Mouse K-Cl cotransporter KCC1: cloning, mapping, pathological expression, and functional regulation

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1Molecular Medicine and Renal Units, Beth Israel Deaconess Medical Center, Boston 02215; 2Department of Laboratory Medicine, The Children’s Hospital, Boston 02115; Departments of 3Medicine, 4Cell Biology, and 5Pathology, Harvard Medical School, Boston, Massachusetts 02215; and 6Department of Clinical and Experimental Medicine, University of Verona, Verona, Italy

Su, Wanfang, Boris E. Shmukler, Marina N. Chernova, Alan K. Stuart-Tilley, Lucia De Franceschi, Carlo Brugnara, and Seth L. Alper. Mouse K-Cl cotransporter KCC1: cloning, mapping, pathological expression, and functional regulation. Am. J. Physiol. 277 (Cell Physiol.): C899–C912, 1999.—Although K-Cl cotransporter (KCC1) mRNA is expressed in many tissues, K-Cl cotransport activity has been measured in few cell types, and detection of endogenous KCC1 polypeptide has not yet been reported. We have cloned the mouse erythroid KCC1 (mKCC1) cDNA and its flanking genomic regions and mapped the mKCC1 gene to chromosome 8. Three anti-peptide antibodies raised against recombinant mKCC1 function as immunoblot and immunoprecipitation reagents. The tissue distributions of mKCC1 mRNA and protein are widespread, and mKCC1 RNA is constitutively expressed during erythroid differentiation of ES cells. KCC1 polypeptide or related antigen is present in erythrocytes of multiple species in which K-Cl cotransport activity has been documented. Erythroid KCC1 polypeptide abundance is elevated in proportion to reticulocyte counts in density-fractionated cells, in bleeding-induced reticulocytosis, in mouse models of sickle cell disease and thalassemia, and in the corresponding human disorders. mKCC1-mediated uptake of 86Rb into Xenopus oocytes requires extracellular Cl−, is blocked by the diuretic (−)-2-n-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-indenyl-5-yl)oxyacetic acid, and exhibits an erythroid pattern of acute regulation, with activation by hypotonic swelling, N-ethylmaleimide, and staurosporine and inhibition by calyculin and okadaic acid. These reagents and findings will expedite studies of KCC1 structure-function relationships and of the pathobiology of KCC1-mediated K-Cl cotransport.

red blood cells; sickle cell disease; thalassemia; SC disease; SAD1

AN EVOLUTIONARILY ANCIENT gene superfamily, the cation-
Cl− cotransporter (CCC) gene superfamily (35, 40) comprises three functionally distinct branches: the Na-K-Cl cotransporters (NKCC) (39, 42, 54) or bumetanide-sensitive cotransporters (15, 20), the Na-Cl cotransporters (NCC) or thiazide-sensitive cotransporters (TSC) (21), and the K-Cl cotransporters (KCC) (22, 26, 41, 46). In addition, CCC members discovered by homology searches in the genomes of yeast (YBR235W) (1), nematodes (26), flies (AA697465 and others), and plants (24) may well yet define additional functional groups within this superfamily.

The NKCC cotransporters have wide roles in transepithelial reabsorption (NKCC2) and secretion (NKCC1) of salt and water (48), as well as central roles in cell volume regulation in response to hypertonic stress (35, 38, 40). Mutations in NKCC have been implicated as one of several causes of Bartter’s syndrome (48, 49, 52). The NCC cotransporters function in transepithelial NaCl reabsorption, and loss-of-function mutations in NCC-TSC have been implicated in Gitelman’s syndrome (50).

Unlike the Cl−-loading NKCC and NCC polypeptides, the KCC polypeptides KCC1 (22), KCC2 (41), and KCC3 (25) serve under prevailing physiological concentration gradients to mediate KCl exit from nonexcitable cells. K-Cl cotransport has been documented in red blood cells (32), neurons (41, 46), heart (44), and (controversially) kidney (23, 47; see Ref. 33 for additional references). In proximal straight tubules, K-Cl cotransport may contribute to reabsorption of filtered Cl− across the basolateral membrane of the tubular epithelial cell into the peritubular space (47). In neurons, KCC2-mediated K-Cl cotransport induced during postnatal development is responsible for the change in Cl− reversal potential that converts γ-aminobutyric acid from an excitatory to an inhibitory neurotransmitter (46). In red blood cells, K-Cl cotransport has been shown to be elevated in sickle cells, where it contributes to the hydrated state of the densest fraction of cells most prone to sickle and to provoke vasoocclusive complications (6, 32). The very broad tissue distribution of KCC1 mRNA (22, 26, 33) has suggested a functional role more widespread than heretofore appreciated.

Therapeutic blockade of pathologically elevated K+ efflux from sickle red blood cells is a means of increasing cell water content to prevent pathological elevation of intracellular Hb S concentration. To achieve this end, the two principal K+ efflux pathways of the erythrocyte must be downregulated or blocked (7). Clinically tolerable prototype inhibitors of one of the pathways, the IK1 Ca2+-gated K+ channel of intermediate conductance (Gardos channel) (45, 51), have already been tested in a mouse model of sickle cell disease (14) and are under clinical test in human subjects (8). Although oral administration of Mg2+ salts has shown promise in effecting in vivo downregulation of the second pathway,

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that of K-Cl cotransport (12), no other clinically useful K-Cl cotransporter inhibitors are available.

