Characterization of Na\(^+\)/HCO\(_3\)\(^-\) cotransporter isoform NBC-3

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Amlal, Hassane, Charles E. Burnham, and Manoocher Soleimani. Characterization of Na\(^+\)/HCO\(_3\)\(^-\) cotransporter isoform NBC-3. Am. J. Physiol. 276 (Renal Physiol. 45): F903–F913, 1999.—Na\(^+\)/HCO\(_3\)\(^-\) cotransporters mediate the transport of HCO\(_3\)\(^-\) into or out of the cell. Two Na\(^+\)/HCO\(_3\)\(^-\) cotransporters (NBC) have been identified previously, which are referred to as NBC-1 and NBC-2. A cDNA library from uninduced human NT-2 cells was screened with an NBC-2 cDNA probe. Several clones were identified and isolated. Sequence analysis of these clones identified a partial coding region (2 kb) of a novel NBC (called here NBC-3), which showed 53% and 72% identity with NBC-1 and NBC-2, respectively. Northern blot analysis revealed that NBC-3 encodes a 4.4-kb mRNA with a tissue distribution pattern distinct from NBC-1 and NBC-2. NBC-3 is highly expressed in brain and spinal column, with moderate levels in trachea, thyroid, and kidney. In contrast with NBC-1, NBC-3 shows low levels of expression in pancreas and kidney cortex. In the kidney, NBC-3 expression is predominantly limited to the medulla. Cultured mouse inner medullary collecting duct (mIMCD-3) cells showed high levels of NBC-1 and low levels of NBC-3 mRNA expression. Subjecting the mutagenized mIMCD-3 cells to sublethal acid stress decreased the mRNA expression of NBC-1 by ~90% but increased the Na\(^+\)-dependent HCO\(_3\)\(^-\) cotransport activity by ~7-fold (as assayed by DIDS-sensitive Na\(^+\)-dependent, HCO\(_3\)\(^-\)-mediated intracellular pH recovery). This increase was associated with ~5.5-fold enhancement of NBC-3 mRNA levels. NBC showed significant affinity for Li\(^+\) in the mutant but not the parent mIMCD-3 cells. On the basis of the widespread distribution of NBC-3, we propose that this isoform is likely involved in cell pH regulation by transporting HCO\(_3\)\(^-\) from blood to the cell. We further propose that enhanced expression of NBC-3 in severe acid stress could play an important role in cell survival by mediating the influx of HCO\(_3\)\(^-\) into the cells.

Recent cloning experiments have identified two NBC isoforms, NBC-1 and NBC-2 (9, 20, 28). NBC-1, which was cloned from human and amphibian kidney, mediates an electrogenic, Na\(^+\)-dependent HCO\(_3\)\(^-\) cotransport that is inhibitable by DIDS (9, 28). Human NBC-1 encodes a 7.6-kb mRNA, whereas amphibian NBC-1 encodes a 4.2-kb mRNA (9, 28). Human NBC-1 shows highest levels of expression in kidney and pancreas with lower levels in brain (9). In the kidney, NBC-1 is predominantly expressed in the proximal tubule (1, 10, 29) and shows adaptive mRNA regulation in rats subjected to HCO\(_3\)\(^-\) loading (10), potassium deprivation (4), or glucocorticoids (3). Pancreatic NBC-1 is a splice variant of kidney NBC-1 and is expressed in both acinar and ductal cells (2). A recent report indicated cloning of a new NBC isoform, NBC-2, from a human retina cDNA library (20). NBC-2 encodes an 8.5-kb mRNA and shows highest expression in testis and spleen and moderate levels of expression in intestine, colon, and muscle (20). Functional properties of NBC-2 have not been described.

The purpose of the current experiments was to identify other possible members of NBC family and study their expression and regulation. Accordingly, a human NT-2 cell cDNA library was screened with NBC-2 cDNA. Positive clones were identified, isolated, and sequenced. The results indicated a novel NBC cDNA isoform, called here NBC-3. NBC-3 is highly expressed in the central nervous system and shows differential regulation vs. NBC-1 in cells subjected to severe acid stress. NBC-3 is likely involved in cell pH regulation by transporting HCO\(_3\)\(^-\) from blood to the cell.

