KCC2a Expression in a Human Fetal Lens Epithelial Cell Line

Peter K. Lauf^{1,4}, Mauricio Di Fulvio^{3,4}, Vinita Srivastava^{1,4}, Neelima Sharma^{1,3,4} and Norma C. Adragna^{1,3,4}

¹Cell Biophysics Group, ²Department of Pathology, ³Department of Pharmacology and Toxicology, ⁴Boonshoft School of Medicine, Wright State University, Dayton

Key Words

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Abstract

The fetal human lens epithelial cell (LEC) line (FHL124) possesses all four K⁺Cl⁻ (KCC) cotransporter isoforms, KCC1-4, despite KCC2 being typically considered a neuronal isoform. Since at least two spliced variants, KCC2a and KCC2b, are co-expressed in cells of the central nervous system, this study sought to define the KCC2 expression profile in FHL124 cells. KCC2a, but not KCC2b transcripts were detected by reverse transcriptase polymerase chain reaction (RT-PCR). Proteins of molecular weights ranging from 95 to 135 kDa were found by Western blotting using non-variant specific anti-KCC2 antibodies directed against two different regions of the KCC2 proteins, and by biotinylation suggesting membrane expression. Immunofluorescence revealed membrane and punctate cytoplasmic staining for KCC2. Low levels of cytosolic α A and α B crystallines, and neuron-specific enolase were also detected contrasting with the strong membrane immunofluorescence staining for the Na/KATPase α 1 subunit. Since the lack of neuron-specific expression of the

KCC2b variant in non-neuronal tissues has been proposed under control of a neuron-restrictive silencing element in the KCC2 gene, we hypothesize that this control may be lifted for the KCC2a variant in the FHL124 epithelial cell culture, a non-neuronal tissue of ectodermal origin.

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Introduction

The lens is a unique structure and the only surface ectoderm derivative in the eye [1, 2]. The remaining ocular structures are either of neuro-ectoderm- or mesoderm-origin. In a distinctive 'trans-differentiation' process, the frontal lens epithelial cells (LECs) on the inner surface of the lens capsule undergo mitosis, degradation of organelles, and elongation to form mature lens fiber cells (LFCs) [3]. This phenomenon begins prenatally, continuing throughout life and resulting in the LFCs being laid in concentric rings [2, 4].

Lens transparency depends on lens fiber integrity maintained by regulation of LEC volume through ion transporters [5, 6] such as the Na⁺/K⁺ pump [7], the Na⁺K⁺2Cl⁻ (NKCC) [8] and K⁺Cl⁻ (KCC) cotransporters [9, 10], the K_{Ca}3.1, IK or Gardos K⁺ channels [11] and other K⁺ and Cl⁻ channels [6]. Of the two NKCC isoforms

known [12], NKCC1 is expressed in the lens [8, 10, 13]. LECs and LFCs also express the Na-Cl cotransporter, NCC [13, 14]. There are four KCC isoforms, KCC1-4 [15, 16], of which KCC1, 3 and 4 were earlier reported to be present in human and rat lens tissue, and in the immortalized human B3-LEC line [9, 10]. The widely distributed KCC1 isoform performs regulatory volume decrease (RVD) in KCC1-transfected, osmotically swollen human embryonic kidney cells [17]. KCC3, with its at least two variants KCC3a and b, also in the human lens and B3-LEC cultures [10], is widespread, has been characterized first in rat vascular smooth muscle cells (VSMCs), and is involved in cell growth regulation [15, 18]. Whereas KCC4 is present in rat LFCs [9] and human lens tissue and B3-LECs [10], it is especially expressed in the kidney, and participates in RVD [19].

Although noticed already in 2001 in rat VSMCs [20], KCC2 has been typically considered a neuron-specific isoform maintaining a low intracellular Cl⁻ concentration required for neurotransmitter activation of Cl⁻ channels [21, 22]. However, it was not detected in rat lenses [9], excised human lens tissue and in the immortalized B3-LEC line [10]. In contrast to these findings, we have recently reported that a fetal, not SV40-transformed human LEC line, FHL124 [23, 24], expresses KCC2 in addition to KCC1, 3 and 4 transcripts, as demonstrated in the previous publication [11].

