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USE OF A NEW POLYCLONAL ANTIBODY TO STUDY THE DISTRIBUTION AND GLYCOSYLATION OF THE SODIUM-COUPLED BICARBONATE TRANSPORTER NCBE IN RODENT BRAIN

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Abstract—NCBE (SLC4A10) is a member of the SLC4 family of bicarbonate transporters, several of which play important roles in intracellular-pH regulation and transepithelial HCO_3^- transport. Here we characterize a new antibody that was generated in rabbit against a fusion protein consisting of maltose-binding protein and the first 135 amino acids (aa) of the N-terminus of human NCBE. Western blotting—both of purified peptides representing the initial ~120 aa of the transporters and of full-length transporters expressed in *Xenopus* oocytes—demonstrated that the antibody is specific for NCBE versus the two most closely related proteins, NDCBE (SLC4A8) and NBCn1 (SLC4A7). Western blotting of tissue in four regions of adult mouse brain indicates that NCBE is expressed most abundantly in cerebral cortex (CX), cerebellum (CB) and hippocampus (HC), and less so in subcortex (SCX). NCBE protein was present in CX, CB, and HC microdissected to avoid choroid plexus. Immunocytochemistry shows that NCBE is present at the basolateral membrane of embryonic day 18 (E18) fetal and adult choroid plexus. NCBE protein is present by Western blot and immunocytochemistry in cultured and freshly dissociated HC neurons but not astrocytes. By Western blot, nearly all NCBE in mouse and rat brain is highly N-glycosylated (~150 kDa). PNGase F reduces the molecular weight (MW) of natural NCBE in mouse brain or human NCBE expressed in oocytes to approximately the predicted MW of the unglycosylated protein. In oocytes, mutating any one of the three consensus N-glycosylation sites reduces glycosylation of the other two, and the triple mutant exhibits negligible functional expression.

Key words: slc4a10, CNS, brain, oocytes, glycosylation.

NCBE belongs to the SLC4 family of bicarbonate transporters, which in humans includes the products of at least 10 genes. NCBE is SLC4A10. The SLC4 transporters play essential roles in intracellular-pH regulation, transepithelial HCO_3^- transport, and (in the case of the erythrocyte) CO_2 transport. Within the SLC4 family are two major groups of HCO_3^- transporters: (1) Na-independent anion exchangers (AEs), including AE1, AE2 and AE3; and (2) Na-coupled HCO_3^- transporters. The latter can be further subdivided into two subtypes: (2a) electrogenic Na-coupled HCO_3^- cotransporters, including NBCe1 and NBCe2; and (2b) electroneutral Na-coupled HCO_3^- cotransporters, including NCBE, NBCn1 and NDCBE. The functional grouping of these eight transporters corresponds rather well with groupings based on amino acid (aa) sequence analyses (for reviews, see refs. Romero et al., 2004; Romero, 2005).

NCBE was first cloned by Wang et al. (2000) from a cDNA library from the mouse insulinoma cell line MIN6. Choi et al. (2002) isolated two splice variants from human brain and kidney, NCBE-A (the ortholog of the mouse clone) and NCBE-B (which contains a 30 aa insert near the middle of the presumed cytoplasmic amino terminal domain). Functional analysis of NCBE in *Xenopus* oocytes and HEK239 cells suggested that NCBE exchanges extracellular Na^+ and HCO_3^- for intracellular Cl^-. Thus, the clone was initially designated as a *N*a$^+$-driven *c*hloride/*b*icarbonate *e*xchanger. When expressed in *Xenopus* oocytes, hNCBE-B has absolute requirements for Na^+ and HCO_3^- (Choi et al., 2002) and is also blocked by DIDS (Choi et al., unpublished observations). However, its Cl^- dependence is unclear (Romero et al., 2004; Romero, 2005).

Giffard et al. (2003) isolated two splice variants, rb1NCBE and rb2NCBE, from rat (r) brain. The first, rb1NCBE, is analogous to human (h) NCBE-B. The second, rb2NCBE, contains an extra 21 aa at the presumed cytoplasmic carboxyl terminus, ending in the consensus PDZ motif ETCL. Recent work (Lee et al., 2006) shows that this PDZ motif of rb2NCBE interacts with ezrin binding protein 50 (EBP50), which in turn binds to the cytoskeleton-anchoring protein ezrin (Bretscher et al., 2000), which binds protein kinase A (PKA). Inhibition of protein kinase A appears to increase the activity of rb2NCBE (Lee et al., 2006).

