Cloning and Expression of Sheep Renal K-Cl Cotransporter-1

Jin J. Zhang¹, Sandeep Misri¹, Norma C. Adragna^{1,3}, Kenneth B.E. Gagnon¹, Robert E. W. Fyffe⁴ and Peter K. Lauf^{1,2}

Cell Biophysics Group, Departments of ¹Pathology, ²Pharmacology and Toxicology, ³Anatomy and Physiology, and ⁴Center for Brain Research, Wright State University, School of Medicine, Dayton, Ohio

Key Words

Sheep • Kidney • KCC1 cDNA • Cloning • Expression
• K-Cl cotransport • N-ethylmaleimide

Abstract

Sheep K-Cl cotransporter-1(shKCC1) cDNA was cloned from kidney by RT-PCR with an open reading frame of 3258 base pairs exhibiting 92%, 90%, 88% and 87% identity with pig, rabbit and human, rat and mouse KCC1 cDNAs, respectively, encoding a ~122 kDa polypeptide of 1086-amino acids. Hydropathy analysis reveals the familiar KCC1 topology with 12 transmembrane domains (TMDs) and the hydrophilic NH₂-terminal (NTD) and COOH-terminal (CTD) domains both at the cytoplasmic membrane face. However, shKCC1 has two rather than one large extracellular loops (ECL): ECL3 between TMDs 5 and 6, and ECL6, between TMDs 11 and 12. The translated shKCC1 protein differs in 12 amino acid residues from other KCC1s, mainly within the NTD, ECL3, ICL4, ECL6, and CTD. Notably, a tyrosine residue at position 996 replaces aspartic acid conserved in all other species. Human embryonic kidney (HEK293) cells and mouse NIH/3T3 fibroblasts, transiently transfected with shKCC1-cDNA, revealed

the glycosylated ~150 kDa proteins by Western blots and positive immunofluorescence-staining with polyclonal rabbit anti-ratKCC1 antibodies. ShKCC1 was functionally expressed in NIH/3T3 cells by an elevated basal Cl-dependent K influx measured with Rb as K-congener that was stimulated three-fold by the KCC-activator N-ethylmaleimide.

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Introduction

Regulation of cell volume is a fundamental property of all cells [1]. To counter the Donnan effect of negatively charged intracellular macromolecules drawing water and ions, animal cells must regulate their volume through primary and secondary active membrane transport mechanisms. Among the latter, the cation chloride cotransporter (CCC) family mediates electroneutral ion movement [2, 3]. Three CCC classes have been identified on the basis of their structures, ligands, and inhibitors: NCC, the thiazide-sensitive Na-Cl cotransporter [4, 5]; NKCC, the loop diuretics-sensitive Na-K-2Cl cotransporter [6]; and KCC, the K-Cl cotransporter [7, 2].

As recently reviewed [2], at least four cDNAs encode multiple KCC polypeptides. The ubiquitous KCC1 cDNA has been cloned from rabbits, rats, humans [8], pigs [9] and mice [10, 11] with gene structures reported for humans [9], mice [10], and *Caenorhabditis elegans* [9]. KCC2 cDNA, found only in brain, has been cloned from human, mouse and rat [12]. Two isoforms of human KCC3 cDNA, KCC3a and KCC3b [13-15], KCC4 cDNA from humans and mice [16], and a *Xenopus* oocyte partial cDNA encoding a KCC isoform of the KCC1/3 pair have also been reported [17].

Whereas KCC1 is considered to be a “house-keeping” isoform [8], KCC2 is only found in neurons maintaining intracellular Cl below electrochemical equilibrium and is also involved in regulating the interstitial K concentration of the central nervous system [18, 19]. To date, only the KCC1 and KCC3 isoforms have been identified in animal erythrocytes [20], and K-Cl COT is pathologically elevated in erythrocytes from patients with sickle cell anemia [21]. Complete or partial ablation of KCC2 causes neonatal death or hyper-excitability and epilepsy in mice [22, 23]. Pathologies of hearing, peripheral nerve function, severe hypertension and renal urine acidification have been found in KCC3 and KCC4 knock-out mice [24, 25]. Abnormalities in KCC3 splicing have been associated with agenesis of the corpus callosum peripheral neuropathy (ACCPN) in humans [26].

Based on data from NKCC1, CCC polypeptides are proposed to span the lipid bilayer with 12 transmembrane domains (TMDs) [27]. Site-directed mutagenesis studies have implicated TMDs 2, 4, and 7 of NKCC1 in ion binding [28]. The N- and C-terminal cytoplasmic domains (NTD and CTD) of the KCCs have also been investigated with respect to their contribution to the overall K-Cl COT activity. The amino acid Tyr(Y)¹⁰⁸⁷ of rat KCC2-CTD and the analogous residue (Y¹⁰⁵⁶) in rabbit KCC1 are each involved in the hypotonic activation of ion transport activity in *Xenopus* oocytes. However, neither residue determines the delivery of the cotransporter to the oocyte plasma membrane or the inhibition of hypotonically-stimulated K-Cl COT by serine-threonine phosphatase antagonists [29]. Removal of most of the CTD from NTD-c-myc-tagged rabbit KCC1 abolished activation by N-ethylmaleimide (NEM) in HEK-293 cells while apparently decreasing, but not abolishing, surface membrane expression [30]. However, deletion of the NTD in mouse KCC1 impaired transport function [31]. Presumably, these truncations interfere with the phosphorylation/dephosphorylation reactions governing K-Cl COT [7, 32].