Increasing numbers of mouse models of sickle cell disease of ever greater similarity to the human disease are being generated (14, 37). We previously documented the presence in mouse erythroid cells of the K1 Ca\(^{2+}\)-gated K\(^+\) channel (14, 45, 51) and K-Cl cotransporter activity (2, 3). Thus the mouse represents a genetically manipulable system in which to study the role of red cell K\(^+\) transporters in the clinical evolution of sickle cell disease and other hemoglobinopathies and in which to explore the function of K-Cl cotransport in the wide range of tissues in which its function is little understood.

For these reasons we set out to clone the mouse erythroid KCC1 (mKCC1) DNA and to characterize its polypeptide product. Here we present the mKCC1 cDNA, the 5'- and 3'-flanking regions of the mouse KCC1 gene, a characterization of three new anti-KCC1 antibodies, the tissue distribution of KCC1 transcript and polypeptide expression, detection of red cell KCC1, and demonstration of its elevated abundance in sickle cells and in other conditions characterized by reticulocytosis. Finally, we express mKCC1 in Xenopus oocytes, where it exhibits several regulatory and pharmacological properties of native erythroid K-Cl cotransport.

**METHODS**

RNA preparation. Total RNA from mouse tissues (freshly resected kidney, stomach, spleen, and colon) and from murine erythroleukemia (MEL) cells was prepared using the RNeasy kit (Qiagen, Chatsworth, CA). Total RNA from ES cells and ES-derived colonies was prepared using the RNAsol reagent (Biotecx, Houston, TX). A mouse tissue total RNA panel (liver, brain, heart, testis, ovary, and embryo) was purchased from Ambion (Austin, TX).

RT-PCR. One microgram of total RNA was reverse transcribed from an oligo(dT) primer with use of the First-Strand DNA Synthesis kit (Ambion). Five percent of the RT reaction volume was used for hot-start PCR in a total reaction volume of 50 µl, using the expand high-fidelity PCR system (EHFPS; Boehringer) or (for Fig. 2B) Taq DNA polymerase (Promega) in the supplier’s recommended buffers (unless otherwise indicated).

We used available nucleotide and amino acid sequences of human (accession no. U55054, GenBank), rabbit (accession no. U55053, GenBank), and rat (accession no. U55051, GenBank) KCC1 to design forward (KCC1.NT) and reverse (KCC1.LCT) oligonucleotides corresponding to the NH\(_2\)- and COOH-terminal peptides of these KCC1 polypeptides. We also designed forward and reverse oligonucleotides (Table 1), some slightly degenerate, corresponding to selected highly conserved internal regions within these KCC1 cDNAs.

PCR mixes lacking only primers were preheated at 82°C for 1 min, then appropriate primers were injected into the mix through mineral oil. The complete reaction mixes were denatured for 3 min at 95°C, then cycled through the following conditions: denaturation for 45 s at 94°C, annealing for 2 min at 60°C [or, as indicated, in the presence of Q-solution (Qiagen) at 52°C], and elongation for 2–6 min at 72°C. Final extension for 10 min at 72°C was terminated by rapid cooling to 4°C after the indicated number of cycles or (for β-actin and β-globin) 25 cycles. PCR products were separated in 1% agarose gels and, as needed, purified with the QIAquick gel extraction kit (Qiagen), then subjected to direct sequencing (ABI 373 DNA Sequencer, Molecular Medicine Unit, Beth Israel Deaconess Medical Center) or subcloning into the “T vectors” pCR2.1 (Invitrogen) or pGEM-EasyT (Promega). PCR products encompassing the full-length KCC1 coding region, as well as selected shorter, overlapping KCC1 PCR products, were purified and sequenced. PCR fragments were sequenced on both strands, and at least two independently amplified PCR fragments were used to confirm each nucleotide position. Only subcloned plasmid fragments without PCR-generated mutations were used for downstream applications.

DNA sequence assembly and analysis were carried out with programs of the University of Wisconsin Genetics Computing Group and the National Center for Biotechnology Information.

5’-Rapid amplification of the cDNA ends. PCR-ready Marathon mouse brain cDNA (Clontech) was used to do the

### Table 1. Oligonucleotides

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<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Position</th>
<th>Reference</th>
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<tr>
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<td>5'--AGGCCCACTCTACACCTGTTG--3'</td>
<td>1-21</td>
<td>U55815††</td>
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<td>KCC1.LCT†</td>
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<td>3262-3235</td>
<td>U55815†</td>
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<tr>
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<td>3605-3586</td>
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</tr>
<tr>
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<td>1395-1415</td>
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<tr>
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* Based on homology with KCC1 cDNAs. † Degenerate. ‡ From indicated EST clones.
Three peptides were synthesized and peptide antibodies. Peptidyl-glycosidase (PNGase F; Promega), SDS-PAGE, N\textsuperscript{35S}-TransLabel (ICN, Costa Mesa, CA) was performed (10) with the 32P-labeled KCC1.NT oligonucleotide (encoding the 

In standard buffer systems, no PCR product was successfully amplified using a range of conditions. Only in the presence of the annealing modifier, Q-solution, was it possible to visualize amplified PCR products by ethidium bromide staining after separation in 1% agarose gel. Diffuse PCR product bands of ~230 bp (experiment a) and ~500 bp (experiment b) were purified from agarose gel and subcloned into PCR2.1 and pGEM-EasyT. Nucleotide sequences were verified by comparison to the corresponding genomic sequence (see below).

