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

HASSANE AMAL, CHARLES E. BURNHAM, AND MANOOCHER SOLEIMANI

Division of Nephrology, Department of Internal Medicine, University of Cincinnati, and Veterans Affairs Medical Center, Cincinnati, Ohio 45267-0585

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.

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 Torrance, CA. Two million human NT-2 cDNA clones were screened with a human 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.
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 GACGAGTCATACAGGAC and CCATGT-GACCCAGTGGT 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 DNA Core, using dye-termination technology. The largest insert (5.5 kb) contained part of the coding region of a variant of NBC-3.

A recent study reported the cloning of a new NBC isoform (we refer to this as NBC-3).1 A recent study reported the cloning of a full-length cDNA that encodes nucleotides 8–2808 and is part of human NBC-2. For NBC-3, a 3-kb Sst1 fragment 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 on RNA isolated from mIMCD-3 cells (−7.6-kb message for NBC-1 and 4.4-kb message for NBC-3). A more specific 700-bp NBC-3 cDNA probe (corresponding to nucleotides 900-1600) also recognized the 4.4-kb message as the only transcript in mIMCD-3 cells.

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 transcription 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′-GAC-CCATACAAGGGG and 5′-CAAGCCAAGCTGAGTTCC.

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 2.438 g/l sodium bicarbonate (GIBCO-BRL) supplemented with 50 U/ml penicillin G, 50 µg/ml streptomycin, and 10% fetal bovine serum. Cultured mlMCD-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-sulfate selection (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 NHE2 isoform in response to sublethal acid stress (5, 30). Briefly, actively proliferative, subconfluent mlMCD-3 cells were treated for 16 h with the mutagen ethylmethylsulfonic acid (EMS) at 500 µg/ml and then subjected to a modified protocol of lethal acid stress (30). Following treatment with EMS, mlMCD-3 cells were grown to confluence, trypsinized, centrifuged at room temperature, and loaded with NH4Cl by incubation for 10 min at 37°C in an 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 NH4Cl-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.

1 While this manuscript was under review, two independent entries into the GenBank (accession nos. AF069512 and AB018282) reported sequences identical to our NBC-3 cDNA. An alignment of our cDNA with these two other cDNAs showed perfect match. The 5′ end of our cDNA sequence shows perfect match to the AF069512 sequence. However, the AB018282 sequence contains an extended 5′ coding region of ~300 nucleotides that replaces the first 63 nucleotides of the two other cDNAs (the current NBC-3 sequence and the AF069512 sequence) and likely represents an alternative splice variant of NBC-3.
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 with cloning cylinder). These results 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). mIMCD-3 cells and cells that were recovered from sublethal acid stress were grown to confluence on coverslips and incubated in the presence of 5 µM BCECF in a Na\(^{+}\)-free solution that consisted of 115 mM TMA-Cl and 25 mM KHCO\(_3\), pH 7.4 (solution A, Table 1). pH was measured in a thermostatically controlled holding chamber (37°C) in a Delta

### 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% CO\(_2\)-95% O\(_2\); solution D was gassed with 100% O\(_2\); pH was adjusted to 7.40 with Tris. TMA, tetramethylammonium; NMG, N-methyl-D-glucamine.

Fig. 1. Nucleotide sequence of the human Na\(^{+}\)-HCO\(_3\)\(^{-}\) 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.
The fluorescence ratio at excitation wavelengths of 500 and 450 nm was utilized to determine \( pH_i \) 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 \( pH_i \) 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 \( pH_i \) recovery from acidosis (5). The initial rate of \( pH_i \) 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 \( pH_i \) recovery. Correlation coefficients for these linear fits averaged 0.98 \( \pm \) 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 LifeTechnologies.