Sheep erythrocytes constitute a well established model to study cellular cation homeostasis since they exhibit a genetically based potassium (K) dimorphism, the high K/low K phenotypes. Low K erythrocytes maintain cellular steady state with Na/K pump and K-Cl COT activities less than one fourth and some ten-fold greater, respectively, than found in high K erythrocytes [for recent reviews see 33, 34]. While the difference in Na/K pump activity has been resolved at least at the kinetic level [34], the nature of the K-Cl COT differences remains elusive. It is intriguing that at least two KCC isoforms (KCC1 and 3) have been shown in both red cell phenotypes by Western blots and immunocytochemistry [20], suggesting a more complex mechanism that needs to be resolved at the molecular level. However, no sequence information on the sheep KCC isoforms is available.

Herein, we report the cloning of a full-length KCC1-cDNA from sheep (sh) kidney (Accession AF515770) and its functional and immunochemical expression in two tissue culture cell lines transiently transfected with shKCC1-cDNA. A preliminary report of this work was presented at the 2003 Experimental Biology Meeting in San Diego [35].

Materials and Methods

Reagents and Solutions

Analytical grade chemicals such as NaCl, MgCl₂, perchloric acid (PCA), dimethyl sulfoxide (DMSO), tris(hydroxymethyl)aminomethane (Tris), N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (Hepes), KCl, NaOH, and fetal bovine serum (FBS) were procured from Fisher Scientific (Fair Lawn, NJ); ultrapure Rb salts from Alfa (Danvers, MA); bovine serum albumin (BSA), CaCl₂, N-ethylmaleimide (NEM), ouabain, sulfamic acid, and 3-[N-morpholino] propane sulfonic acid (Mops) from Sigma Chemical (St. Louis, MO); CsCl, glucose, penicillin, streptomycin, and amphotericin from Invitrogen-Life Technologies, Inc. (Carlsbad, CA).

Balanced salt solution (BSS-NaCl) consisted of 20 mM Hepes/Tris buffer pH 7.4 containing [in mM]: NaCl [132], KCl [5], CaCl₂ [2], MgCl₂ [1], and glucose [10]. In BSS-Na-sulfamate media, methyl-sulfamate substituted for Cl in the K, Rb and Na salts, and gluconate in Ca and Mg salts. Stock solutions of 1M NEM and 0.2 M ouabain were made in DMSO or ethanol and all other chemical reagents dissolved in deionized water.

Molecular biological chemicals and their origins, nature and source of antibodies, and cell lines are listed in the respective sections below.

Sheep KCC1 Cloning and Sequence Analysis

Polymerase chain reactions (PCR) were used to produce several PCR fragments from a sheep bone marrow cDNA library

with primers prepared according to the sequence information of KCC1 from other species. The PCR fragments were sequenced and the sequences aligned with those from other species. The shKCC1-cDNA fragments covered about two-thirds of the full-length KCC1.

The partial sequence information was used to obtain the full length shKCC1-cDNA. Specific shKCC1 primers were designed and used in reverse transcription polymerase chain reaction (RT-PCR) as follows. Total RNA was isolated from a sheep kidney with the RNeasyTM Total RNA Isolation System (Promega, Madison, WI) and ThermoScript (RNase – H) reverse transcriptase (Invitrogen, Carlsbad, CA) was used to synthesize the first strand cDNA with two antisense primers: an anti-sense primer corresponding to nucleotide position 2070 (5'-GCCAGTTCCTGGTGTGAGGAGGCC) and a second anti-sense primer corresponding to nucleotide position 3390 (5'-CCAGGGTGAGGCCAGGCAAAGTG). The sequence number refers to that of rabbit (rb) KCC1.

The first-strand cDNAs were then used as templates and amplified in two different PCR reactions using Platinum Pfx DNA polymerase (Invitrogen, Carlsbad, CA) to produce double-stranded cDNAs under the following conditions: initial denaturation (94°C, 2 min), 40 cycles of denaturation (94°C, 1 min), annealing (58°C, 1 min) and extension (72°C, 4 min), followed by a final extension (72°C, 15 min). The first PCR reaction used the following pair of primers: 5'-ATGCCTCACTTCACCGTG (sense) and 5'-GCCAGTTCCTGGTGTGAGGAGGCC (anti-sense) at nucleotide 2070 and generated a 2070 bp 5' shcDNA fragment from the start codon to nucleotide position 2070. The second PCR reaction used the primer pair 5'-GCCATGGTCATCGCAGGC (sense) at nucleotide 1920 and 5'-CCAGGGTGAGGCCAGGCAAAGTGG (anti-sense) at nucleotide 3390 and generated a 1470 bp 3'-shcDNA fragment from position 1920 to 3390. According to the sequence information from other species, the two overlapping fragments constitute the full-length sheep KCC1 which has about 130 more bp after the stop codon (the stop codon for rbKCC1 is at nucleotide 3259). A Bam HI restriction enzyme sites located within the 150 nucleotide overlap between the two fragments was used to link the two fragments together.

