Molecular cloning and functional characterization of KCC3, a new K-Cl cotransporter

J O A N N E E . R A C E , 1 F A D I N . M A K H L O U F , 1 2 P A U L J . L O G U E , 2 F R E D E R I C H H. W I L S O N , 3 P H I L I P B. D U N H A M , 2 A N D E L I J . H O L T Z M A N 1 4

1Renal Division, Department of Medicine, and 4Department of Biochemistry and Molecular Biology, State University of New York Health Science Center, Syracuse 13210; 2Department of Biology, Syracuse University, Syracuse, New York 13244; and 3Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520

Race, Joanna E., Fadi N. Makhlouf, Paul J. Logue, Frederick H. Wilson, Philip B. Dunham, and Eli J. Holtzman. Molecular cloning and functional characterization of KCC3, a new K-Cl cotransporter. Am. J. Physiol. 277 (Cell Physiol. 46): C1210–C1219, 1999.—We isolated and characterized a novel K-Cl cotransporter, KCC3, from human placenta. The deduced protein contains 1,150 amino acids. KCC3 shares 75–76% identity at the amino acid level with human, pig, rat, and rabbit KCC1 and 67% identity with rat KCC2. KCC3 is 40 and 33% identical to two Caenorhabditis elegans K-Cl cotransporters and ~20% identical to other members of the cation-chloride cotransporter family (CCC), two Na-K-Cl cotransporters (NKCC1, NKCC2), and the Na-Cl cotransporter (NCC). Hydropathy analysis indicates a typical KCC topology with 12 transmembrane domains, a large extracellular loop between transmembrane domains 5 and 6 (unique to KCCs), and large NH2 and COOH termini. KCC3 is predominantly expressed in kidney, heart, and brain, and is also expressed in skeletal muscle, placenta, lung, liver, and pancreas. KCC3 was localized to chromosome 15. KCC3 transiently expressed in human embryonic kidney (HEK)-293 cells fulfilled three criteria for increased expression of K-Cl cotransport: stimulation of cotransport by swelling, treatment with N-ethylmaleimide, or treatment with staurosporine.

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THE FAMILY OF INORGANIC cation-chloride cotransporters (CCC) is comprised of three subfamilies: the Na-Cl cotransporter (NCC), Na-K-Cl cotransporters (NKCC), and K-Cl cotransporters (KCC). All of them mediate electroneutral transport; the stoichiometry of the Na-K-Cl cotransporter is 1 Na:1 K:2 Cl in most, but not all, systems. The cotransporters share the substrate anion and differ in their substrate cations and in their sensitivities to drugs. Na-Cl cotransport is inhibited by the thiazide-derivative diuretics. Na-K-Cl cotransport is inhibited by loop diuretics such as bumetanide and furosemide. K-Cl cotransport is inhibited by bumetanide and furosemide but is orders of magnitude less sensitive to bumetanide than is Na-K-Cl cotransport. K-Cl and Na-K-Cl cotransport are inhibited by [(dihydroindenyl)oxy]alkanoic acid (DIOA) with similar sensitivities (12). K-Cl cotransport is distinguished as the bumetanide-insensitive, DIOA-inhibitable K transport. Thus there is a diagnostic inhibitor (or pair of inhibitors) for each of the three cotransporters. In addition, N-ethylmaleimide (NEM) is a specific activator of K-Cl cotransporter (24). For a useful, succinct review of cation-chloride cotransporters, see Mount et al. (27).

The primary protein structures of a number of cation-chloride cotransporters have been determined. The primary protein structure of the Na-Cl cotransporter, or NCC, was first characterized from flounder urinary bladder (9) and subsequently in rat renal cortex (8). NCC mediates NaCl reabsorption; the mammalian NCC is confined to renal apical membranes. Only one isoform of NCC has been identified. The protein structure of the Na-K-Cl cotransporter (NKCC1) was first described from shark rectal gland (37) and was subsequently characterized from mouse kidney (4) and human colon (32). NKCC1 is widespread among many tissues where it is primarily involved in regulation of cell volume. NKCC1 is found in the basolateral membranes of secretory epithelia, where it promotes secretion of salt and water. In reabsorptive epithelia, NKCC1 promotes regulation of cell volume (27). Another isoform of the Na-K-Cl cotransporter, NKCC2, which is kidney specific, was characterized from rat outer medulla (8) and from rabbit kidney (30). NKCC2 is apparently restricted to apical membranes in kidney, where it is involved in NaCl reabsorption and the concentration of urine by the countercurrent multiplier.