Cloning and amplification of mKCC1 genomic fragments. The mKCC1 gene was cloned by hybridization screening of a commercial high-density arrayed bacterial artificial chromosome (BAC) genomic library in pBeloBAC11 (Research Genetics, Huntsville, AL). Five unique BAC DNA clones containing the complete mKCC1 gene, were characterized by restriction mapping and hybridization analysis (unpublished observations). BAC DNA 87P12 and 93J3 were purified and studied further.

Southern blot hybridization of Hind III-digested BAC DNA with the \textsuperscript{32P}-labeled KCC1.NT oligonucleotide (encoding the KCC1 NH\textsubscript{2} terminus) identified an ~3-kb Hind III fragment containing the 5'-flanking region of the mKCC1 gene, exon 1 and part of intron 1. This fragment was subcloned and sequenced. Physical linkage between the mKCC1 and mouse lecithin-cholesterol acyltransferase (mLCAT) genes was determined by 25-cycle PCR amplification from 100 ng of 87P12 or 93J3 BAC DNA with use of the forward primer KCC1.E22 (from mKCC1 exon 22; unpublished observations) and the reverse primer LCAT.E1R (from the NH\textsubscript{2}-terminal sequence of mLCAT, X54095). Amplification conditions were as for RT-PCR.

cDNA cloning, reconstruction, transcription, and translation of mKCC1. Full-length mKCC1 cDNA amplified by 26-cycle RT-PCR from brain total RNA was subcloned into pGEM-EasyT and sequenced. A plasmid without mutations and containing 17 nt of 5'-untranslated region (UTR) was subcloned into the Xenopus oocyte expression vector pX7T (51), and the recombinant pXmKCC1 was linearized with Xba I to generate template for transcription of capped cDNA from the plasmid's T7 promoter (Megascript, Ambion). cRNA was injected into Xenopus oocytes maintained for at least 2 days at 19°C as previously described (11, 51).

In vitro translation of mKCC1 cRNA in the presence of \textsuperscript{35S}-TransLabel (ICN, Costa Mesa, CA) was performed (10) with the rabbit reticulocyte lysate (nuclease-treated) system (Promega) in the presence of canine pancreatic microsomal membranes (Promega). N-deglycosylation of in vitro translated polypeptide with peptidyl-N-glycosidase (PNGase F; Promega), SDS-PAGE, and autoradiography were carried out as previously described (10, 55).

Generation, purification, and characterization of anti-peptide antibodies. Three peptides were synthesized and HPLC purified by the Massachusetts Institute of Technology Biopolymers Facility. The COOH-terminal peptide encoding mKCC1 amino acids 1074-1085 was synthesized with an acetylated NH\textsubscript{2}-terminal cysteine residue. Two NH\textsubscript{2}-terminal peptides encoding mKCC1 amino acids 1-14 with a COOH-terminal amidated the cysteine residue. One NH\textsubscript{2}-terminal peptide had a free NH\textsubscript{2} terminus, and the other was synthesized with an acetylated NH\textsubscript{2}-terminal residue.

HPLC-purified peptides were reacted via their appended cysteiny1 sulfhydryl groups with the cross-linker 4-(maleimidomethyl)cyclohexane-1-carboxylic acid-N-hydroxysuccinimide ester (Pierce, Rockford, IL) and then coupled to keyhole limpet hemocyanin (Calbiochem). After collection of preimmune sera, peptide-coupled keyhole limpet hemocyanin mixed in Freund's complete adjuvant was used to immunize New Zealand White rabbits subcutaneously, with boosts of antigen compounded in incomplete Freund's adjuvant. Immune sera were affinity purified by passage over a column of Sulfo-link matrix (Pierce) derivatized with peptide antigen via its terminal cysteine. After extensive washing, the remaining bound immunoglobulin was eluted with pH 2.5 acetate and neutralized with Trizma base.

Affinity-purified antibody was used for immunoblot, as previously described (10, 55). Treatment of KCC1-containing membrane samples with SDS-load buffer at >37°C or prolonged storage at 4°C exacerbated SDS-resistant oligomerization. Temperatures above >60°C led to disappearance of monomeric polymeric peptide from the blots.

Heterologous expression of KCC1 cDNA in HEK-293 cells. HEK-293 cells were maintained in DMEM (GIBCO-BRL, Bethesda, MD) containing 10% calf serum. mKCC1 cDNA was subcloned into pcDNA3 (Invitrogen) with a 5'-UTR of 20 nt. Recombinant or nonrecombinant plasmid vector was transfected into HEK-293 cells at 50% confluency by the calcium phosphate method (22). Two days later, cells were harvested for immunoblot analysis.

Plates (~10 cm) of cells were metabolically labeled for 4 h with 200 µCi of \textsuperscript{35S}-TransLabel (NEN-DuPont, Boston, MA) 24 h after transfection. Normal growth medium was then restored for 48 h, and labeled cells were harvested for immunoprecipitation.

Cells were rinsed with Hanks' balanced salt solution, scraped, collected, and extracted with IP buffer containing 1\% Triton X-100, 140 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1% NP-40, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, and 1× complete protease inhibitor (Boehringer, Indianapolis, IN) used according to manufacturer's protocol, then vortexed and incubated on ice for 30 min. Clarified IP buffer extracts were assayed for protein by the bicinchoninic acid method (Pierce). After 2 h of incubation with affinity-purified antibody (2 µl/200 µl extract), samples were incubated for 1 h with protein G-Sepharose (Sigma Chemical), washed extensively with IP buffer, and analyzed by SDS-PAGE scintigraphy.

