A SLC4A10 gene product maps selectively to the basolateral plasma membrane of choroid plexus epithelial cells

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Submitted 9 June 2003; accepted in final form 28 October 2003

Praetorius, J., L. N. Nejsum, and S. Nielsen. A SLC4A10 gene product maps selectively to the basolateral plasma membrane of choroid plexus epithelial cells. Am J Physiol Cell Physiol 286: C601–C610, 2004. First published October 30, 2003; 10.1152/ajpcell.00240.2003.—The choroid plexus epithelium of the brain ventricular system produces the majority of the cerebrospinal fluid and thereby defines the ionic composition of the interstitial fluid in the brain. The transepithelial movement of Na⁺ and water in the choroid plexus depend on a yet-identified basolateral stilbene-sensitive Na⁺-HCO₃⁻ cotransporter. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis revealed the expression in the choroid plexus of SLC4A10 mRNA, which encodes a stilbene-sensitive Na⁺-HCO₃⁻ cotransporter. Anti-COOH-terminal antibodies were developed to determine the specific expression and localization of this Na⁺-HCO₃⁻ cotransporter protein. Immunoblotting demonstrated antibody binding to a 180-kDa protein band from mouse and rat brain preparations enriched with choroid plexus. The immunoreactive band migrated as a 140-kDa protein after N-deglycosylation, consistent with the predicted molecular size of the SLC4A10 gene product. Bright-field immunohistochemistry and immunoelectron microscopy demonstrated strong labeling confined to the basolateral plasma membrane domain of the choroid plexus epithelium. Furthermore, the stilbene-insensitive Na⁺-HCO₃⁻ cotransporter, NBCn1, was also localized to the basolateral plasma membrane domain of the choroid plexus epithelium. Hence, we propose that the SLC4A10 gene product and NBCn1 both function as basolateral HCO₃⁻ entry path-ways and that the SLC4A10 gene product may be responsible for the stilbene-sensitive Na⁺-HCO₃⁻ uptake that is essential for cerebrospinal fluid production.

A CONSTANT AND OPTIMAL COMPOSITION of the cerebrospinal fluid (CSF) is essential for normal neuronal function, because the brain interstitium is supplied with its solutes, water, and micronutrients by the CSF (11). The major site of CSF production is the epithelial cells of the choroid plexus situated in the lateral ventricles and the third and fourth ventricles. From this site, the CSF flows through the ventricular system to the subarachnoid space and is reabsorbed into the bloodstream (for review, see Ref. 7). During the passage, water, nutrients, and waste products exchange through the very leaky ependymal epithelium lining the ventricles and the outer brain surface. Transepithelial movement of Na⁺ is a central element in the production of CSF (11). The apical plasma membrane Na⁺,K⁺-ATPase of the choroid plexus epithelium is directly responsible for the movement of intracellular Na⁺ to the CSF (21, 27). The Na⁺,K⁺-ATPase has a high transport capacity in the continued presence of ATP, which leaves Na⁺ translocation across the basolateral membrane as rate limiting for transepithelial Na⁺ transport.

A basolateral plasma membrane Na⁺/H⁺ exchanger (NHE) was thought to mediate Na⁺ entry into the choroid plexus epithelial cells and to explain the pH dependence of CSF production. The evidence in support for this view was the amiloride sensitivity of the Na⁺-dependent CSF secretion (6, 19), and NHE1 mRNA was accordingly found in the choroid plexus (13). The high concentration of amiloride necessary to affect 22Na⁺ uptake, however, suggests an atypically low sensitivity of NHE1 to the drug (18, 20), which speaks against the involvement of NHE1 in the observed Na⁺ uptake. Moreover, NHE proteins have never been detected directly in the choroid plexus, suggesting a rather low expression of NHE proteins. Finally, Na⁺/H⁺ exchange seemed to be of little significance for choroid plexus pH regulation in the presence of normal Na⁺ and HCO₃⁻ levels (18). These findings suggest the presence of other proteins to explain the basolateral choroid plexus Na⁺ transport.

Conversely, the major component of the Na⁺ extrusion into the CSF was coupled to HCO₃⁻ secretion (17), supporting the finding that apical fluid production is enhanced with increased basolateral HCO₃⁻ concentration in vitro (10). Furthermore, basolateral HCO₃⁻ entry into frog choroid plexus was stillbene sensitive and utilized the Na⁺ gradient as driving force (22). These findings imply a possible role for a basolateral Na⁺-driven HCO₃⁻ uptake mechanism at least in choroid plexus acid/base handling. The strongest evidence for a critical involvement of HCO₃⁻ transport is, however, that pH restoration after acidification of cultured choroid plexus epithelial cells depends on Na⁺ and HCO₃⁻ at the basolateral side and is prevented by the application of a stilbene (18). This mechanism is very likely identical to the reported stilbene-sensitive HCO₃⁻ uptake into the choroid plexus epithelium (12).