The primary protein structures of K-Cl cotransporter subfamily members were the last to be described. Two isoforms have been described. KCC1 is expressed in kidney as well as many other tissues (11, 16), and KCC2 is expressed only in neuronal tissue (29, 31). In the human nephron, mRNA for KCC1 is widely distributed, as shown by in situ hybridization (23). In the thick ascending limb, K-Cl cotransport is localized to basolateral membranes, where, together with ion channels, it mediates reabsorption of salt (13). In nonepithelial tissues, K-Cl cotransport promotes regulation of cell volume. One property of KCC1 consistent with this function is its activation by osmotic cell swelling when stably expressed in HEK cells. KCC2 expressed in HEK cells is not activated by swelling and therefore may not participate in volume regulation. One function proposed for KCC2 is to maintain the low intracellular chloride concentration in neurons necessary for the function of some inhibitory synapses (29). An inhibitory synaptic transmitter increases chloride conductance,
which results in an inward chloride current and hyperpolarization.

We have cloned and characterized a novel cDNA, which we denote KCC3. This novel cDNA encodes a protein predicted to contain 1,150 amino acids. Its topology is typical of previously reported K-Cl cotransporters (11, 29). We have expressed KCC3 transiently in HEK-293 cells and have shown enhanced expression of K-Cl cotransport. The enhanced K uptake requires CI but not Na. It is stimulated by osmotic swelling and by NEM, characteristics of K-Cl cotransport.

MATERIALS AND METHODS

Cloning of the human KCC3 cotransporter gene. A search of the Expressed Sequence Tags database (dbEST) yielded nine human cDNAs (accession nos. H17157, AA324145, N32221, N57318, AA781746, W78961, AA004792, R00066, and T03544) that showed significant homology to KCC1 and KCC2. The first three, which contained partial 3' coding sequences, were purchased (Research Genetics, Huntsville, AL; American Type Culture Collection, Rockville, MD) and sequenced by the dideoxy chain-termination sequencing method (34). The missing 5' segment was identified from a human kidney cDNA library (Clontech, Palo Alto, CA) by Rapid Amplification of cDNA Ends (RACE) as previously reported (7) with the use of an outer reverse human gene-specific primer, HGSP1 (5'-CCTCTGTCTGCTGTTTTGGATAGC-3'), and an adapter primer, AP1 (Clontech), followed by a nested PCR reaction using HGSP2 (5'-GCATGGCGCAAGGCTGCTGGACCTT-3') and AP2 (Clontech). This procedure was repeated with further upstream reverse gene-specific primers, HGSP3 (5'-AATATTTTGTAGACATGGGAGTGTA-3') and HGSP4 (5'-ACACCCATGGAATCCATTCCATTGGG-3'), against AP1 and AP2. The resulting products from five separate reactions were sequenced (to obtain a consensus sequence), and primers from the start site (5'-ATGCATCCTC-CGAAAACCCACCC-3') and the stop site (5'-GGTATT-GAATAATGTTGATCCTACCTGCAACCCCGG-3') areas were used to generate a full-length clone from a human placenta cDNA library (Clontech) by PCR with cloned Pfu Turbo DNA polymerase (Stratagene, LaJolla, CA) in accordance with the manufacturer's instructions. Upon sequence comparison to identify wild type, three identical clones were subcloned into the eukaryotic expression vector pCDNA3 (Invitrogen, Carlsbad, CA). One antisense clone was selected for use as a control in functional expression studies.

Secondary structure predictions and sequence analysis. Sequence analysis and alignments were done with EDITEQ and MEGALIGN sequence analysis software (Lasergene; DNASTAR, Madison, WI). Similarity and identity of various KCC clones were determined with the NCBI BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) and by alignment with the MEGALIGN sequence analysis software (Lasergene; DNASTAR). The phylogenetic tree was constructed as a schematic diagram by means of the unweighted pair-group method with arithmetic mean from MEGALIGN sequence analysis software (Lasergene; DNASTAR). A probable model for transmembrane topology was generated with three programs: SOSUI program [Tokyo University of Agriculture and Technology (http://www.tuat.ac.jp/%7Emitaku/adv_sosui/)]; PhdTopology Predict-Protein Program [EMBL-Hidelberg, Germany (http://www.embl-heidelberg.de/predictprotein/predictprotein.html)]; and Tmpred, prediction of transmembrane regions and orientation program (http://www.isrec.isb-sib.ch/software/TMPRED_form.html). Hydropathy plots were made with the Kyte-Doolittle algorithm with a window of 12 amino acid residues (21). Prediction of hydrophathy was made with the DNA Strider 1.2 software (CEA, France).