KCC1 detection in tissues. Mice were bled retroorbitally or were anesthetized with methoxyflurane (Metofane) and bled by cardiac puncture. Ghost membranes were prepared as previously described. Freshly excised mouse tissues were homogenized on ice with a Polytron in homogenization buffer identical in composition to extraction buffer except for the absence of NP-40. The microsome-containing supernatant from a 30-min centrifugation at 24,000 g was analyzed by SDS-PAGE and immunoblot.

Human blood was obtained according to clinical protocols approved at Beth Israel Deaconess Medical Center, The Children's Hospital, and the University of Verona Hospital. Red cell indexes were measured with autoanalyzers (model H\textsuperscript{+}3 or H\textsuperscript{+}4, Technicon, Tarrytown, NY). Discontinuous Stractan gradient fractionation of red blood cells (5, 6) and red cell membrane preparation by hypotonic lysis (3) were
carried out as previously described. Semiquantitative densitometry of enhanced chemiluminescence immunoblot signals was performed with an Ultroscan XL laser scanning densitometer (LKB-Pharmacia, Kalamazoo, MI) or by analysis of flatbed scans with NIH Image version 1.61.

Fig. 1. Molecular cloning of mouse KCC1 (mKCC1) gene regions flanking protein coding region. A: sequence of 5' flanking region of mKCC1 gene, with aligned available 5' untranslated sequences of KCC1 from human (U55054), pig (AF028807), and rabbit (U55053). Initiator ATG (numbered +1) is boldface, as are mismatches in aligned sequences at bottom. Lowercase g at position +267 indicates most 5' nucleotide of mKCC1 cDNA yielded by 5' rapid amplification of cDNA ends (RACE) PCR. Potential transcription factor binding sites and motifs are labeled as follows: CACCC, bold and underlined; E-box, bold and italic; GATA motif, underlined. TATA box, CCAAT motif pentamer, and MCAT motif are not present. Six formal Sp1 motifs (GGGCGG) at position +212 to +247 overlap with cDNA sequence. A 93-nt perfect direct repeat of unknown function is overlined. Lowercase 23-mer indicates position of oligonucleotide KCC1.IT.

B: sequence of 3' flanking region of mKCC1 gene and its junction with adjacent downstream lecithin-cholesterol acyltransferase (LCAT) gene, aligned with corresponding human sequence (X04981). KCC1 termination codons are underlined and marked with an asterisk. Potential polyadenylation sites are boldface and underlined. mKCC1 3' most defined nucleotide at nt 579 (Ref. 40) and human KCC1 3' most defined nucleotide at nt 507 (Ref. 22) are boldface; 3' most reverse oligonucleotide used in this study (Table 1) is bold italic. Initiation codons for LCAT genes are boldface.

86Rb influx measurements into Xenopus oocytes. Stage V-VI oocytes from mature female Xenopus were dissected and defolliculated as previously described (10, 11, 51). Oocytes were injected with 50 nl of water or the same volume containing 5 ng of mKCC1 cDNA, then maintained at 19°C for
2–10 days. All influx media contained 0.1 mM ouabain to inhibit endogenous oocyte Na-K-ATPase and 5 µM bumetanide to inhibit endogenous oocyte Na-K-2Cl cotransport. Isotonic medium was ND-96, and hypotonic medium was ND-72. Cl⁻-free medium consisted of gluconate salts. For experiments in Cl⁻-free media, oocytes were preincubated for 60 min or overnight before assay in the same medium. For experiments testing effects of alternate media or drug exposure, oocytes were preincubated for 30 min before initiation of 60-min influx assays under the same conditions. In some experiments, drugs were injected at 10-fold the desired final concentration in 50 nl of Na-HEPES, pH 7.4, 10 min before initiation of the influx assays. Influx assays were performed on groups of 6–15 oocytes in 150 µl of influx medium containing 2.5–5 µCi of ⁸⁶RbCl (NEN-Dupont). After completion of the influx period, oocytes were washed through five changes of 50 ml of Cl⁻-free influx medium lacking isotope at 4°C, then subjected, along with aliquots of influx medium, to gamma counting in a Cobra AutoGamma (Packard Instruments, Downers Grove, IL).

RESULTS

Molecular cloning of mKCC1 cDNA. mKCC1 cDNA cloning by RT-PCR was based on homology with published sequences and sequences deposited in the databases. Combination of any forward vs. reverse couple of primers (including KCC1.NT and KCC1.CT; Table 1) allowed amplification of PCR products (including full-length products) of identical mKCC1 cDNAs from spleen, ES cells, and brain with use of EHFPS under standard conditions after 30–32 cycles of PCR.

The resulting cDNA possessed terminal coding sequences derived from synthetic oligonucleotides of non-murine origin. To verify the terminal coding sequences, 5'-RACE and additional 3'-UTR amplification (KCC1.E20F vs. KCC1.3p) were performed. 5'-RACE extended the KCC1 cDNA sequence 5'-ward up to nt −67, further than available for other KCC1 cDNAs (Fig. 1A). The unusually high G + C content of the mKCC1 mRNA 5'-UTR through nt −193 (up to 86%) very likely impedes processivity of RTs. This may explain the failure of RACE-enhanced primer extension to identify the transcriptional initiation site of the mKCC1 mRNA in this and other studies. Oligonucleotide KCC1.IT (containing the terminator codon at nt −207 to 205, upstream of the presumed ATG; Table 1) did not amplify KCC1 products from cDNA but did from genomic DNA (not shown). Thus assignment of the ATG remains presumptive in mKCC1,¹ as in KCC1 of all other species reported to date.