EXPERIMENTAL PROCEDURES

Isolation of human NBC-3 cDNA. A cDNA library (prepared from uninduced, exponentially growing testicular teratocarcinoma neuroepithelial cells; Ntera-2/c1.D1, NT-2 cells, cloned into the ZAP Express vector) was purchased from

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Stratagene (La Jolla, CA) and screened at high stringency using a 1.48-kb 32P-labeled cDNA probe prepared by PCR amplification of an expressed sequence tag cDNA clone (GenBank accession no. AA216661) encoding a part of human NBC-2, purchased from the American Type Culture Collection. The primers GACGAGTCCATACGAGAG and CCATGATA

GACCACAAGCTGAC were used to amplify the NBC-2 probe from the NBC-2 expressed sequence tag clone. Nylon membranes were used to lift plaques in duplicate from plates. DNA was denatured by incubation of the membranes (5–7 min) in 0.5 N NaOH, 1.5 M NaCl. Then the membranes were neutralized by soaking 5 min in 1 M Tris-Cl, pH 8, followed by 5 min in 1 M Tris-Cl, pH 8, 1.5 M NaCl. This was followed by a rinse in 2× SSPE. After ultraviolet cross-linking, the membranes were dried. They were then soaked overnight with gentle agitation in a 65°C prehybridization solution containing 5× Denhardt's BSA, 6× SSPE, 0.1% SDS, and 100 µg/ml carrier DNA. The following day, freshly labeled probe was added, and the hybridization was continued until the next morning. The membranes were then washed twice for 10 min each in 2× SSPE + 0.1% SDS at room temperature. A third wash for 30 min at 65°C in 1× SSPE + 0.1% SDS was followed by a brief rinse in 2× SSPE at room temperature before exposure to film. Following plaque-purification and conversion to plasmid form (pBK-CMV), DNA sequence analysis was performed on six clones at the University of Cincinnati. A second clone contained a 2-kb insert that included the 5' end of the coding region of NBC-3. The other four clones contained smaller inserts that were included in the two larger clones (the 5.5-and the 2-kb clones). The cloned cDNA represents a partial sequence of a new NBC isoform (we refer to this as NBC-3). A recent study reported the cloning of a full-length cDNA that shows complete homology to our NBC-3 (24). However, no functional studies were performed to examine the identity of the cloned cDNA.

Northern hybridization. Total RNA samples (30 µg/lane) were fractionated on 1.2% agarose-formamide gel and transferred to nylon membranes. RNA was covalently bound to the nylon membranes by ultraviolet cross-linking (14). The primers GACGAGTCCATACGAGAG and CCATGATAGACCACAAGCTGAC were used to amplify the cDNA probe from the 5.5-kb cDNA or a 700-bp fragment from the 2-kb cDNA was used as a probe. The respective NBC probes recognize appropriate size mRNAs on Northern blots. Northern blots that were probed with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. For NBC-1, the full-length cDNA was used as a specific probe. For NBC-2, the probe was generated by PCR amplification of an EST cDNA fragment (GenBank accession no. AA216661) that encodes nucleotides 8–2808 and is a part of human NBC-2. For NBC-3, a 3-kb St I fragment from the 5.5-kb cDNA or a 600-bp fragment from the 2-kb cDNA was used as a probe. The RT-PCR products were DIG-labeled using the RT-PCR of NBC-2 on the RNA of mIMCD-3 cells. RNA (2 µg) from control and acid-stressed cells was subjected to RT-PCR using an oligo-dT reverse transcriptase primer and mouse NBC-2-specific primers for the PCR. Amplification across an intron-exon junction ensured selectivity for mRNA vs. genomic DNA. The mouse-specific primers were 5'-GACCTATCAAGTTTGG and 5'-CAAGGCCCCACTGAGTTCTCTC. Cycling parameters were as follows: 30 cycles of 94°C, 30 s; 57°C, 1 min; 72°C, 2 min.