Northern blot analysis. Northern analysis was performed with the Human Multiple Tissue Northern (MTN) Blot I and the Human Cancer Cell line MTN Blot II (Clontech). Each blot contained ∼2 µg poly(A)+ RNA per lane. The human blot I contained RNA from eight tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas), and the human cancer cell line blot II contained RNA from seven cell lines (promyelocytic leukemia HL-60, HeLa cell S3, chronic myelogenous leukemia K-562, lymphoblastic leukemia MOLT-4, colorectal adenocarcinoma SW480, lung carcinoma A549, and melanoma G361). The Northern blots were probed with a 680-bp 3'untranslated cDNA fragment, unique to KCC3, and the human β-actin cDNA control probe. The KCC3 fragment was PCR-amplified with Pfu Turbo DNA polymerase (Stratagene) in accordance with the manufacturer's instructions, with EST N57318 as the template and with (5'-GTCGCGGTTGTTGCAGTGAAGTGATCACCC-3') and reverse primer (5'-GAAGAAGTAGAGGCTGACAGCATGTTCAAGTAGATGATCC-3'). The KCC3 fragment was gel purified, and both probes were random primed (High Prime and Sephadex G Quick Spin Columns; Boehringer Mannheim, Indianapolis, IN) with [α-32P]dCTP (3,000 Ci/mmol, 10 µCi/µl; NEN Life Science Products, Boston, MA). Hybridizations were carried out at 68°C for 1 h. The washing procedure was as follows: continuous agitation at 2 x SSC/0.05% SDS at room temperature with four changes of wash solution, followed by agitation at 0.1 x SSC/0.1% SDS at 50°C for 40 min with one change of wash solution.

Chromosomal localization. The location of the KCC3 gene was determined by radiation hybrid mapping by means of a GeneBridge 4 Radiation Hybrid Panel (Research Genetics). A panel of 93 radiation hybrid clones of the human genome was screened with a 282-bp PCR-generated product with two primers from KCC3, an intronic forward primer (5'-TGTGGTT- GTTATTGCTACCTTTTGGCC-3') and a reverse exonic primer (5'-TGCGTAACTGTATAGAAGGTG-3'). Each 20-µl PCR reaction contained 25 ng radiation hybrid DNA, 0.125 mM dNTPs, 0.5 µM of each primer, 1 U Taq DNA polymerase, and buffer as described in the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN). The conditions were: 95°C, 5 min; 40 cycles at 94°C, 30 s; 60°C, 30 s; and 72°C, 45 s, with an extension of 72°C for 10 min after 40 cycles. Mapping results were obtained by submission of PCR results to the Whitehead Institute website (http://www.genome.wi.mit.edu).

Cell culture. Human embryonic kidney cells (HEK-293) were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and penicillin and streptomycin (100 U/ml each; GibCO, Gaithersburg, MD) at 37°C in a humidified atmosphere with 5% CO2.

Transient transfection. The full-length KCC3 cDNA construct was subcloned into the eukaryotic expression vector pCDNA3 (Invitrogen). Plasmids were transfected into HEK-293 cells by calcium phosphate precipitation (Profection Mammalian Transfection System; Promega, Madison, WI) (17). Optimal transfection efficiency was obtained by the addition of 15 µg of total plasmid DNA to a 55-cm2 plate (Falcon, Lincoln Park, N J) followed by incubation for 20 h without glycerol shock. Cells were then trypsinized and transferred to a 24-well plate at a density of 2 x 106 cells/well and incubated until assayed. Control cells for the flux assays were transfected with pCDNA3 containing antisense DNA of the putative KCC3 gene. The K influx measured in cells transfected...
with the antisense construct and in untreated cells were indistinguishable.

Assay of K-Cl cotransport in HEK-293 cells. These methods were similar to those published earlier (16). Unidirectional K influxes were measured on control and transfected cells at 26°C with the use of 86Rb as a tracer. Cells in 24-well plates were incubated overnight and allowed to reach ~50% confluence. The plates were then washed with saline and incubated overnight and allowed to reach 50% confluence. The wells had been coated with poly-D-lysine (Fisher Scientific, Pittsburgh, PA) to promote adherence of the cells. There was a preincubation period of 30 min in standard medium (16) before measurement of the tracer flux. The desired agents were added during the final 10 min of the preincubation. As desired, the preincubation medium contained 30 µM bumetanide, which inhibited all Na-K-Cl cotransport. The bumetanide-inhibitable flux gave a measure of Na-K-Cl cotransport. Preincubation medium was removed, and flux medium (0.5 ml standard medium containing 2 µCi of 86Rb) was added. The flux period was 4 min. The radioactivities of cell extracts and of a sample of the flux medium were determined in a liquid scintillation counter by Cerenkov radiation. The protein concentration of each sample was determined (16). K influxes are expressed as nanomoles (or picomoles) per milligram protein per minute ± SD.