3'-UTR amplification confirmed the absence of COOH-terminal amino acid difference between mKCC1 and KCC1 from human, rat, rabbit, and pig (AF028807). In addition to the previously noted 12 putative transmembrane spans preceded by ~120 putatively cytoplasmic NH₂-terminal amino acids and followed by ~436 putatively cytoplasmic COOH-terminal amino acids, the four exofacial consensus N-glycosylation sites, and numerous phosphorylation sites, mKCC1 shares with KCC1 of other species two leucine zipper motifs. Although one (amino acid 560–581) resides in putative transmembrane helices 9 and 10, the other (amino acid 697–718) is found ~48 residues beyond the final putative transmembrane helix 12, where it may serve a protein-binding function at the cytosolic face of the protein.

¹The 3,764-nt sequence encoding the mKCC1 cDNA and its deduced sequence of 1,085 amino acids have been deposited with GenBank under accession number AF121118.
The EST database revealed 10 clones encoding parts of mKCC1 (AA521711, AA185691, AA619174, AA200145, AA511202, AA410020, AA410019, AA921466, AA474557, AA921468). During completion of this work, an mKCC1 cDNA sequence (AF047339) from MEL cells of DBA strain origin was published (43). Except for the absence of 5'-noncoding region, AF047339 differs from the present AF121118 only in several 3'-UTR nucleotides that may reflect strain differences.

Cloning of the 5'- and 3'-flanking regions of the mKCC1 gene. Upstream of the presumptive ATG initiation codon of the mKCC1 gene, 1,277 bp of new genomic sequence were found in the plasmids subcloned from the 87P12 and 93J3 BAC genomic DNA clones. This genomic sequence (AF116526) was used to confirm cDNA sequence obtained from 5'-RACE. Several motifs suggesting potential transcription factor binding sites were noted (Fig. 1A), but the extent of the promoter region remains to be determined. Notable in this putative promoter region is a 93-nt direct perfect repeat (overlined).

The human KCC1 gene is part of a tightly packed cluster of at least five genes at chromosome 16q22 (17). The 3'-end of the human KCC1 gene overlaps the promoter region of the human LCAT gene (22). We therefore sought and found evidence for similar physical linkage between the KCC1 and LCAT genes in the mouse. The genomic interval between the coding regions of KCC1 and LCAT was 78% identical in nucleotide sequence between mouse (GenBank AF121128) and human. Mapping of the mouse LCAT gene to chromosome 8 (53) allowed the conclusion that the physically linked mKCC1 gene also maps to chromosome 8 at 53 cM.

Tissue distribution of mKCC1 mRNA and expression in a model of erythroid development. RT-PCR revealed full-length mKCC1 mRNA at highest abundance in whole embryo, heart, brain (including choroid plexus), and ovary, with intermediate abundance in spleen, ES cells, kidney, stomach, testis, and liver. Abundance was lowest among tested tissues in colon (Fig. 2A). This tissue distribution approximates that in rat (22) but is somewhat more widespread than in human (33). KCC1...
mRNA levels did not evidently change on erythroid induction of ES cells, during which mRNA levels of both erythroid differentiation markers β-globin and AE1 increased markedly (Fig. 2B).

In vitro translation and N-glycosylation of mKCC1 polypeptide. As shown in Fig. 3, translation of mKCC1 cRNA in the presence of dog pancreatic microsomes yielded two polypeptides. The relative molecular weight of the larger polypeptide decreased on incubation with PNGase F to comigrate with the lower polypeptide, suggesting that the larger polypeptide carried N-glycans. The glycoproteins synthesized from mKCC1 cRNA (lanes 3 and 4) were indistinguishable from those produced from rabbit KCC1 cRNA (lanes 1 and 2).

Anti-KCC1 antibodies detect expression of rabbit KCC1 and mKCC1 in transiently transfected HEK-293 cells. Antibodies to the NH2-terminal 14 residues of mKCC1 (Fig. 4A) and to the COOH-terminal 12 residues of mKCC1 (Fig. 4B) each recognized with immunospecificity the same complex of polypeptides in HEK-293 cells transiently transfected with rabbit KCC1 cDNA. Polypeptides detected by both antibodies were reduced comparably in molecular mass (Mr) by treatment of membranes with PNGase F before SDS-PAGE and immunoblot analysis (Fig. 4C). Similar behavior was evident when cells transiently transfected with mKCC1 (Fig. 4D) were immunoblotted with antibody to NH2 and COOH termini, as well as with antibody to the acetylated NH2-terminal peptide (Fig. 4D). All immunoreactivity at ~108 kDa extending up to ~130 kDa was specifically competed in the presence of antigen peptide, but not in the presence of irrelevant peptide. Figure 4 also shows that SDS-resistant oligomerization/aggregation was variably present for rabbit KCC1 and mKCC1.

Anti-mKCC1 antibodies immunoprecipitate mKCC1 transiently expressed in HEK-293 cells. The HEK-293 cells transiently transfected with mKCC1 cDNA were metabolically labeled, solubilized in nonionic detergent, and subjected to immunoprecipitation in the presence of irrelevant peptide or peptide antigen. Figure 5 shows that 35S-labeled mKCC1 polypeptide was immunoprecipitated with anti-mKCC1 antibodies in the presence of antigen peptide.