Concomitantly with above studies, an additional splice variant of KCC2, named KCC2a, was found co-expressed in neuronal cells [25]. This KCC2a variant is to be distinguished from the other neuronal KCC2-spliced variant, named KCC2b, the first one cloned and originally known as KCC2 [22]. These KCC2 variants are the result of the election of alternative promoters located upstream of two mutually exclusive first exons: exon 1 (exon a) and exon 2 (exon b). The purpose of the present study was to establish whether KCC2a, KCC2b, or both, are present in these lens-derived primary epithelial cell cultures. By using RT coupled to PCR and splicing-specific sets of primers to detect KCC2a and KCC2b transcripts in FHL-124 cells, expression of the KCC2a variant, but not the neuron-specific KCC2b was established. KCC2 expression was detected in these cells at the protein level by Western blotting (WB), KCC2 positive protein staining in a biotinylated membrane fraction, and by immunofluorescence (IF).

This work was reported as poster presentations at the 25th Ohio Physiological Society meeting 2010, the Experimental Biology Meeting 2011, and the International Lens Research Conference in Kona, HI, 2012.

Materials and Methods

Human Lens Epithelial Cell Culture

FHL124 cells [23, 24] were kindly donated by Dr. John Reddan (Oakland University, MI). According to specifications, culture flasks were coated with liquefied 0.1–0.2 mg gelatin/cm² and subsequently dried for at least 2 h. Cells were grown on gelatin at 37°C in 10% of a 1:1 mixture of heat-inactivated horse serum and 10% FBS. Cells were split after being rinsed with Ca²⁺-Mg²⁺-free phosphate-buffered saline (PBS).

Total RNA extraction, reverse transcription and PCR

Total RNA was isolated from FHL-124 cells with the RNeasy Protect Mini kit (Qiagen, CA), following manufacturers' instructions. RNA concentration was estimated at 260 nm. First-strand cDNA synthesis (reverse transcription, RT) was initiated with 0.5–1.0 µg of total RNA, 200 ng of random hexamers, 500 mM deoxynucleotide triphosphates (dNTPs) (Affymetrix/USB Corp., OH), 10 mM dithiothreitol (DTT), 40 U of recombinant ribonuclease inhibitor (RNase OUT, Invitrogen, CA) and 200 units of SuperScript III Reverse Transcriptase (Invitrogen, CA). The RT reaction was performed at 50°C for 50 min and stopped at 75°C for 15 min. PCR was done with 2 µl of cDNA synthesis (RT reaction) in a final volume of 50 µl containing 0.4 U of Platinum Pfx polymerase (Invitrogen, CA), 0.4 mM dNTPs, 1.5 mM MgSO₄ and 50 pmol of human (h) KCC2 splice variant-specific primer sets. Briefly, hKCC2a- and hKCC2b-specific sense (forward) primers were designed to specifically anneal the exon-exon junction encompassing exons *a* (exon 1) and 3 or exons *b* (exon 2) and 3 (exclusive of hKCC2a or hKCC2b transcripts, respectively). Antisense (reverse) primers for hKCC2a or hKCC2b anneal the exon 7/8 junction or exon 8, respectively. The mRNA regions predicted to be amplified with these primer sets correspond to amino acids 35–228 (hKCC2a) or 14–213 (hKCC2b). It is important to note that the nucleotide sequence starting in exon 3 and ending in exon 8 are identical in both hKCC2a and hKCC2b mRNAs. This region encodes for amino acids 41–228 and 18–214 in hKCC2a and hKCC2b, respectively. The human-specific primer sequences are summarized as follows.

hKCC2a-580 sense (exon *a*-specific in bold, exon 2-specific in italics): 5'-**GGG GGA AGA CGT CAA AGG** TGA T-3' and hKCC2a-580 antisense (exon 8-specific in bold, exon 7-specific in italics): 5'-**AGC CAG CAG GAT TTC GAT** GG-3'; hKCC2b-597 sense (exon *b*-specific in bold, exon 2-specific in italics): 5'-**CAA CCC GGG TGA TGG CAA CC**-3' and hKCC2b-597 antisense (exon 8-specific): 5'-AAG ATG GCC ATG GCT GGG AA-3'. hKCC2a and hKCC2b primers sets were designed to amplify and generate PCR products of 580 bp and 597 bp, respectively. Therefore, hKCC2 primer sets were named as KCC2a-580 and KCC2b-597, respectively (see Fig. 1B for a graphical representation). To detect known splice variants of hKCC2b either in FHL124 cells or in adult human brain cDNA (Zyagen, San Diego, CA), the following sets of PCR primers were used: hKCC2b-s1-504 sense: 5'-GAA GGG AAA GGA GTA TGA TGG CAA GAA CAT-3', hKCC2b-s1-504 antisense: 5'-CAA ACA GAA CAA CGG AGC TGA TGT AGA C-3'; hKCC2b-s2-609 sense: CAA GGA AAG CAG TCC CTT CAT CAA CAG-