The two KCC1-cDNA fragments were cloned into the mammalian expression vector pCMV-script (4278 bp, Stratagene, La Jolla CA) in a two-step process. First, the 2070 bp 5'-shKCC1 fragment and the pCMV-script vector were linearized with Sma I restriction enzyme, generating blunt ends, ligated together with T4 DNA ligase (New England Biolabs, Beverly, MA) and transformed into subcloning efficiency HB 101 competent cells (Invitrogen, Carlsbad, CA). The newly generated plasmid DNA (pCMV2070) was purified with a Qiagen Plasmid Maxi kit (Qiagen, Valencia CA) and the orientation of the 2070 bp fragment in the vector was mapped by Bst XI enzymatic digestion. Second, the pCMV2070 and the 1470 bp PCR product were digested with Bam HI to generate a cohesive end at nucleotide 2011 and Eco RI to produce a cohesive end at the multiple cloning site region within the pCMV-script vector, ligated together with T4 DNA ligase and transformed into MAX Efficiency DH 5 α competent cells (Invitrogen, Carlsbad, CA).

Large amounts of the final construct (pCMVshKCC1) were prepared using the Quiagen Plasmid Maxi Kit, verified by enzymatic digestion with Bam HI and Bst XI restriction enzymes (2070 + 1470 + 4278 $\approx$ 7818 bp) and sequenced by Cleveland Genomics (Cleveland, OH).

Cell Cultures

Two cell lines, the mouse embryonic fibroblast (NIH/3T3) cells and the human embryonic kidney (HEK 293) cells, were selected for the expression of shKCC1. Both cell types were purchased from ATCC (Rockville, MD). The NIH/3T3 cells were grown in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad CA) with 1.5 g/L sodium bicarbonate supplemented with 10% bovine calf serum, penicillin (50 units/ml) and streptomycin (50 μ g/ml). HEK 293 cells were grown in minimum essential medium (MEM) (Invitrogen, Carlsbad CA) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (50 units/ml) and streptomycin (50 μ g/ml). The cells were maintained in a humidified incubator at 37°C in 5% CO₂ and 95% air.

Sheep KCC1 Expression in Two Mammalian Cell Lines

Transient transfection was used to determine the expression of shKCC1. The pCMVshKCC1 plasmid DNA was transiently transfected into NIH3T3 cells and HEK 293 cells by Lipofectamine Plus reagent and Lipofectamine 2000 Reagent (Invitrogen (Carlsbad, CA), respectively).

Two days before transfection, the cells were trypsinized, counted and plated into 12-well plates. According to manufacturer's protocol, 0.8-1.0 μ g of pCMV-script vector and pCMVshKCC1 plasmid DNA were transiently transfected with Lipofectamine Plus (NIH3T3) and Lipofectamine 2000 reagents (HEK293) into each well (60-70% confluent) in antibiotic-free, serum-free medium for 48 h at 37°C under 5% CO₂ and 95% air. The transfection medium was replaced with fresh culture medium prior to incubation for another 24 h and the Rb influx assay used to detect functional shKCC1 protein translation as reported [30]. The expressed protein was extracted using the M-PER protein extraction kit (Pierce Biotechnology, Rockford, IL) and the amount of protein extracted was measured with the bicinchoninic acid (BCA[®]) assay (Pierce Biotechnology, Rockford, IL).

Western Blots and Immunocytochemistry

A) Antibodies. Following a recently established protocol in our laboratory [36], polyclonal rb antibodies were prepared (Wright State University Animal Laboratory Facilities) by injection of New Zealand white rabbits with quindecapetides located within the third extracellular domain (ECL3), sequence 301-405 (VEKHGLPSTDITLGLK), of KCC1 coupled to a non-immunogenic poly-lysine backbone (GeneMed Synthesis Inc., San Francisco, CA) and suspended in sterile saline and Freund's complete adjuvant. The chosen polypeptide sequences for the ECL3 region were unique to rat KCC1, did not cross-react with rabbit, and were selected by the NCBI database protein BLAST program based on several criteria such as epitope accessibility, predicted charge, antigenicity, and post-translational modification. Antibody titers and specificity were

shown by absorption with the respective original peptides using an enzyme-linked immunoabsorbent assay (ELISA) [36]. The immunoglobulins were purified from the polyclonal antisera by binding to and desorption from antigenic peptide-containing sepharose columns.