Transmembrane Na⁺-dependent HCO₃⁻ transport is maintained by proteins belonging to a single family of transporters (3). The SLC4A family includes the electronegative and electroneutral Na⁺-HCO₃⁻ cotransporters (NBCs), the Na⁺-dependent Cl⁻/HCO₃⁻ exchangers (NCBE/SLC4A10 and NDBCE/SLC4A8), and the classic Na⁺-independent Cl⁻/HCO₃⁻ exchangers (AEs). The reported dependence of the SLC4A10 gene product on intracellular Cl⁻ (26) has been disputed by other researchers in an abstract (5). The transporter was characterized as an electronegative Na⁺-HCO₃⁻ cotransporter in the latter study. Nevertheless, there is general agreement that the observed HCO₃⁻ import is Na⁺ dependent and that the transporter is electroneutral and sensitive to a stilbene inhibitor.
Of the aforementioned Na\(^{+}\)-dependent HCO\(_3^{-}\) transporters, the expression of electronegotic NBC1 mRNA has been reported in choroid plexus by in situ hybridization (24). NBC4 mRNA expression also has been demonstrated in homogenates from whole brain (23), and its presence in choroid plexus cannot be excluded because the brain localization of NBC4 has not yet been studied in detail. Furthermore, brain expression of the electroneutral NBCn1 was previously reported by reverse transcriptase-polymerase chain reaction (RT-PCR) and Northern blotting (4) and SLC4A10 and SLC4A8 mRNA also were detected in whole brain preparations by Northern blotting (26, 9). Thus relatively few proteins are candidates for the basolateral Na\(^{+}\)-HCO\(_3^{-}\) transport in choroid plexus epithelial cells. We combined RT-PCR, immunoblotting, and immunohistochemical analysis to identify the basolateral Na\(^{+}\)-HCO\(_3^{-}\) transporters of mouse and rat choroid plexus epithelial cells.

MATERIALS AND METHODS

Experimental animals. Adult male C57bl6 mice (30–80 g) and Wistar rats (250–400 g) from M&B had free access to water and pelleted food. The animals were anesthetized by halothane inhalation, the brain and other organs were dissected, and the animals were killed. Organs were then rinsed in a 4°C saline solution, and the brain was divided into samples of entire cerebellum or cerebrum (including the choroid plexus). For immunoblotting, samples enriched with lateral, third, and fourth ventricle choroid plexuses were scraped from the ventricular system of separate animals with a slim scalpel. The isolates contained ~21% choroidal plexus as validated by electron microscopy (12 of 56 grid fields contained mouse choroid plexus, whereas the remaining tissue represented adjacent glia, neurons, and blood vessels). Alternatively, third and fourth ventricle choroid plexus was isolated by microdissection of 1-mm rat brain slices at 4°C (Tris-buffered saline, pH 7.4). The choroid plexus was detached from the brain parenchyma and the ependyma with forceps under microscopy (12 of 56 grid fields contained mouse choroid plexus, whereas the remaining tissue represented adjacent glia, neurons, and blood vessels). Alternatively, third and fourth ventricle choroid plexus was isolated by microdissection of 1-mm rat brain slices at 4°C (Tris-buffered saline, pH 7.4). The choroid plexus was detached from the brain parenchyma and the ependyma with forceps under microscopy (12 of 56 grid fields contained mouse choroid plexus, whereas the remaining tissue represented adjacent glia, neurons, and blood vessels).

RT-PCR and sequence analysis. Total RNA from fresh tissues was extracted with RNeasy Mini Kits (Qiagen, Germantown, MD), or, for microdissected choroid plexus, mRNA was isolated with a Dynabeads mRNA Direct Micro Kit (Dynal, Oslo, Norway). After DNase treatment (RQ1 RNase-Free DNase, Promega, Madison, WI), the RNA was reverse-transcribed with 2 U/μl reverse transcriptase (Superscript II, Invitrogen, Taastrup, Denmark) in the presence of either poly-T or specific reverse primers for NBC transcripts (gene-specific reverse transcription). PCR (HotStarTag Master Mix, Qiagen) with 10–20% cDNA and 1 pmol of each primer was performed for 30 cycles: hot start at 95°C for 15 min, denaturation at 95°C for 30 s, annealing at 56–60°C (dependent on primer optimum) for 30 s, and elongation at 72°C for 1 min. Negative PCR controls included omission of reverse transcriptase or omission of cDNA. \(^3\)End sequences of SLC4A10 mRNA in cerebrum and choroid plexus were determined by \(^3\)P rapid amplification of cDNA ends (\(^3\)RACE; SMART RACE cDNA amplification kit, BD Biosciences, Erembodegem, Belgium).

PCR for β-actin was performed to validate each batch of template before use. HCO\(_3^{-}\) transporter-specific primers are listed in Table 1. PCR products were submitted to gel electrophoresis separation (2% agarose) and photographed under ultraviolet illumination, and bands of predicted size for each of the PCR products were excised from the gel and purified with a QIAquick Gel Extraction Kit (Qiagen) for nucleotide sequencing (Lark Technologies, Saffron Walden, UK).