Cell culture procedures. mIMCD-3 cells (which were developed from the inner medullary collecting duct cells of simian virus transgenic mice and retain many characteristics of this nephron segment, Ref. 27) were cultured in a 1:1 mixture of Ham's F-12 and DMEM (DMEM-F12) containing 2.5 mM L-glutamine and 2438 g/l sodium bicarbonate (GIBCO-BRL) supplemented with 50 U/ml penicillin G, 50 µg/ml streptomycin, and 10% fetal bovine serum. Cultured mIMCD-3 cells and cells subjected to sublethal acid stress were incubated at 37°C in a humidified atmosphere of 5% CO2 in air. The medium was replaced every other day.

Sublethal acid stress. Cells that were subjected to modified acid-saturation method (sublethal acid stress) were grown as described (5, 30). We have referred to these cells in the past as NHE2d or mutant cells based on the downregulation of NHE-2 isoform in response to sublethal acid stress (5, 30). Briefly, actively proliferative, subconfluent mIMCD-3 cells were treated for 16 h with the mutagen ethylmethylsulfonic acid (EMS) at 500 µM and then subjected to a modified protocol of lethal acid stress (30). Following treatment with EMS, mIMCD-3 cells were grown to confluence, trypsinized, centrifuged at room temperature, and loaded with NH3 by incubation for 10 min at 37°C in a ammonium-containing solution that consisted of 20 mM NH4Cl, 120 mM tetramethylammonium chloride (TMA-Cl), 5 mM KCl, 5 mM glucose, and 5 mM HEPES-Tris, pH 7.40. The cells were then acid-loaded by incubation for 30 min in an NH3-free solution that consisted of 125 mM TMA-Cl, 5 mM KCl, 5 mM glucose, and 20 mM HEPES-Tris, pH 5.5. Thereafter, cells were pelleted.
washed, and incubated for 120 min at 37°C in a solution with a very low concentration of Na\(^+\) (5 mM NaCl) that, in addition, consisted of 120 mM choline chloride, 5 mM KCl, 5 mM glucose, and 20 mM of MES at pH 6.0. The cells were then centrifuged, recovered, and seeded to culture-grade plastic dishes in DMEM-F12 medium (pH 7.40) for 10 days. Cells were subjected while in suspension to two more rounds of acid stress, with each selection event separated from its predecessor by 10 days. The cells were subcultured and passaged at very high dilutions (1:1,000) to isolate individual colonies. A number of individual colonies were isolated from cloning cylinder, then collected and subcultured. One strain (NHE2d) was studied in details for NHE isoform expression (30), \(H_1\)-ATPase activity (5), and the current studies. In addition to the parent cells, three individual colonies from cells not subjected to acid stress were obtained (using trypsinization and passage at very high dilution followed by isolation from cloning cylinder). These strains showed a pattern of NBC expression very similar to the uncloned parent cell line. These results suggest that overexpression of NBC-3 and suppression of NBC-1 in stressed cells could represent true adaptive regulation of these transporters by acid stress. Equally plausible, however, is the possibility that the differential regulation of NBC-1 and NBC-3 could be due to a combination of EMS-induced mutagenesis and acid-suicide.

**Intracellular pH measurement.** Changes in intracellular pH (pHi) were monitored using the acetoxymethyl ester of the pH-sensitive fluorescent dye 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM) as described (6, 34, 35).

**Table 1. Composition of experimental solutions**

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All concentrations are in mM. Solutions A, B, C, E, F, and G were bubbled with 5% CO₂-95% O₂; solution D was gassed with 100% O₂; pH was adjusted to 7.40 with Tris. TMA, tetramethylammonium; NMG, N-methyl-D-glucamine.