3', hKCC2b-s2-609 antisense: 5'-TTA TTC TTC TCT GCC ACC GAC TTG TCC TT-3'; and hKCC2b-s3-643 sense: 5'-CTC CTA CTA CAT GAT TTC CAG GCC TCT G-3', hKCC2b-s3-643 antisense: GCT TTG GAA CTC ATT CCC ACT TAA TTC TTC-3'.

All primers sets are available for testing upon request. PCR conditions were: DNA denaturation at 95°C for 2 min and 40 cycles of 30 sec at each step: denaturation, 95°C; annealing, 56°C and extension, 68°C. Human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) served as positive control. It was PCR-amplified as a PCR fragment of 555 bp by using the following primer set: GAPDH sense: 5'-GTG AAG GTC GGA GTC AAC GGA TTT-3' and GAPDH antisense: 5'-CAC AGT CTT CTG GGT GGC AGT GAT-3'. This primer set was named hGAPDH-555. The RT-PCR products obtained using hKCC2a-specific primers were purified (cleaned-up) from non-used primers and excess nucleotides by enzymatic digestion of an aliquot of the RT-PCR reaction using ExoSapit (Affymetrix/USB Corp., Cleveland, OH), a mixture of exonuclease I and shrimp alkaline phosphatase that digests single-stranded DNA (*i.e.*, unused PCR primers) and dephosphorylates nucleotides (*i.e.*, unused dNTPs) in the PCR reaction. This step is crucial to avoid interference in downstream applications such as DNA sequencing. Once purified, KCC2a fragments were sequenced in both directions [*i.e.*, sense (forward) and antisense (reverse) strands] by using PCR and the very same hKCC2a-specific sense and antisense primers used for original amplification. Sequencing was performed at Agencourt/Beckman Coulter Genomics Inc. (Beverly, MA).

Sodium-dodecyl-laurylsulfate polyacrylamide electrophoresis (SDS-PAGE) and Western Blot

As per manufacturer's instructions, membrane proteins were extracted with the Mem-PER[®] eukaryotic protein extraction reagent kit. The standard WB procedure was conducted at a pH of 8.6 as previously described using for all conditions 7.7% SDS-PAGE [11]. Primary rabbit (rb) anti-rat (rt) KCC2 antibodies and corresponding inhibitory peptides [26] and commercial rb anti-rt KCC2 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used in a 1:250 dilution together with horse radish peroxidase (HRP)-coupled donkey (dk) anti-rb IgG in a 1:4000 dilution. In addition, a polyclonal rb anti-rt KCC2 (B22) antibody, kindly donated by Dr. John Payne, University of California at Davis, was used. This antibody is directed against B22, a C-terminal 112 amino acid long KCC2 peptide attached to a fusion protein [27]. Dilutions of antibodies are indicated in the Figure legends. The blots were exposed for 5 min to Lumi Light Western Blotting substrate, followed by luminescence assay using Fujifilm Image Maker.

Plasma membrane biotinylation

FHL124 cells were grown to confluence in 49 cm² dishes and washed thrice with 5 ml phosphate-buffered saline (PBS) followed by addition of 6.5 ml sulfo-NHS-biotin (0.25 mg/ml). After 30 min incubation at 4°C, 326 µl of 0.5 M Tris/Cl buffer pH 7.4 was added to quench non-reacted biotin. Cells were washed twice in Tris-hydroxy-aminomethane (Tris)-buffered saline (TBS) and then lysed in 1 ml ice cold Pierce M-PER lysis buffer