Table 1. Primers used for detection of SLC4A10 and NBCn1 in brain

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>SLC4A10</td>
<td>GCTTCCTCCAGCTGTGTCGAGC</td>
<td>TCTGTCCTCCAGCTGTGGCAA</td>
</tr>
<tr>
<td>NBCn1</td>
<td>CACAGCTTGATGCTGATG</td>
<td>CTACTCAAGAGTTTCTAGG</td>
</tr>
<tr>
<td>NBC4</td>
<td>GCTGAAAGAGGCTGAGTGAGTAC</td>
<td>ACGCCCTTAAATGGACCCAGAACAGG</td>
</tr>
<tr>
<td>NBCC</td>
<td>ATGGAGAAGCTTCTGGTGGCAC</td>
<td>TCTAGCAGAGAGCATGGC</td>
</tr>
<tr>
<td>BTCR</td>
<td>CACGCTCTGCTGACTACTCC</td>
<td>TGGGAGACAGCTGTCAGTGT</td>
</tr>
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Antibodies. An 18-amino acid COOH-terminal peptide (C-DNSKEKERSFPPSKSSPS) was used for rabbit immunization based on the published mouse SLC4A10 sequence [National Center for Biotechnology Information (NCBI) accession no. AB033759]. The peptide also corresponds to the last 17 amino acids of the predicted COOH terminus of the rat SLC4A10 product, “r1NBCe” (AF439856). Two rabbits were injected with the peptide, and the resulting antisera were affinity purified with the immunizing peptide coupled to an agarose column (Sulfolink, Pierce, Rockford, IL). Previously described antibodies against NBCn1 (25), Na\(^{+}\)-K\(^{-}\)-ATPase α\(_1\)-subunit (14), AE2 (1), and AE4 (15) were also applied for immunolabeling.

Immunoblotting. The protein contents of cerebellum, cerebrum and choroid plexus homogenates were determined with a biarchimonic acid protein assay reagent kit (Pierce) after the suspension of nuclei and unbroken cells was cleared by 4,000 g centrifugation. For deglycosylation, 5 μg of protein was incubated overnight with 1 U/10 μlPNGase F (Boehringer-Mannheim, Germany). Protein samples were adjusted to 1.5% (wt/vol) sodium dodecyl sulfate, 40.0 mM 1,4-dithiothreitol, 6% (vol/vol) glycerol, and 10 mM Tris, pH 6.8 with bromophenol blue. The samples were heated to 65°C for 15 min and stored at −20°C until use. Protein samples of 2–10 μg were separated by 9% polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose membranes, which were blocked by incubation in 5% nonfat dry milk in a phosphate-buffered saline (PBS) solution (PBS-T; in mM: 80 Na\(_2\)HPO\(_4\), 20 NaH\(_2\)PO\(_4\), and 100 NaCl, pH 7.5, with 0.1% vol/vol Tween 20). The membranes were incubated overnight at 5°C with primary antibody in PBS-T. After washing, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) secondary antibody (Dako, Glostrup, Denmark) for 2 h in PBS-T. Excess antibody was then removed by extensive washing, and bound antibody was detected with an ECL chemiluminescence kit (Amersham, Little Chalfont, UK). Semiquantification of the immunoreactive proteins was performed with standard equipment for densitometry. The band intensities were measured within the linear range and corrected for differences in sample loading with Coomassie blue-stained control gels.

Immunohistochemistry. Halothane-anesthetized animals were fixed by retrograde perfusion via the abdominal aorta with 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, and brain and control tissues were removed. The tissues were dehydrated and embedded in paraffin, and 2-μm sections were cut with a rotary microtome (Leica, Heidelberg, Germany). The sections were dewaxed and rehydrated, and endogenous peroxidase was blocked by 0.5% H\(_2\)O\(_2\) in absolute methanol. To reveal antigens, the sections were boiled in 1 M Tris, pH 9, supplemented with 0.5 mM EGTA. Nonspecific binding of immunoglobulin was quenched by incubating the sections in 50 mM NH\(_4\)Cl and blocked in PBS supplemented with 1% bovine serum albumin (BSA), 0.05% saponin, and 0.2% gelatin. The sections were incubated overnight at 4°C with primary antibodies diluted in PBS supplemented with 0.1% BSA and 0.3% Triton X-100. After washing, the sections were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Dako) diluted in PBS supplemented with BSA and Triton X-100. The peroxidase stain was visualized by 0.05% 3,3'-diaminobenzidine tetrahydrochloride dis-
solved in PBS with 0.1% H$_2$O$_2$. Mayer’s hematoxylin was used for countercutting, and the sections were dehydrated and mounted in hydrophobic Eukitt mounting medium (O. Kindler, Freiburg, Germany). Microscopy was performed on a Leica DMRE bright-field microscope equipped with a Leica DM300 digital camera. For immunofluorescence microscopy, the sections were incubated with Alexa488-conjugated goat anti-rabbit secondary antibodies (Molecular Probes, Eugene, OR) in PBS supplemented with BSA and Triton X-100. After washing, sections were mounted with a coverslip on a Leica DMRS confocal microscope with an HCX PL apo × 64 (1.32 NA) objective. The immunofluorescence images were merged with differential interference contrast (DIC) images to reveal the relationship between the tissue structures and the fluorescence labeling.