K-Cl cotransport was defined as the DIOA-sensitive K influx in the presence of 30 µM bumetanide. DIOA is R(+)-(2-n-butyl-6,7-dichloro-2-cyclopentenyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxyacetic acid (Research Biochemicals International, Natick, MA). DIOA inhibits K-Cl cotransport, but not Na-K-Cl cotransport, in red blood cells (10). In HEK cells, it inhibits both Na-K-Cl and K-Cl cotransport (12). Bumetanide at 30 µM did not inhibit K-Cl cotransport in control or KCC3-transfected cells, and DIOA at 50 µM fully inhibited K-Cl cotransport in the presence of bumetanide. The bumetanide-insensitive, DIOA-inhibitable K influx defines “basal” K-Cl cotransport. Additional criteria used to evaluate expression of K-Cl cotransport are described in RESULTS.

Intracellular Mg concentration was reduced with the use of the divalent cation ionophore A-23187 as described earlier (16). A-23187 was demonstrated to not affect Rb permeability (results not shown).

Other pretreatments were as follows: swelling in a medium of 150 mosmol/kgH2O for 10 min (only the NaCl concentration in the medium was reduced); NEM at 0.2 mM for 10 min; staurosporine at 5 µM for 10 min. These treatments were shown to give maximal effects on K-Cl cotransport.

Analysis of results. A nonparametric test was used to determine the statistical significance of differences in cotransport in transfected cells compared with controls. The test was the randomization test of matched pairs, one-tailed, a test with a power efficiency of 100% (35). Fitting of data to curves was carried out with SigmaPlot (Jandel, Corte Madera, CA).

RESULTS

Molecular cloning of KCC3 and amino acid comparisons. A novel K-Cl cotransporter isoform, KCC3, was identified by means of the strategy of homology cloning. A human placenta cDNA library was screened by PCR, and three identical KCC3 clones were isolated. The human KCC3 gene has an open reading frame of 3,453 bp and encodes a putative 1,150-amino acid residue protein with a molecular mass of 127.6 kDa (GenBank accession no. AF116242). The initiation codon, AUG, is the first in an adequate context, as predicted by Kozak (20), and is preceded by an upstream terminator codon.

The human KCC3 protein is 75–76% identical to KCC1 from human, pig, rat, and rabbit and 67% identical to rat KCC2. Human KCC3 shows 40 and 33% identity to the two Caenorhabditis elegans KCCs. The first, CE-KCC1, is a putative 1,003-amino acid residue protein (GenBank accession no. U40798), which shares functional characteristics with mammalian KCC1s (16). The second (GenBank accession no. U23171), CE-KCC2, is a protein of 1,020 amino acids whose predicted topology resembles CE-KCC1 and mammalian KCCs. However, its function has not been characterized. The human KCC3 protein shares ~20% identity with the apical and basolateral Na-K-Cl cotransporters (NKCC2 and NKCC1, respectively) and with the NCC. In contrast, the Na-K-Cl and Na-Cl cotransporters are 50–60% identical (8).

Amino acid sequences from four mammalian homologues of KCC1, rat KCC2, and human KCC3 are compared in Fig. 1. The amino acid residues that are in common among all KCC cotransporters are shaded. The 12 predicted transmembrane domains for KCC3 are indicated by horizontal lines, which are not quite in register among the other proteins. There are some regions of little homology between the KCC1 homologues and KCC2 and KCC3, particularly in the NH2 terminus, in scattered areas in the middle of the COOH terminus, and in the extracellular loop between the fifth and sixth transmembrane domains. In other regions, there is substantial identity, particularly within the transmembrane domains and in the beginning and end of the COOH terminus.

Hydropathy analysis and a topology model of human KCC3. Figure 2 shows a hydropathy plot of the KCC3 determined with the Kyte-Doolittle algorithm (21). Predicted are large hydrophilic NH2-terminal and COOH-terminal domains and 12 transmembrane regions, indicated by arrows. Figure 3 shows a topology model of the human KCC3. The hydropathy and topology of KCC3, like those of the other KCC subfamily members, resemble other proteins of the CCC family. There are 12 putative membrane-spanning helices and large NH2 and COOH termini. There is a large extracellular loop, characteristic of KCCs, between the fifth and the sixth transmembrane regions. This loop contains five potential N-linked glycosylation sites (Asn in positions 383, 402, 415, 421 and 432). There are two consensus cAMP-dependent protein kinase phosphorylation sites (181 and 941), four consensus protein kinase C phosphorylation sites in the COOH terminus (Thr in positions 814, 880, 1010, and Ser 941), and 12 consensus phosphorylation sites for casein kinase II.