containing protease inhibitors and 1 mM EDTA. The lysate, collected in 1.5 ml Eppendorf tubes, was spun for 1 min at 10,000 x g for separation of cytosolic supernatant, and M-PER-lysis buffer insoluble particulate fraction which was washed twice with icecold lysis buffer at 10,000 x g for 1 min. To further solubilize this particulate fraction, most likely containing the notoriously difficult to solubilize membrane transport proteins, the lipid detergent CHAPS was added to yield 0.1% final concentration. After a brief sonication stroke, the sample was centrifuged at 10,000 x g for 1 min and the clear supernatant containing the biotinylated proteins harvested, added to the already washed StreptAvidin and incubated for 1 h at 4°C. The mix was centrifuged for 2 min at 5000 x g, the supernatant discarded, and the remaining resin pellet washed with PBS. The pellet was then mixed with 2 x SDS sample buffer containing DTT to yield 50 mM in order to dissociate streptavidin from the biotin-dithiols, and spun for 2 min at 10,000 x g. The clear supernatant containing the biotinylated plasma membrane proteins were used for polyacrylamide gel electrophoresis (PAGE) and WB (see above).

Immunofluorescence Microscopy

Rabbit antibodies against the sequence of the second extracellular loop (ECL2) of the rat KCC2 protein were from a stock prepared by Gagnon et al. [26], and the polyclonal rb anti-rtKCC2 antibody (B22) mentioned above. Dilutions are indicated in the legends. Primary mouse (ms) anti-neuron-specific enolase (NSE), rb anti-αB and βB crystallin, and a polyclonal rb antibody against a peptide sequence of human Na/K ATPase α1 isoform subunit were procured from Santa Cruz (Santa Cruz Biotechnology, Inc., Santa Cruz, CA. 95060, USA) and used at dilutions indicated in the Figure legend. Secondary antibodies were C3-conjugated rb anti-ms IgG, and Cy3- or FITC-conjugated dk anti-rb IgG, also obtained from Santa Cruz. FHL-124 cells were grown on Lab-Tek chamber culture slides at a density of 6 x 10⁴ cells/well as previously described [26], simultaneously permeabilized and fixed in a freshly prepared 4% paraformaldehyde ± 0.01% saponin solution for 30 min at 4 °C, washed three times with PBS (0.5 ml/well) for 5 min each, incubated at 4°C for 1 h with a nonspecific blocking agent (3%), normal goat serum in PBS, and then incubated overnight at 4°C with primary antibody followed by 2 h incubation with the secondary antibody, a Cy3- or FITC-conjugated dk anti-rb IgG (1:250). Images were obtained with a Nikon E400 epifluorescence microscope under 60 X to 100 X (Oil) objectives using an attached Canon Rebel Xi, and overlays processed with an open source computer program (GIMP).

Results

hKCC2a mRNA expression

The KCC2 isoform, originally reported as of purely neuronal presence [22], is now recognized to be co-expressed in two variants characterized by the inclusion of one of the two mutually exclusive first exons of the KCC2 gene resulting from the election of two different

Fig. 1. Expression of human (h) KCC2s in FHL cells. A. Amplification of endogenous mRNAs encoding for hKCC2a, hKCC2b and glyceraldehyde 3-phosphate dehydrogenase (hGAPDH) by RT-PCR in FHL124 cells. Total RNA was extracted from FHL124 cells and reverse transcribed into cDNA. The transcripts of interest were PCR-amplified using transcript-specific sets of primers. Primer set hKCC2a-580 was designed to amplify the hKCC2a splice variant as an RT-PCR product of 580 bp, whereas hKCC2b-597 amplified hKCC2b as a product of 597 bp. As positive control, hGAPDH transcripts were amplified with a hGAPDH-specific primer set designed to amplify a product of 555 bp. As negative controls, two RT-PCR reactions were