**Immunogold electron microscopy.** Tissue blocks prepared from mouse brain were cryoprotected with 2.3 M sucrose containing 2% paraformaldehyde and rapidly frozen in liquid nitrogen. The samples were freeze-substituted by sequential equilibration over 3 days in methanol containing 0.5% uranyl acetate at temperatures raised gradually from −80 to −70°C, rinsed in pure methanol for 24 h while the temperature was increased from −70 to −45°C, and infiltrated with Lowicryl HM20 and methanol:1:1, 2:1, and, finally, pure Lowicryl HM20 before ultraviolet polymerization for 2 days at −45°C and 2 days at 0°C. Immunolabeling was performed on ultrathin Lowicryl HM20 sections. Sections were pretreated with a saturated solution of NaOH in absolute ethanol (2–3 s), rinsed, and preincubated for 10 min with 0.1% sodium borohydride and 50 mM glycine in 0.05 M Tris, pH 7.4, containing 0.1% Triton X-100. Sections were rinsed and incubated overnight at 4°C in 0.1% sodium borohydride and 50 mM glycine in 0.05 M Tris, pH 7.4, containing 0.1% Triton X-100 with 0.2% milk. After rinsing, sections were incubated for 1 h at room temperature with goat anti-rabbit IgG conjugated to 10-nm colloidal gold particles (GAR.EM10, BioCell Research Laboratories, Cardiff, UK). The sections were stained with uranyl acetate and lead citrate before examination in a Philips Morgagni 268D electron microscope.

**RESULTS**

**Expression of HCO$_3$" transporter mRNA in choroid plexus.** The expression of specific transporter mRNA was analyzed to determine the presence of the SLC4A gene products in the choroid plexus and brain. Two NCBE transcripts were detected from RNA samples from rat cerebrum and cerebellum and in choroid plexus-enriched samples from mouse and rat (Fig. 1A). The ~580-bp product shared 100% nucleotide identity with the rb1 variant of SLC4A10 (accession no. AF439856), whereas the ~490-bp product shared 100% nucleotide identity with the rb2 variant of SLC4A10 (accession no. AF439855). Amplification of the shorter product dominated when mRNA from the microdissected rat choroid plexus was used as template for the RT-PCR. 3′RACE using cerebral and choroid plexus RNA yielded only a single band. The translated 3′ sequence revealed 100% identity to the predicted COOH terminal of the rb1 variant, i.e., the amino acid sequence used for antibody production in the present study.

The expression of NBCn1 mRNA was also detected consistently in mouse choroid plexus-enriched samples as well as in the microdissected rat choroid plexus (Fig. 1B). The amplified sequence of 330 bp contained a variable exon, the so-called “B-cassette”; the splice variant without the B-cassette was not found. An NBC4 transcript was likewise found in the choroid plexus-enriched samples from mouse and in the microdissected rat choroid plexus, but NBC1, NDCBE1, and BTR1 mRNAs were not detected (Fig. 1, C–F, respectively). Identical results were obtained for NBC1 when RT-PCR was repeated with four other choroid plexus isolates. Sequence analysis of representative PCR products from choroid plexus or positive control tissues revealed 100% nucleotide identity to the published sequences for each of NBCn1, NDCBE1, NBC4, NBC1, and BTR1. Interestingly, two NDCBE products were formed in control tissue and sequence analysis revealed alternative splicing in the probed region.

**SLC4A10 gene expression in brain by immunoblotting.** A KLH-conjugated peptide corresponding to the 17 COOH-terminal amino acids of the predicted SLC4A10 gene product was synthesized and used for immunization of rabbits. The unconjugated peptide was blotted onto nitrocellulose membranes to test the ability of the produced antisera to bind the epitope. The antisera recognized the peptide of 2 kDa, illustrated in Fig. 2A, as expected. However, clear bands of ~4, 6, and 8 kDa were also seen, apparently reflecting di-, tri-, and tetramers of the unconjugated peptide. The KLH-conjugated form of the peptide was injected into the rabbits and hence was not tested for polymerization. The antibody was affinity purified and used for immunoblotting of proteins isolated from mouse and rat brain homogenates. Figure 2B shows the binding of the anti-SLC4A10 antibody to the expected ~2 kDa band, as well as to the ~4, ~6, and 8 kDa bands.
SLC4A10 antibody to an ~180-kDa protein of mouse and rat samples enriched with choroid plexus. Preabsorbing the antibody with the immunizing peptide completely prevented binding to the proteins (Fig. 2B). Semiquantitative immunoblotting of protein samples from microdissected rat choroid plexus, entire cerebrum, entire cerebellum, and corresponding samples devoid of visible choroid plexus revealed a 7- to 16-fold (range) stronger expression of the immunoreactive protein in the choroid plexus compared with the other tissues (Fig. 2C; Table 2). Deglycosylation by PNGaseF reduced the molecular mass from 180 kDa toward the expected size of 140 kDa (Fig. 2D), indicating the protein to be glycosylated. Thus the antibodies bind to the sequence-specific SLC4A10-derived peptide and to a brain protein of the expected molecular size. This brain protein seems to be predominantly expressed in the choroid plexus.