The similarities and predicted evolutionary relationships between the various K-Cl cotransporters are represented in the phylogenetic tree in Fig. 4. An early gene duplication event gave rise to mammalian and C. elegans K-Cl cotransport proteins. Two subsequent duplications gave rise to the three mammalian KCC isoforms. The evolutionary distance between rat KCC2 and human KCC3 is much greater than the distance between human and rat KCC1.
Fig. 1. Deduced amino acid sequences for all known mammalian K-Cl cotransporters (KCC): human KCC3 (GenBank accession no. AF116242), rat KCC2 (GenBank accession no. U55816), and four mammalian KCC1s (GenBank accession nos. U55815, human, U55814, rat, U55054, pig, and U55053, rat). Amino acid residues are numbered at left of individual lines. Gaps were added to obtain best alignment. Amino acids that are common in all 6 cotransporters are shaded. Twelve predicted transmembrane domains are numbered and indented for KCC3 by horizontal lines. Alignments were made with Lasergene (DNASTAR) software by means of the clustal method with PAM250 residue weight table. Predictions of transmembrane domains were made as described in MATERIALS AND METHODS.
Tissue distribution of human KCC3. This was examined by Northern analysis (Fig. 5, A and B). Among the human tissues (Fig. 5A), transcripts of ~7.5 and ~4.3 kb were detected in kidney. A transcript of 9 kb was detected in brain and skeletal muscle, whereas transcripts of 9 and 7.5 kb were observed in heart, placenta, lung, liver, and pancreas. The level of expression was most abundant in heart, brain, and kidney. Among the cancer cell lines (Fig. 5B), transcripts of 9 and 7.5 kb were detected in promyelocytic leukemia HL-60, HeLa cell S3, lymphoblastic leukemia MOLT-4, colorectal adenocarcinoma SW480, lung carcinoma A549, and melanoma G361. Transcripts of 7.5 and 4.3 kb were detected in chronic myelogenous leukemia K-562. The integrity of the mRNA was verified in each lane of each blot with human b-actin probe (Fig. 5, A and B). Band intensity of this control was generally uniform for each tissue and cell line.

Analysis of tissue distribution of the KCC3 cotransporter in dbEST showed sources of KCC3 cDNA from infant brain (accession nos. H17157, AA324145, TO3544), placenta (N32221, N57318), testis (AA781746), and fetal liver and spleen (W78961, AA004792, R00066).

Chromosomal localization of KCC3. KCC3 was localized to chromosome 15. The distance between KCC3 and chromosome 15 marker D15S144 was 7.58 centi-Rays; the marker is proximal to the centromere. This corresponds approximately to chromosomal location 15q12 or 13. The gene for KCC1 is at chromosomal location 16q22.1 (22). The gene for NKCC2 is on chromosome 15 in a cluster of loci at 15q15–21 (36).

Functional studies. K-Cl cotransport was measured in cells transiently transfected with KCC3 and in control cells (see MATERIALS AND METHODS). Three types of studies were carried out on KCC3, first to confirm that KCC3 is a K-Cl cotransporter and second to obtain some preliminary information on the functional characteristics of the cotransporter: 1) the sensitivity of KCC3 to the inhibitors bumetanide, furosemide, and DIOA alone and in selected combinations; 2) the dependence of K influx on extracellular Cl and Na, and the apparent Michaelis constants (Kₘ) of KCC3 for K and Cl; and 3) regulation of cotransport: cells expressing KCC3 were subjected to various treatments known to alter K-Cl cotransport in red blood cells and KCC1 in HEK cells (17), and the effects on cotransport were evaluated and compared with the effects on endogenous K-Cl cotransport in HEK cells.

Function: inhibitors. Bumetanide, furosemide, and DIOA were tested for their effectiveness against KCC3...
in dose-response experiments. The inhibitors were tested alone and/or in selected combinations on cells transiently transfected with KCC3 cDNA and pretreated with 0.2 mM NEM, known to stimulate K-Cl cotransport in red blood cells (24) and in HEK cells (11). Bumetanide was tested on cells not NEM treated, in which the major pathway for K influx is Na-K-Cl cotransport. The IC₅₀ for inhibition of K influx was 0.3 µM. (The mean Na-K-Cl cotransport flux in these experiments was 0.35 ± 0.06 nmol·mg protein⁻¹·min⁻¹ (n = 4).) Next, bumetanide was tested on NEM-treated cells, in which the major K influx pathway is through the K-Cl cotransporter. The IC₅₀ for inhibition of K influx was ~100 µM. At 30 µM bumetanide, the concentration used throughout this study, there was ~10% inhibition, presumably as a result of inhibition of Na-K-Cl cotransport. The analysis of this curve at low bumetanide concentrations was confounded by the slight and inexplicable ~10% stimulation of K influx by 10 µM bumetanide. In KCC1-transfected HEK cells treated with NEM, a similar inexplicable phenomenon was observed (12; Fig. 3):