Fig. 5. Immunoprecipitation of recombinant rbKCC1.
HEK-293 cells transiently transfected with rbKCC1 cDNA were labeled with 35S-TransLabel for 4 h, then whole cell NP-40 lysates (500 µg protein) were subjected to immunoprecipitation with indicated antibodies in presence of excess irrelevant peptide (lanes 1, 3, 5, and 6) or peptide antigen (lanes 2 and 4), and then to SDS-PAGE scintigraphy. AcNH2term, acetylated NH2 terminus; COOHterm, COOH terminus. Arrows, KCC1 polypeptides.

mRNA levels did not evidently change on erythroid induction of ES cells, during which mRNA levels of both erythroid differentiation markers β-globin and AE1 increased markedly (Fig. 2B).

In vitro translation and N-glycosylation of mKCC1 polypeptide. As shown in Fig. 3, translation of mKCC1 cRNA in the presence of dog pancreatic microsomes yielded two polypeptides. The relative molecular weight of the larger polypeptide decreased on incubation with PNGase F to comigrate with the lower polypeptide, suggesting that the larger polypeptide carried N-glycans. The glycoproteins synthesized from mKCC1 cRNA (lanes 3 and 4) were indistinguishable from those produced from rabbit KCC1 cRNA (lanes 1 and 2).

Anti-KCC1 antibodies detect expression of rabbit KCC1 and mKCC1 in transiently transfected HEK-293 cells. Antibodies to the NH2-terminal 14 residues of mKCC1 (Fig. 4A) and to the COOH-terminal 12 residues of mKCC1 (Fig. 4B) each recognized with immunospecificity the same complex of polypeptides in HEK-293 cells transiently transfected with rabbit KCC1 cDNA. Polypeptides detected by both antibodies were reduced comparably in molecular mass (Mr) by treatment of membranes with PNGase F before SDS-PAGE and immunoblot analysis (Fig. 4C). Similar behavior was evident when cells transiently transfected with mKCC1 (Fig. 4D) were immunoblotted with antibody to NH2 and COOH termini, as well as with antibody to the acetylated NH2-terminal peptide (Fig. 4D). All immunoreactivity at ~108 kDa extending up to ~130 kDa was specifically competed in the presence of antigen peptide, but not in the presence of irrelevant peptide. Figure 4 also shows that SDS-resistant oligomerization/aggregation was variably present for rabbit KCC1 and mKCC1.

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Fig. 6. Endogenous KCC1 polypeptide expression in mouse tissues. A: all 3 antibodies recognize mKCC1 in CD-1 mouse choroid plexus (sample pooled from 4 mice, representative of 4 similar experiments). B: KCC1 signal intensity increases in proportion to protein loading; 10 µl corresponds to 4% of the total choroid plexus harvested from both lateral and the IVth ventricles of one mouse. C: mouse KCC1 is detectable at lower levels in a wide range of other tissues (100 µg protein/lane). Arrows, KCC1 polypeptides.
precipitated by antibodies to NH₂ and COOH termini of the polypeptide and that antibodies to acetylated NH₂ terminus (lanes 3–5). Note that anti-COOH terminus preferentially detects an upper band, anti-acetylated NH₂ terminus preferentially detects a lower band, and anti-NH₂ terminus detects both. PNGase F reduces molecular mass (Mᵣ) of upper and lower bands (lanes 4 and 5).

**B:** red cell KCC1 abundance is similar in CD1, C57BL6, and SAD1 mice, as well as in rat (50 µg/lane, NH₂-terminal antibody).

**C:** red cell KCC1 abundance in 100 µg of ghost protein from pooled red blood cells of 2 pairs of C57BL6 mice before (lanes 1 and 3) and after repeated bleeding (lanes 2 and 4) leading to reticulocytosis (NH₂-terminal antibody).

**D:** KCC1 from density-fractionated mature erythrocytes of mice with β-thalassemia detected with NH₂-terminal antibody (lane 1, bottom fraction; lane 2, reticulocyte-enriched (top) fraction; lane 3, whole blood). One of 2 similar density fractionations from 4 pairs of β-thalassemic mice.

**E:** red cell KCC1 abundance in 100 µg of ghost protein from pooled red blood cells of 3 pairs of SAD1 mice before (lanes 1, 3, and 5) and after repeated bleeding (lanes 2, 4, and 6) leading to reticulocytosis. Detected with NH₂-terminal antibody. Arrows, KCC1 polypeptides.

**Fig. 8.** KCC1 in erythrocytes of other animal species.

**A:** pig red cell KCC1 (100 µg membrane protein/lane) carries an N-glycan (lanes 2 and 4) and is detected by anti-NH₂-terminal (lane 1) and anti-acetylated NH₂-terminal antibody (lanes 2 and 4), but not by anti-COOH-terminal antibody (lane 3). B: KCC1 from sheep red blood cells (25 µg membrane protein in lane 1, 5 µg in lane 2) and rabbit red blood cells (100 µg membrane protein in lane 3) are detected by COOH-terminal antibody and (not shown) with lower sensitivity by NH₂-terminal antibodies. Sheep red blood cell K⁺ content was not assessed. Arrows, KCC1 polypeptides.
protein load-dependent manner (Fig. 6B). Antibody to mKCC1 COOH terminus detected more strongly than did the other antibodies an additional band of higher Mr, (Fig. 6A). mKCC1-related polypeptide was also detected at high levels in lung and at lower levels in other organs (heart ~ spleen > kidney ~ liver > testis; Fig. 6C), in reasonable agreement with the KCC1 mRNA tissue distribution in mouse (Fig. 2A) and in rat (22).