**Immuno localization of a SLC4A10 gene product in mouse and rat brain.** Brain sections were stained with affinity-purified antibodies to map the cerebral and cerebellar distribution of the SLC4A10 gene product in mouse and rat. Strong immunoreactivity was observed in the third ventricle choroid plexus of mouse and rat brain by light microscopic analysis of immunoperoxidase-stained sections (Fig. 3, A and B, respectively). The labeling was restricted to the basolateral plasma membrane domains of the epithelial cells of the mouse and rat choroid plexus when visualized at high magnification (Fig. 3, C and D, respectively). Preabsorbing the antibody with the immunizing peptide completely prevented staining of mouse and rat choroid plexus in the consecutive brain sections (Fig. 3, E and F, respectively). The surrounding tissue did not label for SLC4A10 with fluorescence double-labeling with Na\(^+\),K\(^+\)-ATPase antibodies, as shown in Fig. 3G. The basolateral localization of the SLC4A10 gene product was confirmed by higher-resolution immunofluorescence confocal microscopy. The apical Na\(^+\),K\(^+\)-ATPase was clearly expressed in a membrane domain opposite to the SLC4A10 gene product (Fig. 3H). The staining intensities and immunolabeling patterns seemed identical in the choroid plexus of the lateral, third, and fourth ventricle, whereas staining was absent in all other parts of the mouse and rat brain including the ciliated ventricular or brain surface ependyma (not shown).

The SLC4A10 gene products are likely to be transmembrane proteins as deduced from the predicted amino acid sequence, but a close membrane association can normally not be confirmed by light microscopy. Therefore, immunoelectron microscopic analysis was conducted with the anti-SLC4A10 antibodies on Lowicryl-embedded brain tissue. Distinct immunogold labeling was seen in close relation to the basolateral plasma membrane of mouse choroid plexus epithelial cells (Fig. 4A), whereas the apical plasma membrane was unlabeled (Fig. 4B). An identical labeling pattern was found in the rat choroid plexus (not shown). The gold particles were always found on the intracellular side of the plasma membrane, indicating that the COOH-terminal epitope faces the cytoplasm. The highly folded membrane processes between neighboring epithelial cells were more strongly labeled than the basal and lateral surfaces. The sparse labeling of the basal and lateral plasma membrane was confined to small protrusions of these

Table 2. Densitometric analysis of rat brain SLC4A10 immunoblotting

<table>
<thead>
<tr>
<th>Protein Sample</th>
<th>Antibody A</th>
<th>Antibody B</th>
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<tbody>
<tr>
<td>Cerebrum, +pl.c.</td>
<td>16.8</td>
<td>22.6</td>
</tr>
<tr>
<td>Cerebellum, +pl.c.</td>
<td>19.0</td>
<td>26.9</td>
</tr>
<tr>
<td>Cerebrum, -pl.c.</td>
<td>8.3</td>
<td>12.8</td>
</tr>
<tr>
<td>Cerebellum, -pl.c.</td>
<td>6.4</td>
<td>14.8</td>
</tr>
</tbody>
</table>

Rat cerebral and cerebellar bands from Fig. 2C were quantified relative to bands obtained with microdissected choroid plexus. +pl.c., samples containing choroid plexus; -pl.c., denotes samples without visible choroid plexus (see MATERIALS AND METHODS). Band densities were corrected for SDS-gel protein loading.
membranes. In contrast, the nucleus, mitochondria, vesicles, and cytosol were all negative for SLC4A10 gene product labeling by electron microscopy, as were other cells and structures of the choroid plexus. Figure 4C shows the same epithelial cell at lower magnification to demonstrate the structural orientation of the details in Fig. 4, A and B. Hence, the antibody specifically labeled the choroid plexus and staining was exclusively associated with the basolateral plasma membrane.