Fig. 1. Nucleotide sequence of the human Na⁺-HCO₃⁻ cotransporter NBC-3 cDNA (see RESULTS). Stars indicate the ends of the 700-bp probe used for Northern hybridizations. Sequence has been assigned GenBank accession no. AF107099.
Scan dual-excitation spectrofluorometer (PTI, South Brunswick, NJ). The monolayer was then perfused with the appropriate solutions (Table 1). The fluorescence ratio at excitation wavelengths of 500 and 450 nm was utilized to determine pH values in the experimental groups by comparison to the calibration curve that was generated by KCl/nigericin technique. The fluorescence emission was recorded at 525 nm. The Na\(^{+}\)-HCO\(_3\) cotransporter activity was determined as the initial rate of the DIDS-sensitive, Na\(^{+}\)-dependent pH recovery (dpHi/dt, pH/min) in a HCO\(_3\)-containing solution (Table 1, solution C) following an acid load induced by NH\(_3\)/NH\(_4\)\(^{+}\) loading (Table 1, solution B) and withdrawal (Table 1, solution A). The experiments were performed in the presence of 1 mM amiloride to block the Na\(^{+}/H^{+}\) exchanger activity. Glucose was deleted from the solutions to minimize the contribution of H\(^{+}\)-ATPase to pH recovery from acidosis (5). The initial rate of pH recovery (dpHi/dt, pH/min) following intracellular acidosis was calculated by fitting to a linear equation the first 30 s of the time course of pH recovery. Correlation coefficients for these linear fits averaged 0.98 ± 0.01. The representative and bar graph experiments were compiled from data from a single acid-stressed clonal line.

Materials. DMEM-F12 medium was purchased from Life Technologies. BCECF was from Molecular Probes. Amiloride, DIDS, nigericin, and other chemicals were purchased from Sigma Chemical. \(^{32}\)P was purchased from New England Nuclear (Boston, MA). The Gibco-BRL RadPrime DNA labeling kit was purchased from Life Technologies.

**RESULTS**

Partial sequence of NBC-3 cDNA. Figure 1 shows the partial nucleotide sequence (encompassing 2 kb) and conceptual translation of NBC-3. The sequence shows an in-frame stop codon prior to the initial methionine indicating that the clone contains the 5' end of an NBC-3 cDNA coding region. The remainder of the 5.5-kb sequence (not shown) did not correlate with any known gene and is likely to be the second half of a cDNA double insert. A separate 2-kb NBC-3 cDNA clone was identified that spans the entire coding region of the 5.5-kb cDNA and contains the 5' end of NBC-3 cDNA clone. The absence of a cDNA region encoding the terminal transmembrane regions common to other members of this gene family suggests that the 3' end of the message is missing from both of the cDNA clones (see Footnote 1). An analysis of the amino acid translation of NBC-3 shows 72% identity with NBC-2 and 53% identity with NBC-1. Black boxes indicate conserved domains among the NBC isoforms. Uppercase letters indicate areas of divergence.
the open-reading frame shows 72% and 53% identity with NBC-2 and NBC-1, respectively, as shown in Fig. 2.

Northern blots. Two human multiple tissue Northern blots (Clontech) were probed with a 32P-labeled NBC-3 cDNA. Figure 3A shows that a 4.4-kb mRNA in human brain hybridized strongly with the probe, indicating a high level of NBC-3 expression in the central nervous system. A moderate band was detected in kidney and skeletal muscle. The expression of NBC-3 was very faint in the pancreas. In addition to the 4.4-kb band, two other transcripts at 9 and 3 kb were also detected in the brain. Figure 3B shows strong expression of the 4.4-kb mRNA in human spinal column followed by adrenal gland and lymphoid tissue. A moderate band was detected in thyroid and trachea. Similar to the brain, two other transcripts at 9 and 3 kb were also detected in the spinal column and adrenal gland. Figure 3C examines the expression of NBC-3 in rat kidney cortex and medulla. As indicated, NBC-3 mRNA is predominantly expressed in the medulla, with NBC-3-to-GAPDH mRNA ratio being 2.8 ± 0.3-fold higher in the medulla vs. cortex (Fig. 3D) (n = 4 for each group, P < 0.04).