B) Cell extraction. Wild type or transfected cells in 100 mm culture plates were washed thrice with and collected in 1 ml ice-cold phosphate buffered saline (PBS) in a conical tube and spun at 14,000 rpm for 2 min. The cell pellet was lysed with 250 μ l of M-PER™ reagent (Pierce Chemicals, Rockford, IL) in presence of the protease inhibitors (μ g/ml) aprotinin (3), leupeptin (1), pepstatin A (1), and phenylmethylsulphonyl fluoride (10). The lysates were mixed with equal volumes of 2 X SDS (sodium dodecyl lauryl sulfate) electrophoresis sample buffer and boiled for 5 min. Approximately 20 μ g of protein sample was applied to each lane of a 12% polyacrylamide gel in a Protean XLi vertical gel electrophoresis system (Bio-Rad, Hercules, CA) at 35 mA for 5 h. The protein bands were transferred to a polyvinylidene difluoride (PVDF) membrane overnight at 10 volts and then identified by a rabbit anti-rtKCC1-CTD against 77 amino acids (943-1020) of the rat KCC1 CTD [30, kindly provided by Dr. Eric Delpire, Vanderbilt Medical Center, Nashville, TN] and detected using horseradish peroxidase (HRP)-coupled donkey anti-rbIgG (Jackson Immunoresearch Laboratories, West Grove, PA,) and ECL detection reagents for Western blots (GE Healthcare/Amersham Biosciences, Piscataway, NJ). Due to the large antigen used to generate this anti-KCC1-CTD antibody, multiple epitopes exist against which the polyclonal antibodies are directed, facilitating cross-reaction with CTD of KCC1 from different species such as human, rabbit, rat and sheep.

C) Glycanase-F treatment of the protein extracts from both NIH/3T3- and HEK293 cells transfected with vector + full length sheep KCC1 was performed as follows: A 200 μ g of protein sample was mixed with 250 mM phosphate buffer (pH 7.5) containing 2% SDS and 1 M 2-mercaptoethanol and heated at 100 °C for 5 min in a microcentrifuge tube. After cooling on ice, 15% (v/v) TRITON X-100 was added and mixed, to which 2.0 μ l of the glycanase F solution was introduced followed by incubation at 37 °C for 3 h. The glycanase F digests along with the untreated samples were then run on SDS-PAGE.

D) Immunocytochemistry was performed as described elsewhere [25]. Briefly, NIH/3T3 and HEK293 cells were grown at 10⁴ cells/well, in normal growth medium in Lab-Tek® Chamber-Slides™ (NUNC), after an initial poly-D-lysine coat for improved cell-to-slide adhesion. The cells were then incubated in a 5% CO₂-95% air incubator at 37°C until reaching ~80% confluence, fixed and permeabilized with 2% formaldehyde/0.01% saponin for 5-20 min followed by overnight exposure to the primary rabbit anti-rtKCC1-ECL3 antibody, washing and blocking with 3% goat serum, and a final incubation with 1/100 diluted Cy3-coupled goat anti-rabbit IgG. Immunofluorescence was examined using appropriate filters on a Nikon Labophot epifluorescence microscope with a 40X (N.A. 0.75) objective. Images were obtained using a digital color camera (Spot2 camera; Diagnostic Instruments, Sterling Heights, MI), and were analyzed using SPOT Advance, Diagnostic Instruments, Sterling Heights, MI).

Rb Influx

The degree of functional shKCC1 protein expression was assayed by influx measurements using Rb as K-congener. The method, published earlier [30], is briefly described here. HEK293 and NIH/3T3 cells, grown to about 80% confluence in 12-well plates, were transiently transfected with shKCC1 cDNA as described above. About 48 h after transfection, the plates were removed from the incubator, the supernatants aspirated and the cells washed three times with 1 ml of balanced salt solution (BSS) containing either NaCl or Na-sulfamate and 1% BSA at 37°C. Supernatants were aspirated and replaced with 37°C pre-warmed BSS-Cl or BSS-sulfamate solutions with 0.5 mM NEM at 37°C for 10 min, in a pre-incubation medium identical to that used during the flux but without Rb. Immediately thereafter, the media were removed and fresh 37°C pre-equilibrated Rb influx medium was added containing 10 mM Rb, 20 mM Tris-Hepes buffer pH 7.4, 10 mM glucose and Na up to 300 mOsm with either Cl or sulfamate as the anion. To inhibit Rb uptake through NKCC and the Na/K pump, respectively, 5 μ M bumetanide and 1 mM ouabain were present. Rb uptake was stopped after 6 min by aspiration of the supernatants and four washes of ice-cold 10 mM Tris/Cl-buffered (pH 7.4) MgCl₂ solution. Intracellular ions were extracted by 5% perchloric acid and measured with a Perkin Elmer 5000 atomic absorption spectrophotometer. Total cellular protein was dissolved in 0.1 N NaOH for protein assay by the BCA method®. From the initial Rb uptake at 6 min, Rb influx was calculated in nmol Rb per mg protein $\times$ 6 min. Thus, K-Cl COT is the calculated difference between Rb influx in the presence of Cl minus that in sulfamate. Values are expressed as mean $\pm$ SD (n = 4). Significance was accepted at the p<0.05 level.