performed, one with water (H₂O) instead of total RNA and another one where the enzyme reverse transcriptase was omitted (-RT). The primer set used in both control reactions (H₂O and -RT) was hGAPDH-555. B. Positive controls for hKCC2a and hKCC2b mRNA amplification using total cDNA from human brain. C. Graphic representation of the relevant region of the hKCC2 gene (top) and its two splice variant cDNAs, hKCC2a and hKCC2b (bottom). The 5'-untranslated regions (5'-UTR) of both hKCC2a and hKCC2b cDNAs are indicated as grey boxes (note that exons a and b also encode for the first methionine in each protein). Note that exons a and b are predicted to encode the first 40 or 17 amino acids of the hKCC2a and hKCC2b proteins, respectively. The relative positions of the specific PCR primer sets used to amplify hKCC2a or hKCC2b are shown as arrowheads atop the annealed exons (see Materials and Methods for details). D. Sequence analysis. Protein sequences and relative numbering (*i.e.*, with respect to the first methionine) which are relevant to each hKCC2 variant are also indicated (*i.e.*, PGE...). The 580 bp RT-PCR product amplified from FHL124 cells was purified and sequenced in both directions by PCR with the same PCR primers (sense and antisense) used in the original amplification. Shown is the sequence chromatogram corresponding to the region encompassing exon a and exon 2 of the hKCC2a transcript.

promoters. The human KCC2a variant includes exon 1 (also known as exon a) and encodes unique 40 N-terminal amino acids [25]. The KCC2b variant, the original rat neuron-specific variant characterized by Payne et al. [22], does not contain exon 1, but exon 2 (also known as exon b) [25]. Using total RNA from human FHL-124 cells, and specific sets of primers designed to amplify hKCC2a or hKCC2b, we found that hKCC2a is expressed in FHL124 LECs, while hKCC2b was virtually undetected under identical amplification conditions (Figs. 1, 2). Expression of the two variants was distinguished based

on their separate and unique first exons. To provide additional proof of the hKCC2a mRNA presence as the sole hKCC2 transcript in FHL124 LECs, we searched for the expression of all known full-length hKCC2b transcripts that have been cloned from different neuronal sources and posted in GenBank. We have classified these hKCC2b variants based on their DNA sequences and spliced exons. *In silico* analysis of these hKCC2b transcripts shows that the hKCC2b pre-mRNA may be subject to splicing events involving several exons, *i.e.*, exons 4-9 in hKCC2b-s1 (AK294059) or exons 3-20 in

Fig. 2. Genomic organization of the human KCC2 (SLC12A5) gene, splice variants and expression in FHL124 cells. **A.** Schematic, non-scale representation of the hKCC2 gene (Gene ID: 57468, NT_011362, position 14846021-14885281). This gene is organized in 27 coding exons (filled boxes) arranged in ~40 kb of genomic DNA sequence. Exon numbering is relative to the first coding exon of the gene (*i.e.*, exon 1, a). Introns are represented with V-shaped lines connecting exons, alternative splicing are also represented this way. Grey boxes denote 3' or 5'-UTR regions. Dotted lines represent alternative promoters predicted based upon known full-length hKCC2 cDNAs posted in GenBank. Potential initiation sites for transcription located upstream exon 1 and 2, generate at least two different transcripts named here hKCC2a and hKCC2b. **B.** Representation of full-length hKCC2 transcripts and different hKCC2b splice variants posted in GenBank. Except for hKCC2a, which has not been cloned yet from human sources, accession numbers for hKCC2b transcripts are indicated. These variants were aligned and analyzed *in silico* using Geneious v5.5.3 and the MAFFT v6.814b algorithm to show similarities among them. As shown, at least three hKCC2b

mRNAs encode for shorter variants. They are characterized by splicing of exons 4-9 (hKCC2b-s1, AK294059), 3-20 (hKCC2b-s2, AK295096) or by election of an alternative stop codon located in exon 8 (hKCC2b-s3, AK098371). These transcripts are predicted to encode proteins of 123.5 kDa (hKCC2b), 95.8 kDa (hKCC2b-s1), 36.8 kDa (hKCC2b-s2) and 33.6 kDa (hKCC2b-s3). **C.** The primer sets used to detect hKCC2b transcript variant expression in FHL124 cells are represented as opposite arrowheads connected by a line on top of each relevant transcript. **D.** RT-PCR of hKCC2b variants and hGAPDH from total RNA extracted from FHL124 cells. RT-PCR primers used were designed to amplify known hKCC2b mRNA splice variants using two sets of PCR primers for each one of them: hKCC2b-s1-504, hKCC2b-s2-609 and hKCC2b-s3-643. Shown in the gel is the lack of detection of any of the hKCC2b transcripts in human lenses, but expression of hGAPDH. As negative control, a single RT-PCR reaction was performed using H₂O instead of total RNA. The primer set used in control reactions was hGAPDH-555. **E.** Expression of hKCC2b-s variants in human brain. As control of RT-PCR reactions, hKCC2b-s1, hKCC2b-s2 and hKCC2b-s3 mRNAs were amplified from total RNA obtained from human brain. Faint bands of predicted sizes corresponding to hKCC2b-s1 and hKCC2b-s2 (504 bp and 609 bp, respectively) are detected whereas strong expression of hKCC2b-s3 is evident (643 bp).