Effect of acid stress on the expression of NBC isoforms in mIMCD-3 cells. The expression and regulation of NBC isoforms in the kidney inner medulla was next examined. Normal cultured mIMCD-3 cells were subjected to sublethal acid stress and examined. The Northern blot in Fig. 4A indicates high levels of NBC-1 mRNA expression in control (parent) mIMCD-3. Figure 4A further indicates that cells subjected to acid stress showed significant reduction in NBC-1 mRNA expression. Figure 4B demonstrates that NBC-1 mRNA levels were decreased by ~90% in acid-stressed cells (n = 4 for acid stress; n = 5 for control cells; P < 0.001).

To correlate the expression of NBC-1 with its function, control mIMCD-3 cells were grown to confluence on coverslips, loaded with BCECF, and monitored for pH\textsubscript{i} recovery from intracellular acidosis. In the presence of amiloride (to block Na\textsuperscript{+}/H\textsuperscript{+} exchange), switching to a Na\textsuperscript{+}- and HCO\textsubscript{3}\2-containing solution resulted in rapid pH\textsubscript{i} recovery in mIMCD-3 cells (Fig. 5A). This recovery was absolutely HCO\textsubscript{3}\2-dependent, as shown by the lack of a significant pH\textsubscript{i} recovery in the absence of HCO\textsubscript{3}\2 (Fig. 5B). Indeed, pH\textsubscript{i} recovery from acidosis in the absence of HCO\textsubscript{3}\2 was 0.011 ± 0.002 pH/min (n = 4), a value not significantly different from zero. The Na\textsuperscript{+}-dependent HCO\textsubscript{3}\2 cotransport was completely abolished in the presence of 300 µM DIDS (Fig. 5A). The results, summarized in Fig. 5C, indicate that the rate of Na\textsuperscript{+}-
n stressed cells (The rate of pH_i recovery was 0.676
0.051 pH/min, and decreased to 0.027
in the presence of 300 µM DIDS,
Fig. 6).

C

n 0.005 pH/min (nearly completely inhibited by 300 µM DIDS (Fig. 6,
and

methylammonium.

B

Northern hybridization.

HCO3

stressed inner medullary collecting duct mIMCD-3 cells. NBC-1
isoform. Figure 7 is a Northern blot analysis and indicates that NBC-3 mRNA expression was enhanced
significantly in cells subjected to acid stress, with
mRNA levels increasing by -5.5 ± 0.8-fold (Fig. 7B; P < 0.01 vs. control cells, n = 4 for each group).
Northern hybridization did not detect NBC-2 mRNA
expression in either control cells or cells subjected
to acid stress (data not shown). An attempt was then
made to examine the expression of NBC-2 in 1MCD
cells by RT-PCR according to EXPERIMENTAL PROCEDURES
(Fig. 7C). Lanes 1 and 2 in Fig. 7C are control mIMCD-3
cells, with and without reverse transcriptase, respecti-

Fig. 4. A: NBC-1 Northern hybridizations in control and acid-
stressed inner medullary collecting duct mIMCD-3 cells. NBC-1
mRNA levels were suppressed by ~90% in cells subjected to sublethal
acid stress. Top: NBC-1 Northern hybridization. Bottom: GAPDH
Northern hybridization. B: NBC-1/GAPDH mRNA ratio. TMA, tetra-
methylammonium.

 dependent HCO3-mediated pH_i recovery was 0.076 ±
0.005 pH/min (n = 7) and decreased to 0.012 ± 0.002
pH/min in the presence of 300 µM DIDS (n = 5).