Results

Cloning and Sequencing of Sheep KCC1-cDNA

The sheep-specific KCC1 primers were based on sequence information of PCR products first generated from a sheep bone marrow cDNA library as discussed in Materials and Methods. Sequence analysis indicated that shKCC1-cDNA, obtained by ligation of the 5'-2070 bp and the 3'-1470 bp fragments and subsequently identified for the coded sizes by Bam HI and Bst XI enzyme digestion, exhibited about 92% and 90% identities with pig (*sus scrofa*) and rabbit (*oryctolagus cuniculus*) KCC1, respectively. The shKCC1-cDNA encodes a 1086-residue polypeptide from its start codon at nucleotide 1, ATG to the stop codon at #3259. As shown in the amino acid alignment of Figure 1 and Table 1, there was 87%–88% homology (BLAST) of the primary protein structure of KCC1 between sheep and pig, rabbit, rat, mouse and man, and close to 97% based on ClustalW. The translated (unglycosylated) protein has a predicted mass of 122 kDa and a theoretical pI value of 6.2, largely due to an excess

CCC Protein	Species	Identity
KCC1	Sus scrofa	88%
KCC1	Oryctolagus cuniculus	87%
KCC1	Ratus Norvegicus	87%
KCC1	Homo Sapiens	87%
KCC1	Mus musculus	87%
KCC1	C. elegans	48%
KCC2	Homo Sapiens	62%
KCC2	Mus musculus	63%
KCC2	Ratus Norvegicus	63%
KCC3	Homo Sapiens	70%
KCC3	Mus musculus	70%
KCC3a	Homo Sapiens	70%
KCC3a	Mus musculus	70%
KCC3b	Homo Sapiens	68%
KCC3b	Mus musculus	67%
KCC4	Homo Sapiens	67%
KCC4	Mus musculus	63%
NKCC	Shark Rectal Gland	44%

Table 1. Sheep KCC1 Protein Homology with Other CCCs (BLAST).

of 108 asparagines and glutamic acids over 99 arginines and lysines. Based on the Dense Alignment Surface (DAS) hydrophobicity analysis shown in Figure 2A [37], 12 TMDs and a cytoplasmic NTD and CTD are predicted (Table 2) with 5 protein kinase C (PKC) and 8 casein II kinase consensus sites and 4 major N-glycosylation sites within the ECL3.

Figure 2B depicts and Table 2 details the predicted membrane topology based on the von Heijne algorithm [38, 39]. Note the expected presence of a large ECL3 between TMD 5 and 6 (119 amino acids) which is conserved throughout all KCC isoforms. Figure 2B also reveals that the first ten TMDs are certain, whereas the last 2 TMDs remain putative and are located further downstream toward the C-terminus than proposed in other species, producing a second large loop, ECL6 (105 amino acids) (see Discussion). Table 3 highlights the specifics of the 12 amino acid differences between sheep and all other species compared in Figure 1. Two of the sheep-specific amino acids were located in the ECL3, one in ICL4, and 3 each in ECL6 and the CTD, respectively. Notably, a tyrosine residue at position 996 replaces an aspartic acid conserved in all other species.

Topology	Start No.	End No.	Size (amino acids)
NTD	1	118	118
TMD 1	119	139	21
ECL 1	140	152	13
TMD 2	153	173	21
ICL 1	174	199	26
TMD 3	200	220	21
ECL 2	221	252	32
TMD 4	253	273	21
ICL 2	274	275	2
TMD 5	276	296	21
ECL 3	297	415	119
TMD 6	416	436	21
ICL 3	437	454	18
TMD 7	455	475	21
ECL 4	476	499	24
TMD 8	500	520	21
ICL 4	521	575	55
TMD 9	576	596	21
ECL 5	597	615	19
TMD10	616	636	21
ICL 5	637	719	83
TMD 11	720	740	21
ECL 6	741	845	105
TMD 12	846	866	21
CTD	867	1086	220

Table 2. Topology of Cytoplasmic, Extracellular and Transmembrane Domains in Sheep KCC1. ICL = intracellular (cytoplasmic) loops, ECL= extracellular loops, TMD= transmembrane domains, CTD= C-terminal domain, NTD= N-terminal domain.

Sheep KCC1 cDNA Expression

Figure 3 shows Western blots of mouse NIH/3T3 and HEK293 cells with rabbit anti-rtKCC1-CTD as the primary and HRP-conjugated donkey anti-rabbit IgG as the secondary antibody. After transfection with the competent KCC1-cDNA containing vector, the endogenous band at ~150 kDa was amplified consistent with overexpression of KCC1 in these cells. Also shown in the figure is the effect of Glycanase F treatment of the protein samples. An additional smaller band of ~120 kDa was observed which is consistent with the predicted mass for the unglycosylated translated protein [8]. In sheep erythrocytes, we showed evidence for a larger KCC1 molecular mass (~180 kDa) which may be attributed to higher glycosylation levels than in other tissues [20].