By RNA blot analysis, NCBE is expressed at high levels in brain and at low levels in pituitary, testis, kidney and ileum (Wang et al., 2000). By *in situ* hybridization, NCBE is expressed in the CNS very early in development, in a pattern consistent with neuronal expression (Hübner et al., 2004). The same study showed that NCBE is expressed in the peripheral nervous systems and in non-neuronal tissues, such as the choroid plexus, the dura and some epithelia. Praetorius et al. (2004)

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Abbreviations: aa, amino acid; AE, anion exchanger; AP, affinity purification; AQP1, aquaporin 1; CB, cerebellum; Ct, C terminus; CX, cerebral cortex; EGFP, enhanced green fluorescent protein; E18, embryonic day 18; GST, glutathione transferase; HC, hippocampus; MBP, maltose-binding protein; MW, molecular weight; NGS, normal goat serum; Nt, N terminus; P, postnatal day; pH_i, intracellular pH; RT, room temperature; SCX, subcortex; SNK, Student-Newman-Keuls.

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developed an antibody generated against an 18-aa peptide corresponding to a portion of the C terminus (Ct) of mouse NCBE. An immunocytochemistry study has demonstrated that NCBE as well as NBCn1 is localized to the basolateral membrane of choroid-plexus epithelial cells, whereas NBCe2 is localized to the apical membrane (Bouzinova et al., 2005; Praetorius et al., 2004). Thus NCBE and NBCn1 may mediate net base influx into choroid-plexus epithelial cells, whereas NBCe2 may facilitate HCO_3^- and Na^+ transport from the cells into the cerebrospinal fluid, and thereby play an important role in cerebrospinal-fluid production (Bouzinova et al., 2005). However, so far there is no evidence at the protein level for the expression of NCBE in neurons.

In the present study, we characterized a new antibody directed against the amino terminus of hNCBE. By Western blotting, the antibody is specific for NCBE versus NDCBE or NBCn1 heterologously expressed in *Xenopus* oocytes. In mice and rats, the antibody detects a ~150-kDa band in cerebral cortex (CX), subcortex (SCX), cerebellum (CB), and hippocampus (HC)—including microdissected mouse CX, CB, and HC preparations that were free of choroid plexus. By immunocytochemistry, NCBE is present at the basolateral membrane of choroid plexus in both fetus and adult. In rat HC neuronal cultures, Western blotting demonstrates NCBE as a ~150-kDa band. Moreover, immunocytochemistry demonstrates the presence of the protein on the plasma membrane of both cultured and freshly dissociated neurons. Treatment with PNGase reduces the molecular mass to ~127 kDa in mouse brain tissue as well as oocytes heterologously expressing NCBE. Mutating the three consensus glycosylation sites eliminates glycosylation in oocytes.

EXPERIMENTAL PROCEDURES

Antibodies

A recombinant fusion protein consisting of maltose-binding protein (MBP) and the first 135 aas of human NCBE (GenBank accession: AY376402) in the cytoplasmic N terminus (Nt), was expressed in *E. coli* on a pMAL™-2 vector (New England Biolabs, Ipswich, MA, USA), and the fusion protein was purified by affinity chromatography. A rabbit was injected with the purified MBP-fusion protein and the resulting antiserum was used for the present study.

For affinity purification (AP) of the NCBE antiserum, we used a CarboxylLink™ Kit (Pierce, Rockford, IL, USA) following the instructions of the manufacturer. Briefly, a His-tagged peptide consisting of the first 123 aa of NCBE (see below) was covalently linked to a column of CarboxylLink™ Coupling Gel. The column was equilibrated with 100 mM PBS, pH 7.4. The crude antiserum diluted in PBS was applied to the column for incubation at room temperature (RT) for 2 h, and then the column was washed with PBS to remove the unbound proteins. The bound antibody was eluted with 100 mM glycine, pH 2.8, and the eluted antibody was neutralized by adding 1/10 volume of 1 M Tris–HCl.

A monoclonal antibody against His-tag was purchased from Novagen (San Diego, CA, USA). A monoclonal antibody against glutathione transferase (GST) was purchased from Chemicon International (Temecula, CA, USA). A polyclonal antibody against aquaporin 1 (AQP1) was purchased from Alpha Diagnostics International (San Antonio, TX, USA).