**Immunohistochemical localization of additional HCO$_3^-$ transporters in brain.** The localization of other HCO$_3^-$ transport proteins was examined on paraformaldehyde-fixed mouse and rat brain with previously characterized antibodies. The basolateral membrane domain of choroid plexus epithelial cells stained with an anti-NBCn1 antibody in both mouse and rat brain as illustrated in Fig. 5, A and B. A similar labeling pattern was observed with a second anti-NBCn1 antibody (not shown). The NBCn1 immunolabeling was intensified near the highly
folded basal interface between neighboring cells of the choroid plexus, similar to the localization of the SLC4A10 gene product. AE2 was previously demonstrated in the basolateral plasma membrane of choroid plexus cells (1, 16), and anti-AE2 antibody was applied to explore whether the labeling of plasma membrane subdomains was a general feature of HCO3−/H+ transporters in the choroid plexus. Indeed, AE2 labeling also seemed to be intensified toward the basal intraepithelial interface of mouse and rat choroid plexus (Fig. 5, C and D, respectively). With regard to AE4, antibodies stained the apical domain of ciliated ependymal cells of mouse and rat third ventricle (Fig. 5, E and F) but did not label the choroid plexus. The labeling of positive control sections from the same species processed in parallel with the brain sections using the same antibody dilutions is also shown in Fig. 5 (insets). Positive control sections show labeling of duodenal villus cells (Fig. 5A), renal medullary thick ascending limbs (Fig. 5B), and intercalated cells of the collecting duct (Fig. 5, E and F).
Antibodies against AE1, kidney type NBC1, NHE1, NHE2, and NHE3 did not label the choroid plexus (not shown). For the remaining proteins of interest, NDCBE1- and NBC4-reliable antibodies have not yet been developed.

DISCUSSION

The current study aimed to define candidate proteins involved in choroid plexus transepithelial movement of \( \text{Na}^+ / \text{H}^+ \) and \( \text{HCO}_3^- / \text{H}^+ \). The SLC4A10 gene product and the electroneutral \( \text{Na}^+ / \text{HCO}_3^- \) cotransporter NBCn1 were both expressed in choroid plexus as assessed by RT-PCR analysis and immunoblotting. Immunohistochemical analysis localized both proteins to the basolateral plasma membrane domain of the epithelial cells.

**SLC4A10 gene expression in choroid plexus.** SLC4A10 mRNA was previously shown by Northern blot analysis mainly to be expressed in brain, although it was also detectable in testis, kidney, ileum, and pituitary gland (26). The SLC4A10 mRNA expression in the mouse and rat brains was confirmed by RT-PCR and sequence analysis. Interestingly, RT-PCR products were detected corresponding to two splice variants of the SLC4A10 mRNA. This variation seems to be specific to the brain, because only one PCR product appears when, e.g., kidney cDNA is probed with the same primers (unpublished observations). The small PCR product obtained with the microdissected rat choroid plexus clearly dominated the reaction and corresponds to the “rb2NCBE” variant. However, there are at least two variable regions in the SLC4A10 gene, the 5’ end and the fragment we probed for in this study. Because very little is known regarding the possible combinations of these variations, it cannot be concluded that similarity to rb2NCBE in the shown PCR analysis is accompanied by the expression of the rb2NCBE 5’ end (corresponding to the COOH terminal). In fact, 5’RACE analysis indicated that only the rb1NCBE 5’ end is expressed in rat brain whereas the rb2NCBE 5’ end is found in other organs (unpublished observations). Hence, it is most likely that a rb1NCBE COOH-terminal peptide-derived antibody would stain brain structures selectively. The significance of the variable splicing and whether all mRNA combinations result in protein expression remain to be examined.

Rabbit antisera were produced with a peptide sequence specific to the SLC4A10 gene product. The peptide corresponds to the last 17 amino acids of the predicted COOH terminus of rb1NCBE. The peptide is also identical to a
14-amino acid sequence located 35–21 amino acids from the predicted rb2NCBE COOH terminus. However, binding to the latter protein seems to be negligible, because no other SLC4A10-positive organs label with the antibody. The affinity-purified antibodies were characterized by immunoblotting of various mouse and rat brain preparations. First, the specificity of the formed bands was ensured by the disappearance of immunoreactivity when the antibodies were preabsorbed with the immunizing peptide. Second, immunoblotting revealed an immunoreactive rat brain protein that seemed much more abundant in the isolated choroid plexus than in the reminder of the cerebrum and cerebellum by semiquantitative immunoblotting. This detection of a SLC4A10-derived protein in the choroid plexus is strongly supported by the expression of the corresponding mRNA as described above. The greater abundance of the SLC4A10-derived protein in the isolated choroid plexus is likely underestimated because the removal of all choroid plexus cells from the ventricular system is difficult if not impossible when whole cerebral and cerebellar samples are required. Isolation of more cortical brain samples as choroid plexus-free controls would not have helped to clarify the issue, because the SLC4A10-derived protein might still have been expressed in deeper brain regions. Therefore, it is conceivable, but not proven, that the detected SLC4A10 gene product is confined to the choroid plexus in brain.