~15% stimulation of K efflux at 1 µM bumetanide. The conclusion from these experiments on bumetanide is that 30 µM bumetanide inhibits all of Na-K-Cl cotransport and little or none of K-Cl cotransport in cells transfected with KCC3 and treated with NEM.

Next, DIOA and furosemide dose-response curves were determined on cells transiently transfected with KCC3 and treated with 30 µM bumetanide and 0.2 mM NEM. The mean IC₅₀ for inhibition of K influx by DIOA was 9.3 ± 1.5 µM (n = 4); for furosemide the mean IC₅₀ was 103 ± 3 µM (n = 3). (The mean K-Cl cotransport flux in these experiments was 0.35 ± 0.07 nmol·mg protein⁻¹·min⁻¹ (n = 4). There was considerable variability in the estimates of KCC3 in these experiments and others below, perhaps owing to different extents of transfection.) Therefore, DIOA is a better inhibitor of KCC3 than furosemide; 50 µM DIOA inhibits virtually all of K-Cl cotransport.

**Function: Cl and Na dependence.** Figure 6 shows K influxes into NEM-treated transfected cells in normal, Na-free, and Cl-free media. The substitute ions were choline for Na and gluconate for Cl.

**Function: activation of transport by K and by Cl.** Figure 7 shows the K influx into KCC3-transfected cells as a function of the concentrations of K (Fig. 7A) and of Cl (Fig. 7B) in the media. The cells had been pretreated with NEM. As K concentration was increased, concentration of Na ([Na⁺]) was decreased. The substitute ion for Cl was gluconate. Apparent Kₐ values were estimated by a nonlinear least-squares iterative fit of the data to a hyperbolic function (SigmaPlot).

The apparent Kₐ for K, 10.1 mM (9.5 mM in Fig. 7A, 10.7 mM in another identical experiment), is lower than the Kₐ for KCC1, >25 mM (11), and slightly higher than the Kₐ for KCC2, 6.6 mM (29). In these experiments on the kinetics of K influx, apparent Kₐ
values for K were also determined for NEM-treated nontransfected cells. The results were not very clean; the $K_m$ values were 29 and 46 mM, with large asymptotic SEs. Despite the uncertainty of these results, the apparent $K_m$ for K is probably more than twofold lower for KCC3 than for endogenous K-Cl cotransport of HEK cells.

The apparent $K_m$ of KCC3 for Cl in Fig. 7B is 51 mM. There was considerable variability in estimates of this value. The mean from 12 experiments was $32 \pm 4$ mM; estimates ranged from 6 to 60 mM. Nevertheless, it is clear that the apparent $K_m$ for Cl of KCC3 is lower than that of KCC1 (>50 mM) (11) or KCC2 (101 mM) (29). Unsuccessful attempts were made to determine the apparent $K_m$ for Cl in control NEM-treated cells. All that could be concluded is that it is very high.

Function: regulation. K influx was measured under the following six conditions chosen to look at aspects of regulation of K-Cl cotransport, and to confirm that KCC3 is a K-Cl cotransporter: 1) measurement of K-Cl cotransport as the bumetanide-insensitive, DIOA-inhibitable K influx, called basal K-Cl cotransport; 2) activation of cotransport by hypotonic cell swelling (5); 3) stimulation of cotransport by NEM (24) (no other membrane transporter has been reported to be stimulated by NEM); 4) stimulation of cotransport by reducing cell Mg concentration with A-23187 (reducing cell Mg ([Mg]) apparently stimulates cotransport by reducing the concentration of Mg-ATP, the substrate for a kinase that inhibits K-Cl cotransport (6)); 5) stimulation of cotransport by the protein kinase inhibitor staurosporine (1) (no other membrane transporter has been reported to be stimulated by staurosporine); and 6) secondary activation of Na-K-Cl cotransport, observed to accompany expression of KCC1 (12, 16).