Anti-mKCC1 antibodies detect KCC1 and/or cross-reactive polypeptides in mouse and rat erythrocytes. Mouse erythrocytes displayed a broad mKCC1 band at 135–150 kDa (Fig. 7A), which reduced to ~115 kDa on N-deglycosylation (lane 5). Antibody to mKCC1 COOH terminus (lane 2) preferentially recognized the upper portion of this range, whereas antibody to mKCC1 unmodified NH₂ terminus (lane 1) recognized the entire band and that to mKCC1 acetylated NH₂ terminus (lane 3) recognized the lower portion more strongly than the upper. We also evaluated red cell KCC1 expression in a mouse model for sickle cell disease, the C57BL6-derived SAD1 mouse (14). Erythrocytes from wild-type C57 and from SAD1 mice exhibited similar levels of KCC1 polypeptide, as did red blood cells from outbred CD1 mice and from rat (Fig. 7B). Because reticulocyte counts are similar in C57 and SAD1 mice, reticulocytosis was induced by a bleeding protocol that reduced hematocrit values to <40% and increased the reticulocyte count to values of 10–18%. Reticulocytosis in C57 (Fig. 7C) and SAD1 mice (Fig. 7E) was accompanied by elevated levels of KCC1 polypeptide in whole blood. Stractan fractionation of erythrocytes from thalassemic mice revealed that mKCC1 polypeptide was present in greater abundance in less-dense than in more-dense cells (Fig. 7D). In this particular experiment, membranes from the less-dense fraction exhibited KCC1 immunoreactivity 8-fold greater than membranes from whole blood and 16-fold greater than membranes from more-dense cells.

KCC1-related proteins in erythrocytes of other species. K-Cl cotransport has been studied extensively in sheep (16, 31), rabbit (27), and (less extensively) dog (19) and pig erythrocytes (28). KCC1 polypeptide from sheep and rabbit erythrocytes (Fig. 8B) and from dog erythrocytes (not shown) resembled that in mouse and rat. In contrast, KCC1 exhibited a higher Mr, in pig than in other species, even after removal of N-glycans. Although reactive with both NH₂-terminal antibodies, pig KCC1 was not recognized by antibody to KCC1 COOH terminus (Fig. 8A). The additional band at 220 kDa not present in red blood cells of other species was detected only by antibody to KCC1 free NH₂ terminus, but not by antibody to the acetylated NH₂-terminus.⁴

KCC1-related proteins in human erythrocytes. Abundance of KCC1 polypeptide was low in AA erythrocytes from individuals of Caucasian or African origin but substantially higher in patients with Hb SS or SC disease (Fig. 9, A and B). As also observed in mouse red blood cells, antibody to COOH terminus preferentially recognized the upper portion of a broad, multicomponent band (Fig. 9B), all of which exhibited reduction of

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⁴The identity of the >220-kDa band present in membranes only from pig erythrocytes, and recognized only by antibody to mKCC1 free NH₂ terminus, but not by antibody to acetylated NH₂ terminus or to COOH terminus, remains uncertain. The band could represent an NH₂ terminal variant of KCC1, a variant form of KCC3 or of another isoform, or another immunologically related protein.
mKCC1 cDNA and gene flanking regions. mKCC1 cDNA is very similar to that of other cloned species, not only in the coding region, but in the 3' non-coding region as well. This similarity is understandable in the 3' UTR, which, in mouse as in human (22) and in pig (17, 26), overlaps with the promoter of the LCAT gene (Fig. 1B). LCAT deficiency causes fish-eye disease, a dyslipidemic syndrome invariably associated with hemolytic stomatocytic anemia, corneal opacities, accelerated atherosclerosis, and proteinuric renal dysfunction (29, 30). Interestingly, almost all reported LCAT mutations associated with fish-eye disease are point mutations.

mKCC1 mRNA is expressed in a wide range of tissues (Fig. 2A), and KCC1-immunoreactive polypeptide is also found widely (Fig. 6). Little is known of the transcriptional regulation of KCC1. Pellegrino et al. (43) reported that mKCC1 mRNA levels are upregulated during DMSO-induced erythroid differentiation of MEL cells. Although we have reported increased IK1 mediated 86Rb uptake was insensitive to block by 5 µM bumetanide (not shown).

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DISCUSSION

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In no species has the true initiator Met or the transcriptional initiation site been defined, secondary to failure of 5' RACE to transcribe through the 5' UTR of any KCC1 mRNA. Amplification of cDNA with the KCC1 oligonucleotide was unsuccessful. Thus the translational initiation site may reside between KCC1 IT and the currently defined ATG site. Alternatively, an additional upstream exon may remain undefined, potentially contributing coding sequence. Most likely, however, is a technical failure of RT.

Two notable features of the 5'-flanking region of the mKCC1 may reflect a possible role in transcriptional or translational regulation of polypeptide expression. The first feature is the remarkably high GC content near the putative initiator ATG. The second feature is the long, perfect, direct repeat upstream of the GC-rich
region, containing multiple CACCC motifs, possibly contributing to mKCC1 promoter function. The KCC1 direct repeat has no homolog within the database. Although direct repeat elements have been frequently observed in promoters and often contribute to transcriptional regulatory activity, the length of this repeat is unusual. The extended stretch of very high GC content in the 5' UTR of the mKCC1 cDNA suggests the possibility of posttranscriptional regulation, but this remains untested.

mKCC1 was mapped by physical linkage to the immediately adjacent LCAT gene, itself previously mapped to mouse chromosome 8, 53 cm. This linkage is consistent with known synteny of this portion of mouse chromosome 8 with the region of human chromosome 16q22.1 in which human KCC1 and LCAT are also close neighbors. The mechanism by which transcription of such closely juxtaposed genes as KCC1 and LCAT might be regulated is not understood but is likely conserved across mammalian species. Maintenance of the KCC1 gene in the transcriptionally active state might interfere with transcription of its downstream neighbor gene, LCAT, the promoter of which is embedded within the transcription unit of the KCC1 gene. Indeed, LCAT expression exhibits a tissue distribution considerably more restricted than for KCC1, with predominant expression in liver (53).

