homologous to a Ct sequence found in all variants of NBCn2 and in NDCBE-A/C. The consequence of cassette III omission from NBCn1 has not been directly addressed, although it does not seem necessary for basal NCBT activity as evidenced by (1) the robust Na/HCO_3 cotransport activity mediated by NBCn1-B and NBCn1-E, both of which lack cassette III (Choi et al., 2000; Cooper et al., 2005), and (2) the lack of functional effect upon removal of cassette III homologous sequence from NDCBE-A (Parker et al., 2008a).

Inhibitory domain of NDCBE-B/D

Uniquely among human NCBT-encoding genes, *SLC4A8* encodes two alternative Ct, each with its own 3′-UTR (Parker et al., 2008a). The shorter of these Ct terminates with a unique 17 amino acid sequence (black boxes in Fig. 4) that is inhibitory to the functional expression of NDCBE in *Xenopus* oocytes (Parker et al., 2008a). Thus, this sequence may represent a second AID in NDCBE.

Binding motif for PDZ domains

Except for NBCe2, all human NCBTs have variant products with either a 'short' or a 'long' alternative Ct. In most cases, the longer of each alternative Ct terminates in the consensus PDZ domain-binding motif 'ET[C/S/T]L'. Such a feature is well recognized in variants of NBCe1 (Bevensee et al., 2000), NBCn1 (Pushkin et al., 2003) and NBCn2 (Giffard et al., 2003) but is more obscure for NBCe2. The human *SLC4A5* gene has the potential to encode an 'ETTL'-Ct ending, orthologous to that which concludes a predicted zebrafish NBCe2 variant (GenBank protein accession number XP_387688). However, when included in mammalian *SLC4A5* transcripts, this sequence forms part of the 3′-UTR (M.D.P., unpublished). No full-length mammalian cDNAs reported to date include the 'ETTL'-Ct in their open reading frames.

Deletion of the PDZ domain ligand from NBCn1 does not affect the functional expression of NBCn1 in HEK293 cells (Park et al., 2002), but it is important for the inclusion of NBCn1 in membrane protein clusters. The PDZ domain ligand 'ETSL' at the Ct of NBCn1 has been reported to mediate interaction with (1) CFTR *via* the PDZ scaffolding protein NHERF1 (Na–H exchanger regulatory factor-1, aka EBP50) in mouse submandibular gland (Park et al., 2002), (2) the H-pump *via* NHERF1 in rat kidney (Pushkin et al., 2003), (3) usherin and VLGR1b (very large G-protein coupled receptor 1b) *via* harmonin in rat cochlear hair cells (Reiners et al., 2005), and (4) the NMDA receptor subunit NR2A *via* the postsynaptic scaffolding protein PSD-95 in rat brain (Rajbhandari et al., 2008). It must be noted that NBCn1 is basolaterally distributed in many epithelial cell types (e.g. Gresz et al., 2002; Praetorius et al., 2004; Vorum et al., 2000) so interactions (1) and (2) – which require an apical NBCn1 presence – may not be universally relevant.

Another NCBT, NBCn2-C, shows an increased association with the actin cytoskeleton in astrocytes compared with NBCn2-B, presumably by virtue of its PDZ domain-binding sequence (Giffard et al., 2003). The interaction between NBCn2-C and actin is likely mediated by the PDZ scaffolding protein NHERF1 and the membrane/cytoskeleton-linking protein ezrin (Lee et al., 2006).

Concluding remarks

Our increasing knowledge of the diverse roles of NCBTs and their variants is shedding some light on the necessity for their seemingly bewildering structural diversity. It is likely that further NCBT variants remain to be cloned. Moreover, we are still far from a complete understanding of the variants already described. Although the molecular consequences of many of these variations have been reported, studies directly addressing their physiological consequences are still in their infancy. A more complete sense of the temporo-spatial distribution of each NCBT variant – as well as the development of splice variant-specific knockout mice – should provide vital clues. Finally, we eagerly await studies of the

regulatory mechanisms that dictate the inclusion or exclusion of certain modules from an NCBT transcript.

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