In the next series of experiments, NBC activity in
cells subjected to acid stress was assayed in a manner
similar to Fig. 5. Figure 6A shows representative pH_i
tracings from both parent and stressed cells and demon-
strates that the Na+-dependent HCO3 cotransport into acid-loaded cells
was significantly increased in stressed cells compared with the parent
cells (dpH_i/dt was 0.610 ± 0.038 pH/min in stressed
cells, n = 7; and 0.085 ± 0.005 pH/min in control cells, n = 6, P < 0.0001). The pH_i recovery from acidosis was
nearly completely inhibited by 300 µM DIDS (Fig. 6, B
and C), consistent with the presence of NBC activity in
stressed cells (The rate of pH_i recovery was 0.676 ±
0.051 pH/min, and decreased to 0.027 ± 0.005 pH/min
in the presence of 300 µM DIDS, n = 5 for each group,
Fig. 6C). The minimal pH_i recovery from acidosis in
the presence of DIDS likely represents a contribution by
H+-ATPase, as it was also evident in the absence of
HCO3 in the media. Depleting the intracellular Cl-- by
incubating the cells in Cl--free media (only Cl--free
solutions were used for the duration of experiments,
solution G, Table 1) did not reduce the rate of Na+-
dependent HCO3 cotransport into acid-loaded cells
(Fig. 6D), indicating that the enhanced activity is not
due to the Na+-dependent Cl--/HCO3 exchanger [The
rate of pH_i recovery from acidosis was 0.630 ± 0.023
pH/min in Cl--free solution (n = 4), a value not
different from pH_i recovery in Cl--containing media].
HCO3 dependence of pH_i recovery from cell acidosis in
stressed cells was confirmed by the lack of a significant
recovery from acidosis in the absence of HCO3 in the
media (Fig. 6E). pH_i recovery from acidosis in the
absence of HCO3 was only 0.01 ± 0.002 pH/min (n = 4).

The above experiments indicate that sublethal acid
stress decreases the mRNA expression of NBC-1 but
increases the Na+-dependent HCO3 cotransport activity.
This clearly raises the possibility that enhanced
NBC activity in acid-stressed cells is due to another
NBC isoform. Figure 7A is a Northern blot analysis and indicates that NBC-3 mRNA expression was enhanced
significantly in cells subjected to acid stress, with
mRNA levels increasing by -5.5 ± 0.8-fold (Fig. 7B;
P < 0.01 vs. control cells, n = 4 for each group).

2 To determine the presence of a mouse homolog of human NBC-3
in the mIMCD-3 cells, an RT-PCR experiment was designed to detect,
amplify, and sequence such a homolog. Accordingly, cDNA was
prepared from mIMCD-cell total RNA (1 µg) using SuperScript
reverse transcriptase (Life Technologies) and an oligo-dT primer
according to standard procedures. The following PCR primers were
amplified, and sequenced, using the amplification primers as sequencing prim-
ers. Several amplification products were detected by gel electropho-
resis, and one band of ~600-bp was excised from the gel, reamplified,
and sequenced, using the amplification primers as sequencing prim-
ers. Clear sequence electropherograms were obtained, indicating the
amplification of a pure product. The nucleotide sequence of the PCR
product was 87% identical to the human NBC-3 sequence. The mouse
amino acid sequence was 94% identical to the human sequence.
Taken together with the Northern blot analysis described in this
article, this demonstrates that mIMCD-3 cells express NBC-3.
hybridization, we conclude that NBC-2 mRNA levels are very low in control and acid-stressed cells.

Functional characterization of NBC-1 and NBC-3. The above experiments demonstrate high levels of expression of NBC-1 and NBC-3 along with a DIDS-sensitive Na\(^{+}\)-dependent HCO\(_3\)\(^{-}\)cotransport activity in control and acid-stressed cells, respectively. Given very low mRNA levels for NBC-2 and NBC-3, these data suggest that NBC activity in control mIMCD-3 cells is likely mediated via NBC-1. The very low levels of NBC-1 and NBC-2 mRNA in acid-stressed cells strongly suggest that most of the NBC activity in these cells is mediated via NBC-3. To characterize the NBC activity in control mIMCD-3 and stressed cells further, the interaction of Li\(^{+}\) with NBC in both cell lines was studied. As shown in Fig. 8A, control mIMCD-3 cells showed little pH\(_i\) recovery from intracellular acidosis in the presence of Li\(^{+}\) (Table 1, solution E). Switching from the Li\(^{+}\)-containing solution to the Na\(^{+}\)-containing solution (Table 1, solution C) caused a rapid rise in pH\(_i\) recovery (Fig. 8A). As indicated and summarized in Fig. 8B, the rate of HCO\(_3\)\(^{-}\)-dependent pH\(_i\) recovery from acidosis was very low in the presence of Li\(^{+}\) (0.008 ± 0.002 pH/min, n = 5) but was significantly high in the presence of Na\(^{+}\) (0.09 ± 0.009 pH/min, n = 5). These results indicate that NBC activity in control mIMCD-3 cells (likely mediated via NBC-1) has low affinity for Li\(^{+}\).