An immunoreactive band was formed with a slightly smeared appearance by using choroid plexus membrane fractions. The detected protein was ~30 kDa larger than expected from the NCBE amino acid sequence. Both observations suggest that the immunoreactive protein is normally glycosylated. The SLC4A10 product has seven asparagine residues in extra-cellular loops that are putative sites for N-glycosylation, and, indeed, deglycosylation resulted in formation of a single immunoreactive band close to the expected size. In conclusion, the antibody binding is consistent with recognition of a SLC4A10 gene product, which seems to be a glycosylated protein predominantly expressed in the choroid plexus.

Immunohistochemical analysis showed a remarkable staining pattern for the two NCBE antibodies: an intense labeling was confined to the choroid plexus epithelial cells and was exclusively associated with the basolateral plasma membrane domains. The staining intensified toward the basal infoldings under conventional microscopy, a feature that was confirmed by immunoelectron microscopy. The highly folded processes between neighboring epithelial cells stained far more for the SLC4A10 gene product than for the straight basal and the lateral surfaces. This particular localization could simply be a function of the larger surface area in these folded regions. Nevertheless, this is both a site of possible intracellular communication and a narrow passage into the lateral intracellular space of the tight junction area and likely limits the diffusion of solutes into this space. Clearly, extensive studies of the possible differential ionic distribution in the cytosol, the basal fluid compartment, the lateral intracellular space, and the apical fluid compartment are warranted to uncover the significance of this finding.

Interestingly, the gold particles on the electron micrographs were all situated on the cytosolic side of the plasma membrane, implying that the COOH terminus of the SLC4A10 gene product is intracellular. This is seemingly a common feature for all described proteins of the HCO3− transporter gene family and for most transmembrane ion transport proteins, to our knowledge. The NH2 and COOH termini of HCO3− transporters tend to be highly variable from cell type to cell type. In relation to this phenomenon, there has been much speculation as to whether the variations in putative phosphorylation sites within these amino acid sequences reflect a differential acute regulation of transporter function or of protein trafficking. To date, there is no clear overview on this topic. Nevertheless, the abundant expression of the SLC4A10 gene product in the basolateral plasma membrane suggests that the protein may play a role in the reported stilbene-sensitive Na+-dependent HCO3− uptake into choroid plexus epithelial cells.

**NBCn1 and other HCO3− transporters of choroid plexus.** Other HCO3− transporters of potential relevance to choroid plexus epithelia include the electroneutral NBCn1, the electronegenic NBC1 and NBC4, as well as the Cl−-dependent NBC1 and the anion exchangers AE2 and AE4. The detection of NBCn1 mRNA in the isolated choroid plexus was substantiated by the localization of the NBCn1 protein to the basolateral surface of the epithelial cells. The amplified mRNA sequence contained a variable exon, the so-called B-cassette (4). Although many tissues express NBCn1 mRNA both with and without the B-cassette, alternative splicing of this sequence does not seem to occur in the choroid plexus. The B-cassette contains a highly possible phosphorylation site and could

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**Fig. 6.** Revised model of the Na+ and acid/base equivalent transport in choroid plexus epithelia. Apical Na+–K+–ATPase creates an inward electrochemical Na+ gradient by pumping Na+ into the cerebrospinal fluid (CSF) in exchange for K+. This drives basolateral electroneutral Na+ uptake by Na+/H+ exchange (NHE), Na+–HCO3− transport (SLC4A10 gene product and NBCn1), as well as the apical Na+ uptake by Na+–K+–2Cl− cotransport (NKCC1). Cl− (and/or HCO3−) efflux by CIC-2 is also facilitated by the inside negative membrane potential (set by the Na+–K+–ATPase). The basolateral alkaline extruder, AE2, is driven by difference in cross-membrane pH. Carbonic anhydrase (CA) contributes to accumulation of intracellular HCO3− by catalyzing the conversions of CO2/H2O to HCO3−/H+. NBC4 is also possibly localized to the epithelial cell plasma membrane (not shown).
possibly be involved in NBCn1 regulation in the choroid plexus.

Staining with the NBCn1 and AE2 antibodies intensified corresponding to the folded basal-lateral domain of the epithelial cells. Hence, this distribution appears to be common for at least three basolateral HCO$_3^-$ transporters. Again, it is likely that the extensive expression of these transporters in the basal infoldings is a function of the larger surface area in these folded regions. On the other hand, one could speculate that this localization would enable the HCO$_3^-$ transporters to control, e.g., the pH and Na$^+$ or Cl$^-$ content of the intracellular lateral spaces.

Brain NBC1 is mainly expressed in glial tissue and contains a unique COOH-terminal amino acid sequence compared with the renal type (8, 2). Immunohistochemical evidence for NBC1 in choroid plexus has not been presented to date, but mRNA encoding NBC1 has been detected in the choroid plexus by in situ hybridization (24). In light of the epithelial origin, it is possible that the choroid plexus contains an epithelial NBC1 form like the renal NBC1 rather than the glial NBC1 form. Therefore, brain sections were probed with an antibody against the common COOH terminal of both epithelial forms of NBC1, the renal and the pancreatic NBC1. We found no immunolabeling for NBC1 in the choroid plexus, and in contrast to the earlier report (24), the choroid plexus in the present study did not contain detectable NBC1 mRNA in any of the several isolates of choroidal plexus. It is therefore possible that the referred in situ hybridization may have been caused by unspecific probe binding.