Antibodies to mKCC1 detect recombinant and native polypeptide. This work has characterized three affinity-purified polyclonal rabbit antibodies generated to mKCC1 terminal peptides. Each recognizes recombinant mouse and rabbit KCC1 in immunoblot, immunoprecipitation, and (not shown) immunocytochemical assays and displays immunospecificity as judged by peptide antigen competition. The COOH-terminal antibody was at the outset predicted to recognize KCC1 (22) and KCC2 (41). However, the recent publication of the human KCC3 sequence (25) has also revealed that the COOH-terminal epitope of KCC1 differs from the corresponding region of KCC3 in only 1 of 12 residues. In addition, the first six NH2-terminal residues of KCC1 and KCC3 are identical, as are two of the following eight that comprise the NH2-terminal antigen selected to raise antibodies in the present study. Thus the antibody to KCC1 COOH terminus may (likely) also detect KCC3. The antibody to KCC1 NH2 terminus must also be considered able to detect KCC3 until directly tested. However, the antibodies to KCC1 NH2 terminus will not cross-react with the distinct NH2-terminal sequence of KCC2. Indeed, the differential preferences of the NH2- and COOH-terminal antibodies suggest the possibility that, in choroid plexus and perhaps also in erythrocytes, the "upper and lower" portions of the broad KCC1 band may represent at least two KCC gene products. KCC3 may also contribute to the immunoblot signals detected in kidney and heart (Fig. 6), as well as in other tissues, including erythroid tissues, in which KCC3 mRNA has not yet been examined. The EST database includes additional sequences more closely related to KCC1 than to NKCCs or to NCC. Thus the complete spectrum of target antigens for these antibodies used for any mode of detection remains to be determined.

KCC1-related polypeptide in erythrocytes. KCC1-related immunoreactivity is present at higher levels in erythrocytes of mouse, rat, pig, rabbit, and dog than in human AA erythrocytes. KCC1 likely encodes most if
not all of this protein, since basal K-Cl activity in human erythrocytes is very low but is activated by hypotonic swelling. In contrast, human KCC3 was reportedly not activated by hypotonic swelling of HEK-293 cells (25) (but data were not presented).

Reticulocytes exhibit elevated levels of K-Cl cotransport activity (18). KCC1 levels in erythrocyte membrane correlate with reticulocyte count. Erythrocytes from the SAD1 mouse model of sickle cell disease do not exhibit increased levels of KCC1-like polypeptide, in concert with the absence of reticulocytosis. Reticulocytosis produced by repeated bleeding of SAD1 mice or C57 parental strain mice led to increased levels of KCC1-like polypeptide. The light-density fraction of erythrocytes was the richest in KCC1 polypeptide, also consistent with its enrichment in reticulocytes.

K-Cl cotransport activity is elevated in red blood cells of individuals with sickle cell disease (6, 9, 36), thalassemia (13), and disease associated with other mutant Hb (5, 9, 36). Human erythrocytes from individuals with the reticulocytic anemic states of sickle cell disease, Hb SC disease, and β-thalassemia intermedia exhibited uniformly elevated KCC1-like immunoreactivity compared with normal AA erythrocytes. As was true for mice, human red blood cells of the lowest density exhibited highest levels of KCC1. These data are the first to suggest that elevated red cell K-Cl cotransport activity in these conditions is associated with increased abundance of KCC1 polypeptide in the membrane of circulating erythroid cells (reticulocytes and mature red blood cells) but do not rule out additional regulatory alterations such as reported previously for volume dependence of activation (5) or kinetics of inactivation (9).

Because K-Cl cotransport activity contributes to red cell dehydration and because red cell dehydration is thought to exacerbate sickle cell disease via increasing intracellular concentration of Hb S, inhibition of erythroid K-Cl cotransport activity has been proposed as an adjunct therapy for sickle cell disease (7, 8, 12). We are examining the KCC1 gene as a candidate modifier gene for sickle cell disease.

Functional expression of mKCC1 in Xenopus oocytes. The Xenopus oocyte is a useful expression system in which to study recombinant KCC1 function. Recombinant mKCC1 accumulates to high level in oocytes and exhibits posttranslational processing superficially similar to that in HEK-293 cells. However, oocytes exhibit lower endogenous K-Cl cotransport activity than HEK-293 cells and lack immuno-cross-reactive polypeptide. Oocytes also appear to be equipped with the signal transduction pathways needed to manifest all examined regulatory patterns of erythroid K-Cl cotransport, including nearly absent basal activity; activation by hypotonic swelling, NEM, and staurosporine; inhibition by calyculin and okadaic acid; and inhibition by DIOA, but not by low concentrations of bumetanide. Additional regulators such as acid pH, urea, temperature, and oxygen tension are under investigation.

The development of immune reagents and an additional expression system for mKCC1 will allow us to define the structural determinants within the mKCC1 polypeptide that confer on K-Cl cotransport the ability to respond to each of the many types of acute regulation exhibited by this complicated protein. The yet to be addressed roles of membrane trafficking in the subacute regulation of mKCC1 polypeptide can now also be studied in the Xenopus oocyte, as well as in cultured cells.

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