Figure 4 compares the immunofluorescence in vector alone and in vector-plus-KCC1 cDNA-transfected cells (both the HEK293 and NIH3T3 cells) after labeling with primary polyclonal rabbit anti-rtKCC1-ECL3 antibodies and secondary Cy3-labeled goat-anti-rabbit IgG. Expression of shKCC1 increased the red fluorescence

Fig. 1. Alignment of shKCC1 amino acid sequences with those of other species. Red residues are identical in all species. Gray bars with numbers below the sequences denote the transmembrane domains (TMDs). Underlined blue amino acids are sheep specific. Potential N-glycosylation and consensus sites for phosphorylation by kinase II and protein kinase C are indicated by stars and vertical blue and orange bars, respectively.

Fig. 2. Evaluation of amino acid sequence of shKCC1 for membrane topology. A. Dense Alignment Surface (DAS) hydropathy plot of shKCC1. The peaks above the strict cutoff are the presumed TMDs numbered 1-12. B. Proposed membrane topology modified according to a program provided by TOPPRED®. Shown are the proposed TMDs 1-12 and cytoplasmic N- and C-terminal domains, the intracellular loops ICL 1-5 and the extracellular loops ECL 1-6.

in HEK293 cells (compare B with A) as well as in NIH3T3 cells (compare D with C) which was not only distributed within the cytoplasm but also distinctly at the plasma membrane boundaries.

Figure 5 shows expression of functional shKCC1 protein in the Rb uptake measured at 6 min in NIH/3T3 cells 48 h after transfecting with either vector alone (V) or with vector plus shKCC1-cDNA (VKCC) in untreated controls (-C) or NEM-treated (-N) cells. Transfection with vector plus shKCC1 caused a significant increase of Rb influx in Cl (striped columns, compare V-C with VKCC-C) which in VKCC-C was significantly reduced when Rb influx was measured in sulfamate (hatched columns). Consequently, the calculated K-Cl COT (black columns) was not significantly different from zero in the vector-transfected controls V-C but was much greater in VKCC-C, the vector plus shKCC1-transfected cells. Upon exposure to 0.5 mM NEM, primarily the Rb influxes in Cl responded with activation (striped columns of V-N and VKCC-N). Hence, there was some NEM-activation of endogenous mouse KCC to levels seen in controls transfected with shKCC1 (compare V-N with VKCC-

C). In contrast, in NEM-treated shKCC1-transfected cells, K-Cl COT was almost three-fold greater than in the vector-transfected cells (compare VKCC-N with V-N and V-C). In addition to the changes in K-Cl COT, it is evident that NEM also enhanced the Rb influx in sulfamate media suggesting participation of channel-mediated fluxes in the overall activity. Although we have not followed up this phenomenon further, we have seen in previous work that NEM stimulates channel-mediated K fluxes (cation-nonselective?) in HEK 293 cells [30].

These data then suggest that transfection with shKCC1 *per se* reveals a major additional K-Cl COT activity that is characteristically enhanced by NEM.

Discussion

We describe here for the first time the cloning and expression of KCC1 from sheep kidney. In addition to the potential role the transporter may have in red cell cation dimorphism as indicated in the Introduction, we were interested in the sequence of this transporter

Fig. 3. Western blot analysis of HEK293- and NIH3T3-transfected cells with pCMV vector and pCMV vector + full-length shKCC1 using a rb anti-rt KCC1-CTD antibody (provided kindly by Eric Delpire) with HRP goat anti-rb IgG as secondary antibody. Lane 1-4 HEK 293 and lanes 5-8 NIH3T3 cell extracts. Lanes 1 & 6: vector only; Lanes 2, 3 & 5,7: vector + shKCC1-transfected; Lanes 4 & 8: vector + shKCC1-transfected and Glycanase-F deglycosidase treated. Absorption of anti-rtKCC1-CTD with its antigenic maltose-77 amino acid fusion peptide removed KCC1 specific staining [30].

Fig. 4. Immunohistochemical evidence for shKCC1 protein expression in NIH/3T3 cells. As described in “Material and Methods,” cells were first exposed to polyclonal rabbit anti-rat KCC1-ECL3 antiserum followed by Cy3-labeled goat anti-rabbit IgG. DAPI-staining was superimposed to visualize nuclei. A and C display pCMV vector only in HEK293 and NIH3T3 cells, respectively, and B and D, pCMV vector plus full length shKCC1-transfected HEK293 and NIH3T3 cells, respectively. Scale bars 3μm.

because low K (LK) sheep erythrocytes possess both KCC1 and KCC3 isoforms [20] and display a basal K-Cl cotransport mechanism that can be readily activated by cell swelling, thiol modification by NEM, Mg-depletion and other maneuvers [7, 33, 34, 40]. For a convenient

start, we chose kidney from a genetically most likely LK sheep as the mRNA source rather than the difficult to isolate erythrocyte precursors. The amino acid sequence of shKCC1 exhibits significant homology to KCC1s from other species (>86%) with an overall amino acid identity

Table 3.