An NBC4-specific mRNA sequence was amplified from the isolated choroid plexus. This is potentially important, because NBC4 is a stilbene-sensitive Na$^+$-HCO$_3^-$ cotransporter and, hence, may be a candidate as a basolateral HCO$_3^-$ transporter alongside the SLC4A10 gene product. However, it is uncertain whether NBC4 resides in the basolateral plasma membrane of the epithelial cells or whether other cells in the microdissected tissue express NBC4. In addition to the epithelial cells, these samples contain blood-filled fenestrated capillaries and the surrounding connective tissue. Unfortunately, the finding of NBC4 mRNA in the choroid plexus could not be verified and the protein could not be localized by antibody binding because of the lack of reliable antibodies.

The presence of additional known or putative Na$^+$-HCO$_3^-$ transporters in the choroid plexus is unlikely, because the observed AE4 immunoreactivity in brain seemed to be restricted to the ciliated ependymal cells of the ventricular system and because NDCBE1 and BTR1 mRNA were not found in the isolated choroid plexus. In conclusion, a SLC4A10 gene product seems to be accompanied by NBCn1 and AE2 in the basolateral plasma membrane of choroid plexus epithelial cells and NBC4 is possibly also expressed in the choroid plexus.

Cellular HCO$_3^-$ uptake by the SLC4A10 gene product is stillbene sensitive (25), whereas NBCn1 is relatively stillbene insensitive (4). Therefore, the previously observed Na$^+$-dependent HCO$_3^-$ uptake (22, 18) most likely reflects the function of the SLC4A10 product rather than NBCn1. Thus, as in other tissues, NBCn1 may transport HCO$_3^-$ into the cell simply to counteract intracellular acid challenges or, perhaps, volume changes. In addition to cellular pH regulation, we propose that the SLC4A10 product could contribute to the pH homeostasis of the CSF and thereby be centrally involved in transepithelial Na$^+$, HCO$_3^-$, and fluid secretion. The Na$^+$-HCO$_3^-$ transporters are shown on the revised model of choroid plexus epithelial ion transport (Fig. 6), which is based on the present data, previous localization reports, and transport studies. According to the model, at least three proteins can function as basolateral base importers or acid excluders in the choroid plexus epithelium: the SLC4A10 gene product, NBCn1, and NHE. Na$^+$ is transported to the ventricular lumen by the apical Na$^+$-K$^+$-ATPase, and the outward HCO$_3^-$ transport is likely to occur via ClC-2 or by a yet-undetected electrogenic HCO$_3^-$ exit mechanism. It is noted that NBC4 could be located anywhere in the plasma membrane of the epithelial cells or in the blood vessels. Therefore, it is possible that NBC4 participates in basolateral, stilbene-sensitive Na$^+$-HCO$_3^-$ transport in the choroid plexus epithelium.

In summary, cell physiological transport studies have provided compelling evidence that CSF production depends on basolateral, Na$^+$-driven, stilbene-sensitive HCO$_3^-$ transport. The present study demonstrates a very strong and selective expression of a SLC4A10 gene product in the basolateral plasma membrane of choroid plexus epithelial cells. The specific epithelial localization of the N-glycosylated protein, together with its mode of transport and its stilbene sensitivity, suggest that the SLC4A10 gene product might be the previously described basolateral Na$^+$-HCO$_3^-$ transporter of the choroid plexus. Finally, the identification of these candidate proteins permits more extensive investigations of the Na$^+$ and HCO$_3^-$ handling in the choroid plexus epithelium. Studies in transgene or gene knockout mice are likewise warranted to fully characterize the specific role of the SLC4A10 gene product or, perhaps, NBCn1 and NBC4 in CSF production.

ACKNOWLEDGMENTS

We thank Mette F. Vistisen, Lotte V. Holbech, Zhila Nikrozi, and Ida M. Jalk for skilled technical assistance. The anti-AE2 antibody was kindly provided by S. Alper, Department of Medicine, Harvard Medical School, Beth Israel Deaconess Medical Center, Boston, MA, and the anti-AE4 antibody was generously supplied by K. Ishibashi, Department of Pharmacology, Ichi Medical School, Minamikawachi, Tochigi, Japan. Christian Aalkjaer of the Institute of Physiology, University of Aarhus, is thanked for valuable discussions and comments to the manuscript.

GRANTS

The Water and Salt Research Center at the University of Aarhus was established and supported by the Danish National Research Foundation (Dansmarks Grundforskningsfond). The European Commission (Contract no. QLK3-CT-2000-0078) and the Human Frontier Science Program also provided support for this study.

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AJP-Cell Physiol • VOL 286 • MARCH 2004 • www.ajpcell.org