The interaction of NBC with Li\(^{+}\) in acid-stressed cells was next tested. As shown in Fig. 9A, exposing the cells to a Li\(^{+}\)-containing solution (Table 1, solution E) caused significant recovery from intracellular acidosis. As indicated and summarized in Fig. 9B, the rate of HCO\(_3\)\(^{-}\)-dependent pH\(_i\) recovery from acidosis was 0.327 ± 0.025 pH/min in the presence of Li\(^{+}\) (n = 7) and 0.618 ± 0.033 in the presence of Na\(^{+}\) (n = 7; P < 0.0001). The Li\(^{+}\)-dependent HCO\(_3\)\(^{-}\)cotransport was completely inhibited in the presence of 300 µM DIDS (Figs. 9C, representative pH\(_i\) tracings). Figure 9D is the summary of the results and indicates that the rate of Li\(^{+}\)-dependent HCO\(_3\)\(^{-}\)-mediated pH\(_i\) recovery from cell acidosis was 0.327 ± 0.0025 pH/min (n = 7) and decreased to 0.011 ± 0.003 pH/min in the presence of 300 µM DIDS (n = 5). These results indicate that Li\(^{+}\) inhibits NBC activity in control mIMCD-3 cells, likely mediated via NBC-1.

In several experiments, Li\(^{+}\) caused a weak HCO\(_3\)\(^{-}\)-dependent pH\(_i\) recovery in control mIMCD-3 cells when the nadir pH\(_i\) was reduced to ≤5.8 (by longer incubation in the NH\(_4\)\(^{+}\) containing solution). The Li\(^{+}\)-dependent pH\(_i\) recovery reached a plateau at pH\(_i\) ≈6.2. This suggests that the interaction of NBC-1 with Li\(^{+}\) in mIMCD-3 cells is likely dependent on the pH\(_i\); at pH\(_i\) > 6.2 or higher, NBC-1 does not mediate Li\(^{+}\)-dependent HCO\(_3\)\(^{-}\)cotransport.
can substitute for Na⁺ on NBC in acid-stressed cells, with Li⁺ showing less ability to mediate HCO₃⁻ cotransport. The NBC in control or acid-stimulated cells showed no affinity for K⁺ (data not shown).

**DISCUSSION**

The NBC-3 cDNA clone was identified by screening a NT-2 cell cDNA library with NBC-2 cDNA. Figure 1 shows the partial nucleotide sequence of NBC-3. Figure 2 shows significant homology with both NBC-1 and NBC-2. Tissue distribution studies of NBC-3 (Fig. 3) show a pattern of expression distinct from either NBC-1 and NBC-2. Unlike NBC-1 and NBC-2, NBC-3 is highly expressed in brain and spinal column. Contrary to NBC-1, NBC-3 shows low level of expression in pancreas and kidney cortex. NBC-3 and NBC-1 are expressed in cells from renal inner medulla and show differential expression in EMS-mutagenized mIMCD-3 cells subjected to sublethal acid stress; NBC-1 is suppressed, whereas NBC-3 is enhanced. NBC-3 and NBC-1 show different affinities for Li⁺; NBC-3 likely mediates Li⁺-dependent HCO₃⁻ cotransport, whereas NBC-1 does not.

NBC-3 shows highest expression in brain and spinal cord, suggesting that this isoform may be the dominant Na⁺-dependent HCO₃⁻ cotransporter in human astro-
cytes (16). This is in contrast to NBC-1 and NBC-2, which show either low expression or no expression, respectively, in brain (9, 20). In rat and in contrast to human, NBC-1 shows high level of expression in brain (10), indicating species differences with regard to the tissue distribution of NBC isoforms.