Amino Acids Different in Sheep KCC1 Protein		
Amino Acids in other Species		Amino Acids in Sheep
<i>N-terminal domain (NTD):</i>		
#37	S (Serine) in hm, ms, rt, pg; P (Proline) in rb.	A (Alanine)
#63	Y (Tyrosine) in hm, ms, rb, rt; C (Cysteine) in pg.	H (Histidine)
#108	T (Threonine) in hm, rb, pg; G (Glycine) in ms, rt.	A (Alanine)
<i>Extracellular loop (ECL) 3:</i>		
#304	V (Valine) in hm, ms, rb, rt, pg;	I (Isoleucine)*
#351	D (Aspartic acid) in hm, ms, rb, rt, pg;	E (Glutamic acid)*
<i>Intracellular loop (ICL) 4:</i>		
#532	A (Alanine) in hm, ms, rb, rt, pg;	T (Threonine)*
<i>Extracellular loop (ECL) 6:</i>		
#758	V (Valine) in hm, ms, rb, rt, pg;	A (Alanine)*
#790	S (Serine) in hm, ms, rb, rt, pg;	T (Threonine)*
#810	T (Threonine) in hm, ms, rb, rt, pg;	N (Asparagine)*
<i>C-terminal domain (CTD):</i>		
#966	Y (Tyrosine) in hm, ms, rb, rt, pg;	S (Serine)*
#996	D (Aspartic acid) in hm, ms, rb, rt, pg;	Y (Tyrosine)*
#1013	D (Aspartic acid) in hm, ms, rb, rt, pg;	N (Asparagine)*

*denotes occurrence only in sheep.

Fig. 5. Functional overexpression of K-Cl COT mediated by shKCC1 in NIH/3T3 cells before and after transfection with vector alone or vector plus shKCC1 cDNA. K influx was measured in NIH/3T3 cells with Rb as K congener in Cl and sulfamate flux media two days after transient transfection with vector alone or with vector plus shKCC1. The black columns are the K-Cl COT rates calculated from the difference of the Rb influx in Cl (striped columns) and sulfamate (cross hatched columns). V-C: Vector alone control; V-N: Vector alone after NEM-treatment; VKCC-C: Vector plus shKCC1 control; VKCC-N: Vector plus shKCC1 after NEM treatment as described in "Materials and Methods." N=4±SD (error bars).

between KCC1 and KCC3 of 70% of different species and less than 45% with NKCC (Table 1).

Based on the alignment of the predicted KCC1 amino acid sequence of sheep and additional species (Figure 1), it is evident that shKCC1 differs by 12 amino

acids, 9 of which are found only in sheep (Table 3), two residues in ECL3, one in ECL4 between TMD 8 and 9, and 6 within the previously recognized [27] cytoplasmic carboxy-terminal (CTD) region of the molecule (however, see discussion below). Notably, a tyrosine residue at

position 996 replaces an aspartic acid residue conserved in all other species. The functional significance of this mutation, i.e. possible phosphorylation by a Y-kinase, needs to be further addressed in the future, in particular, the role this difference may play in the genetic red blood cell cation dimorphism [33, 34]. Clearly, further sequence studies are necessary on KCC1cDNAs from established HK and LK animals.

The predicted N-glycosylation sites (amino acid residues 91, 312, 331, 347, 361, 439 and 1013), the eight casein II kinase (amino acid residues 24, 298, 327, 734, 836, 942, 966 and 1051) and five protein kinase C (PKC) consensus sites (residues 438, 748, 814, 944 and 977) identified in Figure 1 are discussed below in terms of a predicted quarternary membrane topology of shKCC1. Also predicted are two leucine zipper sites at 560 – 581 and 697 – 718, various putative N-myristoylation sites at residues 119, 149, 178, 198, 208, 239, 294, 367, 370, 395, 403, 433, 454, 489, 510, 568, 622, 667 and 785, and a cell membrane attachment sequence (RGD sequence) at residues 14–16, suggesting that the initial part of the cytoplasmically located NTD may interact with the plasma membrane and perhaps with other membrane surface proteins.

The Kyte-Doolittle hydropathy plot has been used previously to establish the NKCC membrane topology [27], which, in turn, appears to have supported models for the descriptions of the KCC membrane topologies [12, 20, 28]. However, in determining the membrane topology of shKCC1, we have tested several programs with the shKCC1 amino acid sequence such as TOPREDTM, TMHMMTM and TMPredTM yielding a membrane topology different from that predicted previously for the various KCCs cloned thus far (internal CTD and NTD, one large extracellular loop between TMD 5 and 6). Previous immunocytochemical evidence for the presence of both NTD and CTD in the cytoplasm was established by anti-c-myc and anti-rbKCC1 CTD antibodies in HEK293 cells transfected with a c-myc-tagged rbKCC1-cDNA [30]. Keeping these different algorithms in mind, we have based our proposed transmembrane topology for shKCC1 shown in Figure 2B on a) the display of the cytoplasmic, extracellular and transmembrane domains by the TOPPED[®] program (Table 2), b) the DAS hydrophobicity plot (Figure 2A), and c) the presence of the 4 N-glycosylation sites in ECL3. The DAS analysis for shKCC1 gives 10 certain and 2 uncertain TMDs. Notably, the location of TMDs 11 and 12 are further downstream toward the C-terminus with TMD 11 separated from TMD 10 by 83 amino acids of