Table 1 summarizes the results of seven experiments on K-Cl (and Na-K-Cl) cotransport in control cells and cells transiently transfected with KCC3 cDNA. In control cells, swelling, NEM, and reduced cell [Mg] stimulated endogenous KCC by two- to threefold. In contrast with earlier results with HEK cells (16), staurosporine did not stimulate KCC. In KCC3-transfected cells, the results were different in several respects: swelling, reducing cell [Mg], and staurosporine all stimulated KCC 2- to 5-fold, whereas NEM stimulated 26-fold.

The critical comparisons are between control and transfected cells that received the same treatments. There was no significant enhancement of basal KCC in transfected cells. There was also no significant enhancement of KCC in cells with reduced cell [Mg] (there was enhanced cotransport in transfected cells in a majority of the experiments, but the increase was not significant). There was a small but statistically significant enhancement in swollen cells. There was a nearly fivefold enhancement in staurosporine-treated cells, and a ninefold enhancement in NEM-treated transfected cells. There was no secondary stimulation of Na-K-Cl cotransport in transfected cells; indeed there was a statistically significant inhibition of NKCC. With KCC1-transfected cells, there was secondary stimulation of NKCC (16). The above results provide further support for KCC3 being a K-Cl cotransporter, and provide clues about the regulation of the cotransporter.

DISCUSSION

Molecular structure. A novel K-Cl cotransporter, KCC3, was identified by means of the strategy of homology cloning. KCC3 shares predicted features of secondary protein structure with other members of the CCC family. These are the large NH$_2$ and COOH termini and probably 12 transmembrane domains. [It has been suggested recently (26) that cation-chloride cotransporters may possess 10 rather than 12 membrane spanners, owing to the very short loops between putative spanners 9 and 10 and also 11 and 12.]

In addition, KCC3, like all proteins in the CCC family, has $\sim$1,100 amino acid residues. KCC3 shares high amino acid sequence identity (76 and 67%, respec-
tively) with KCC1 and KCC2. KCC3 has a large extracellular loop between the fifth and sixth transmembrane domains, which is characteristic of the KCC proteins. These molecular characteristics show that KCC3 is a member of the CCC family and the KCC subfamily, and that KCC3 is a novel isoform.

Tissue distribution. The distribution of KCC3 is broad like that of KCC1 (11), but not KCC2, which is limited to neuronal tissues (29). KCC3 was most abundant in kidney and heart, followed by brain and skeletal muscle. The three different length transcripts suggest that there are alternatively spliced variants of KCC3. Like KCC1 (33), KCC3 is expressed in erythroleukemic cells and therefore probably is expressed in mature erythrocytes.

Function. The results presented here establish that the gene KCC3 codes for a K-Cl cotransporter. K influx into HEK cells expressing the KCC3 protein required Cl in the medium, but not Na. It was weakly inhibited by bumetanide and furosemide.

There are activities in common between KCC1 and KCC3. Both are stimulated by NEM and by hypotonic cell swelling (the latter small but significant for KCC3) (11, 16). KCC1 and KCC3 are both stimulated by staurosporine (16).

There is one significant difference in the activities of KCC1 and KCC3. In HEK cells expressing KCC1, there is a secondary stimulation of Na-K-Cl cotransport (12, 16). This was recently attributed, at least in part, to a reduction in intracellular concentration of Cl ([Cl]i) caused by the enhanced K-Cl cotransport (12). Reduced [Cl]i is well known to stimulate Na-K-Cl cotransport (2, 25). In striking contrast, in cells transiently transfected with KCC3, there was no stimulation of Na-K-Cl cotransport; indeed there was statistically significant inhibition (Table 1). The explanation for this inhibition is not readily apparent. It is known that elevated [Cl]i and intracellular [Na]i (2), cell swelling, and dephosphorylation of the cotransporter (14) inhibit Na-K-Cl cotransport. It is unlikely that enhanced K-Cl cotransport causes elevated [Na]i or [Cl]i or cell swelling. Therefore the inhibition of NKCC by KCC3 may be a consequence of dephosphorylation of the NKCC protein, perhaps because of competition between KCC3 and the Na-K-Cl cotransporter for regulatory enzymes (cf. Ref. 12), but this is purely speculative.

A few words of explanation are in order about the relation between the stimulatory effects of NEM and staurosporine and regulation of cotransport. When K-Cl cotransport is stimulated by swelling, there is good evidence that the primary event is the inhibition of a volume-sensitive protein kinase, allowing a phosphatase to activate cotransport by dephosphorylation (6). It has been proposed that NEM stimulates cotransport.