Construction of an enhanced green fluorescent protein (EGFP)–tagged full-length transporters in a *Xenopus* expression vector

Creating EGFP "donor" cDNA. Our starting material was NBCe1-A-EGFP.pGH19)—that is, hNBCe1-A cDNA (GenBank accession: NM_003759) cloned into a pGEM-based vector between the 5′ and 3′ UTRs of the *Xenopus* β-globin gene. The C terminus (Ct) of NBCe1-A is connected via the 5 aa linker "SPVAT" to the Nt of EGFP (Toye et al., 2006). In this construct the EGFP is flanked at both the 5′ end (i.e. within SPVAT) and 3′ end by *Age*I restriction sites. We eliminated the *Age*I site at the 3′ flank of EGFP (ACCGGT to ACCCGT) using the Quickchange® Site-Directed Mutagenesis Kit (Stratagene, Cedar Creek, TX, USA). This enabled the isolation of the EGFP cDNA cassette from the construct by digestion of the *Age*I site at the 5′ flank of EGFP and the *Xho*I site at the 3′ flank of EGFP located within the pGH19 vector sequence. The isolated cassette was *Age*I-EGFP-*Xho*I.

Creating NBC-like "acceptor" cDNA. Our starting materials were (*1*) NCBE-B.pGH19—that is, hNCBE-B cDNA (SLC4A10; GenBank accession: AY376402; this construct lacks the 30 aa cassette A and has a short Ct) cloned into pGH19 (Trudeau et al., 1995), (*2*) NDCBE1.pGH19—that is, hNDCBE1 cDNA (SLC4A8; GenBank accession: NM_004858) cloned into pGH19 and (*3*) NBCn1-B.pGH19—that is, rNBCn1-B cDNA (SLC4A7; GenBank accession: NM_058211) cloned into pGH19 (Choi et al., 2003). We introduced the sequence TGCTC*ACCGGT*A (i.e. Cys and Ser codons and an *Age*I restriction site) at a position immediately prior to the termination codon for each construct, using the Quickchange mutagenesis kit according to the manufacturer's protocol. The Ser represents the first residue of the SPVAT linker. The resulting constructs were NCBE-B/*Age*I.pGH19, NDCBE1/*Age*I.pGH19 and NBCn1-B/*Age*I.pGH19.

Creating NBC-like-EGFP.pGH19. The vector sequence between the *Age*I site (i.e. 3′ end of the NBC-like sequence) and *Xho*I site of NCBE-B/*Age*I.pGH19, NDCBE1/*Age*I.pGH19 and NBCn1-B/*Age*I.pGH19 was excised by enzymatic restriction and replaced with *Age*I-EGFP-*Xho*I to create NCBE-B-EGFP.pGH19, NDCBE1-EGFP.pGH19 and NBCn1-B-EGFP.pGH19 that is, NBC-like cDNA cloned into pGH19 between the 5′ and 3′ UTRs of the *Xenopus* β-globin gene and linked to a Ct EGFP via the 6 aa linker "CSPVAT" between the NBC-like sequence and EGFP.

cRNA injection of oocytes

Xenopus oocytes were prepared as previously described (Goldin, 1992). Briefly, female *Xenopus laevis* were anesthetized in a solution of 0.2% tricaine (ethyl 3-aminobenzoate methanesulfonate or MS-222, catalog# A5040, Sigma-Aldrich, St. Louis, MO, USA). Ovarian lobes were removed, placed in 0-Ca solution (98 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.5), cut into small pieces, and washed five times with 0-Ca solution. Oocytes were dissociated by enzymatic digestion with 2 mg/ml of type IA collagenase (Sigma-Aldrich) in 0-Ca solution. Stage V–VI oocytes were selected and kept at 18 °C in OR3 medium.

We made cRNA encoding the three transporters described in the previous section: (1) hNCBE-B with and without a Ct-EGFP tag, (2) rNBCn1-B with a Ct-EGFP tag, and (3) hNDCBE with a Ct-EGFP tag. Capped cRNAs encoding the above transporters were injected into *Xenopus* oocytes, which we incubated for 4–5 days at 18 °C before collecting them for membrane protein preparation.

Site-directed mutagenesis of NCBE

The putative extracellular loop between the TM5 and TM6 of hNCBE has three potential N-glycosylation motifs (^{677}NGTL, ^{687}NISA and ^{697}NLTV). The three Asn residues were mutated to