intracellular loop (ICL) 5, and TMD 12 from TMD 11 by 105 amino acids in ECL6. This model depicts the hydrophilic cytoplasmic CTD of shKCC1 by more than 200 amino acids shorter than previous models proposed for other KCC1s [8, 9, 12], removing 2 of the PKC consensus sites (amino acids 748 and 814) and 3 of the casein II kinase sites (amino acids 734, 738 and 836) from participation in potential cytoplasmic phosphorylation reactions. Direct phosphorylation of other KCCs at any of these residues including the NTD-based casein II kinase site #24 has not yet been reported. In our hands the KCC1 sequences of sheep and other animals were independent of several algorithms used meaning that indeed earlier models need some revision. For example in our earlier work [30], we based the truncation of rbKCC1 by 425 amino acids (at bp 2011) on the earlier model provided [8, 12] which therefore, according to our present model of KCC1, also included removal of TMD 11 and 12, ECL6 and the CTD. Hence, it would be of interest to know whether truncation of just the 225 amino acid long CTD of sheep KCC1 would abolish the stimulation of K-Cl COT by NEM.

Western blots with rb anti-rtKCC1-CTD followed by donkey anti-rabbit IgG (Figure 3) show that HEK293 (lane 1) and NIH/3T3 (lane 5) cells transfected with vector alone stained weakly for a 150 kDa protein, consistent with the reported molecular weights of KCC1 [8, 30], and much stronger after transfection with pCMV vector plus shKCC1 cDNA (Lanes 2 and 3 for HEK293, Lanes 6 and 8 for NIH3T3 cells). This means that a) the endogenous human (HEK293) and mouse (NIH/3T3) KCC1s are cross reacting with the polyclonal rb antibody directed against the broad rtCTD segment of 77 amino acids (943-1020) and b) the additional staining in vector plus shKCC1 transfected cells is due to cross reactivity of the antibodies with the shKCC1 isoform. We have previously found that pre-absorption of this antibody with the maltose-77 amino acid fusion peptide removed the staining in control and rbKCC1-cDNA transfected HEK 293 cells [30]. A smaller band is also observed upon Glycanase F treatment of the cell extracts, indicating that the glycosylated products were at least partially deglycosidated under our conditions (Figure 3, Lanes 4 and 7 for HEK293 and NIH3T3 cells, respectively).

Immunostaining of NIH3T3 cells and HEK293 cells transfected with vector or vector plus KCC1 revealed a result qualitatively similar to the Western blots, although a different antibody was used. The primary antibody, directed against a quindecapetide selected within the ECL3 of rtKCC1 and visualized by Cy3-tagged goat anti-

rabbit IgG, reacts basically with the external aspect of both the endogenous mouse and shKCC1, i.e., the transporters that actually should have reached the plasma membrane. However, we permeabilized the membranes with saponin in order to stain all KCC1 proteins under cellular production in these transiently transfected cells. Figure 4 shows the direct immunofluorescence of the endogenous and the translated shKCC1 protein, respectively in HEK293 and NIH3T3 cells. Clearly, vector plus KCC1-transfected cells stained more intensely than vector-only-transfected cells commensurate with shKCC1 expression.

The final evidence for expression of a functional shKCC protein is the demonstration of an increase in Cl-dependent K flux, i.e., K-Cl COT as depicted in Figure 5. Clearly, the endogenous mouse KCC in vector-alone transfected cells was activated by NEM, a finding consistent with mouse KCC1 expression studies [11]. However, K-Cl COT was significantly higher in cells transfected with shKCC1 versus controls and exhibited a three-fold increase in activity after NEM pre-treatment. As shown previously for HEK293 cells transfected with rbKCC1, replacement of Cl with sulfamate provides a means for calculating the Cl-dependent Rb influx. However, NEM also enhanced Rb influx in the presence of sulfamate, suggesting that either unknown K channels were stimulated together with K-Cl COT or some cotransport of Rb occurred with sulfamate.

In summary, the present study shows that shKCC1 cDNA has been cloned from sheep kidney and expressed in mammalian cells. Protein primary sequence alignment corresponds 87-88% with that of five other species: mouse, man, rat, rabbit and pig. There were nine sheep-specific amino acid substitutions with an otherwise remarkable sequence conservancy of KCC1 throughout these species,

and with 1 sheep-specific mutation of an amino acid conserved in other species. The shKCC1 protein probably spans the plasma membrane 12 times with a downstream shift of TMD 11 and 12 compared to previous models of other KCCs. Thus, the large ECL 6 is generated with a defacto shortening of the length of the CTD by some 200 amino acids. The expressed protein was visualized by Western blots and immunofluorescence and its functionality documented by the presence of an elevated K-Cl COT that responded to the characteristic stimulation by NEM.

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