**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 codon 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 areas of identical amino acids in all 3 isoforms. Uppercase letters indicate conserved domains among the NBC isoforms. Lowercase 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 mMCD-3 cells. The expression and regulation of NBC isoforms in the kidney inner medulla was next examined. Normal cultured mMCD-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) mMCD-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 mMCD-3 cells were grown to confluence on coverslips, loaded with BCECF, and monitored for pHi recovery from intracellular acidosis. In the presence of amiloride (to block Na⁺/H⁺ exchange), switching to a Na⁺- and HCO₃⁻-containing solution resulted in rapid pHi recovery in mMCD-3 cells (Fig. 5A). This recovery was absolutely HCO₃⁻ dependent, as shown by the lack of a significant pHi recovery in the absence of HCO₃⁻ (Fig. 5B). Indeed, pHi recovery from acidosis in the absence of HCO₃⁻ was 0.011 ± 0.002 pH/min (n = 4), a value not significantly different from zero. The Na⁺-dependent HCO₃⁻ 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⁺-
The rate of pH\textsubscript{i} recovery from acidosis was 0.630 ± 0.023 pH/min in Cl\textsuperscript{-}-free solution (n = 4), a value not different from pH\textsubscript{i} recovery in Cl\textsuperscript{-}-containing media. HCO\textsubscript{3} dependence of pH\textsubscript{i} recovery from cell acidosis in stressed cells was confirmed by the lack of a significant recovery from acidosis in the absence of HCO\textsubscript{3} in the media (Fig. 6E). pH\textsubscript{i} recovery from acidosis in the absence of HCO\textsubscript{3} 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\textsuperscript{+}-dependent HCO\textsubscript{3} 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).

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 IMCD 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, respectively. Lanes 3 and 4 are acid-stressed cells, with and without reverse transcriptase, respectively. Lane M is a 1-kb DNA molecular mass ladder (Life Technologies). As demonstrated, the mouse-specific primers amplified a single 279-bp product in both cell lines but failed to amplify a 1,018-bp genomic sequence (Fig. 7C). These results are consistent with the mRNA expression of NBC-2 in mIMCD-3 cells. Coupled with the fact that NBC-2 mRNA expression was not detected by Northern hybridization.

**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, tetramethylammonium.

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 designed (based on the known human sequence) to amplify a 601-bp product (Fig. 7C).

The amino acid sequence was 94% identical to the human 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\(^+\).\(^3\)

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\(^+\)

\(^3\)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\textsubscript{3}\textsuperscript{−} 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\textsuperscript{+}-dependent HCO\textsubscript{3}\textsuperscript{−} 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\textsubscript{3}\textsuperscript{−} from lumen to the blood. Given the important role of IMCD in HCO\textsubscript{3}\textsuperscript{−} reabsorption, it is likely that in mIMCD-3 cells, NBC-1 is functioning in an efflux mode, resulting in the exit of HCO\textsubscript{3}\textsuperscript{−} 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\textsuperscript{+}-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\textsuperscript{+}-dependent HCO\textsubscript{3}\textsuperscript{−} influx has also been observed in fibroblasts subjected to proton suicide (13, 22). In PS120 cells, which are NHE-deficient fibroblast cells (25), Na\textsuperscript{+}-dependent Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchange is enhanced compared with PS127 cells, which overexpress NHE-1 (13, 22). As a result of overexpression of this Na\textsuperscript{+}-dependent HCO\textsubscript{3}\textsuperscript{−} influx pathway, PS120 cells survive acidic pH in the presence but not the absence of HCO\textsubscript{3}\textsuperscript{−} 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 AEs, indicating that NBC-3 is a new member of the superfamily of bicarbonate transporters to which both anion exchangers and Na$^+$-HCO$_3^-$

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**Fig. 8.** Interaction of lithium (Li$^+$) with the NBC in control mIMCD-3 cells. A: cells were acidified and then exposed to a Li$^+$-containing solution (solution E, Table 1). HCO$_3^-$ was present during the entire duration of the experiment. Amiloride, 1 mM, was present to block Li$^+$/H$^+$ exchange. B: cells showed little HCO$_3^-$-mediated pH$_i$ recovery from intracellular acidosis in presence of Li$^+$ compared with Na$^+$ (n = 5 for each group).
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 HCO₃⁻ into the cells.

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Address for reprint requests and other correspondence: M. Soleimani, Univ. of Cincinnati Medical Center, 231 Bethesda Ave, MSB 5502, Cincinnati, OH 45267-0585 (E-mail: Manoocher.Soleimani@uc.edu).

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