NBC-1 expression in the rat kidney is limited to the cortex (1, 10, 29), whereas in the mouse kidney, it is expressed in both cortex and medulla (11), consistent with functional studies. NBC-1 mRNA expression, although absent in rat medullary thick ascending limb and IMCD cells under baseline condition (10), shows heavy induction in potassium depletion (4), indicating that this cotransporter may mediate enhanced HCO$_3^-$ reabsorption in these two nephron segments in certain pathophysiological states. NBC-3 expression in the rat kidney is predominantly observed in the medulla (Fig. 3, C and D), a finding distinct from NBC-1 (1, 10, 29).

Functional studies in cultured IMCD cells have localized the Na$^+$-dependent HCO$_3^-$ cotransporter to the basolateral membrane (19), confirming earlier studies in proximal tubule and other epithelial cells (7, 8, 16–18, 26, 30). It is therefore logical to conclude that both NBC-1 and NBC-3 are expressed on the basolateral membrane of mIMCD-3 cells. Based on the clonal origin of mIMCD-3 cells, we suggest that both these isoforms are present on the basolateral membrane of the same cells rather than different cells. A definitive answer, however, should come from immunocytochemistry studies utilizing specific antibodies.

NBC-1, which is predominantly expressed in the kidney proximal tubule cells, functions in an outwardly directed mode under physiological conditions, resulting in transvectorial transport of HCO$_3^-$ from lumen to the blood. Given the important role of IMCD in HCO$_3^-$ reabsorption, it is likely that in mIMCD-3 cells, NBC-1 is functioning in an efflux mode, resulting in the exit of HCO$_3^-$ across the basolateral membrane under physiological conditions. Induction of NBC-3 in cells that survive the sublethal acid stress (more than 99% of the EMS-mutagenized mIMCD-3 cells subjected to acid stress died; Refs. 5 and 30) suggests that this isoform likely functions in the influx mode under this condition, leading to increased cell pH in severe acidosis. The expression and activity of NHE-1 and H$^+$-ATPase, two known acid extruders, are also increased in acid-stressed cells (5, 30). This likely defends the cells against severe acidosis by transporting acid out of the cells.

Enhancement of Na$^+$-dependent HCO$_3^-$ influx has also been observed in fibroblasts subjected to proton suicide (13, 22). In PS120 cells, which are NHE-deficient fibroblast cells (25), Na$^+$-dependent Cl$^-$/HCO$_3^-$ exchange is enhanced compared with PS127 cells, which overexpress NHE-1 (13, 22). As a result of overexpression of this Na$^+$-dependent HCO$_3^-$ influx pathway, PS120 cells survive acidic pH in the presence but not the absence of HCO$_3^-$ in the media (13, 22). Taken together, these results, along with the results in
Figs. 6 and 7, strongly suggest that induction of NBC-3 likely prevents severe intracellular acidosis by transporting HCO\(_3\) into mIMCD-3 cells subjected to modified acid-suicide selection.

In basolateral membrane vesicles isolated from rabbit kidney cortex, NBC shows considerable affinity for Li\(^+\); however, its affinity was fivefold lower than for Na\(^+\) (33). It is worth mentioning that, although mouse NBC-1 shows no affinity for Li\(^+\) (Fig. 8), human NBC-1 interacts with Li\(^+\) (63). Given the high degree of cDNA homology between mammalian NBC-1 isoforms (human and rat; see Refs. 9, 10, 28, 29), comparison of mouse and human NBC-1 cDNAs could yield possible clues regarding the Li\(^+\)-binding site(s) of NBC-1.

In conclusion, a novel Na\(^+\)-HCO\(_3\) cotransporter (NBC-3) has been cloned. Its partial cDNA sequence shows both the homology and the divergence from NBC-1, NBC-2, and AE1, indicating that NBC-3 is a new member of the superfamily of bicarbonate transporters to which both anion exchangers and Na\(^+\)-HCO\(_3\)
cotransporters belong. NBC-3 shows distinct patterns of tissue expression and shows differential expression compared with NBC-1 in response to sublethal acid stress. On the basis of relatively widespread distribution of NBC-3 and its induction in acid stress, we propose that NBC-3 is likely involved in cell pH regulation by mediating the influx of HCO$_3^-$ into the cells.

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