Table 1. Regulation of K-Cl cotransport (KCC) in control HEK and in cells transiently transfected with human KCC3

| Criteria                        | KCC or NKCC, pmol·mg protein\(^{-1}\)·min\(^{-1}\) | Control | KCC3 | P     |
|---------------------------------|--------------------------------------------------|---------|------|-------|
| Basal KCC                       | 22 ± 4                                           | 22 ± 2  | NS   |       |
| Swelling-activated KCC          | 79 ± 6                                           | 100 ± 10| 0.016|
| NEM-stimulated KCC             | 58 ± 6                                           | 518 ± 40| 0.008|
| Low cell [Mg]-stimulated KCC   | 41 ± 3                                           | 43 ± 6  | NS   |       |
| Staurosporine-stimulated KCC   | 19 ± 3                                           | 77 ± 9  | 0.008|
| NKCC                            | 250 ± 11                                         | 209 ± 11| 0.016|

Results are means ± SE from 7 experiments of the same design. See text for explanation of criteria for evaluating regulation of KCC. Control and KCC3 transfections, measurements of cotransport, and calculations of P values for significant differences between means were carried out as described in MATERIALS AND METHODS. HEK, human embryonic kidney; NKCC, Na-K-Cl cotransport; NEM, N-ethylmaleimide; [Mg], concentration of Mg; NS, not significant.

Fig. 7. Activation of K influx by increasing concentrations of K ([K]; A) and Cl ([Cl]; B). Cells had been transfected with KCC3 and pretreated with 0.2 mM N-ethylmaleimide for 10 min. A: apparent \( K_m \) for K was 9.5 ± 1.4 mM. In another experiment carried out the same way, apparent \( K_m \) was 10.7 ± 2.0 mM (errors are asymptotic SEs from curve-fitting procedure). B: apparent \( K_m \) for Cl was 51 ± 9 mM. In 12 experiments of the same design (including this one) mean \( K_m \) was 32 ± 4 mM.
port by inhibiting the volume-sensitive kinase (18). Stauroporin is also a protein kinase inhibitor of high potency and broad specificity. However, it was shown that this agent stimulates K-Cl cotransport not by inhibiting the volume-sensitive kinase, but rather by inhibiting a kinase that in turn inhibits the activating phosphatase (1). More recent work suggests that the kinase regulating the phosphatase is one, possibly two, Src family kinases, Fgr and/or Hck (5). The physiological input to these tyrosine kinases is unknown.

The question arises, what is the physiological role of KCC3? It has a broad distribution among tissues, so there may be more than one role. It is only weakly volume sensitive in HEK cells; it is possible, but not known, that it is more volume sensitive in other cells. If so, KCC3 may be involved in volume regulation and in transepithelial transport of salt and water. In the heart, skeletal muscle, and brain, where KCC3 is highly expressed, another function is possible: regulation or buffering of K and Cl concentrations in cells and in the interstitial space. Generally the driving force caused by the chemical potential gradients of K and Cl leads to a net outward KCl flux. However, the cotransporter functions in both directions. With an elevated external concentration of K or abnormally low [Cl−], the K-Cl cotransporter would mediate a net KCl influx. The bidirectionality of the cotransporter would permit it to serve the buffering capacity. With higher affinities for K and Cl at the external membrane surface than KCC1, KCC3 may be better suited to such a regulatory function than KCC1. The affinity for K at the intracellular surface may be higher than the external surface, as it is in human red blood cells (19). A similar function has been proposed for KCC2 in the brain (29). For none of the isoforms of KCC are affinities for K and Cl at the intracellular membrane surface known; knowledge of these affinities would afford a clearer view of the possibilities of an ion concentration regulatory function for KCCs. The input to the signal transduction pathway controlling K-Cl cotransport may well involve the Src family tyrosine kinases discussed above.

Recently discovered KCCs. While this paper was undergoing final revision, two reports were published on new KCC3s. In the first, a KCC3 was reported (15). This KCC3 has 1,099 amino acid residues and is 93.2% identical to our KCC3, virtually all of the difference being in the 90 amino acids of the NH2 terminus of our KCC3. The chromosomal locations of the genes for the two KCC3s were at least very similar (15q13). We conclude that our KCC3 and that of Hiki et al. (15) are splice variants of one another. The splice variants are likely to have functional differences; it is unknown what that difference might be.

In the second, more recent, report, KCC3 and KCC4 were presented (28). KCC4 has 1,150 amino acid residues, the same as our KCC3. The protein sequences are essentially identical; the only difference was at position 802 (KCC4, Glu; our KCC3, His). Because KCC4 is probably a splice variant of the KCC3 of Hiki et al. (15) (as is our KCC3), we suggest that the KCC4 of Mount et al. (28) be called KCC3 and their KCC3 be called KCC4. Mount et al. (28) did no functional characterization of their KCC4, only of their KCC3.

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