hKCC2b-s2 (AK295096) and the inclusion of an alternative stop codon located in exon 8 (hKCC2b-s3, AK098371). The genomic organization of the hKCC2 gene and its hKCC2 transcripts are represented in Figs. 2A, B. To determine hKCC2b mRNA expression pattern in FHL124 LECs, we designed a set of specific primers for each hKCC2b-s transcript (Fig. 2C) and used them

in RT-PCR experiments. As shown in Fig. 2D, none of the hKCC2b-s transcripts could be detected in FHL124 LECs, suggesting that hKCC2b mRNAs may not be expressed in these cells. On the contrary, hKCC2b-s3 and very low transcript levels of hKCC2b-s1 and KCC2b-s2 were detected in human brain (Fig. 2E) supporting the idea that FHL124 cells may lack expression of KCC2b

Fig. 3. Western blot of KCC2 in FHL 124 cells. A. KCC2 staining at approximately 135 kDa, and a second, more diffuse band at ~95 kDa performed with commercial rb anti-rtKCC2 primary antibody (1:250 dilution) and HRP-conjugated IgG dk anti-rb secondary antibody (1:500); displayed are two identical lanes per condition. B. A blocking peptide for primary anti-KCC2 abolished staining at either molecular weight; Displayed are two identical lanes per condition. C. KCC2 staining with anti-KCC2(B22) (1:5000) directed at a fusion protein with the 112 C-terminal amino acids of KCC2 [27].

mRNAs. Together, our results indicate that FHL124 LECs express hKCC2a as the sole variant of KCC2.

Western blots of KCC2 expression

To confirm the presence of KCC2 in FHL-124 cells by WB, a commercial rb anti-rtKCC2 antibody was used because a peptide of the corresponding epitope was available, and the blotting compared to that obtained with rb anti-KCC2(B22) from Payne's laboratory [27]. The WB in Fig. 3A shows in two duplicate lanes a distinct protein band at the molecular weight (MW) of ~135 kDa within a broad positive smear with anti-KCC2 which is consistent with the reported molecular mass of KCC2 [27]. Additional staining was seen at the 90-95 kDa region, most likely representing a KCC2 fragment reacting with the commercial KCC2 antibody. As expected, absorbing the antibody with blocking peptides abolished the immunological signal at either of these molecular weights (Fig. 3B, duplicate lanes). An ~80 kDa band was also stained but was not diminished by prior antibody absorption with the peptide suggesting presence of an unrelated cross-reacting protein of non-specific nature and hence this band was omitted from the display in Fig. 3. This decision was also justified by the finding in Fig. 3C that anti-KCC2(B22), failed to detect this peptide. In agreement with the specific banding obtained with the commercial KCC2 antibody (Fig. 3A) and the reported one obtained from neuronal tissues [27], we detected several protein bands of MWs ranging from 100 to 135 kDa. Based on the immunological presence of KCC2 proteins detected with two different KCC2 antibodies it is concluded that FHL124 cells do express KCC2 at the protein level. Therefore, in combination with the data in Fig. 1, the available anti-KCC2 antibodies most likely detected KCC2a but not KCC2b.

To discern whether KCC2 had actually reached the plasma membrane, intact FHL124 cells were biotinylated (see Materials and Methods) and the externally

Fig. 4. Western blot of biotinylated streptavidin-agarose-isolated plasma membranes from FHL124 cells (two identical lanes A and B) KCC2 in plasma membranes of FHL124 cells was localized with commercial KCC2 primary antibody (1:200 dilution) and secondary HRP-conjugated dk anti-rb IgG (1:500). A ~95 kDa protein band was the main KCC2 antibody-positive protein band and corresponded to that blocked by the peptide in Fig. 3. Arrow indicates trace of a high molecular weight protein not visible in the reproduction here.

biotinylated membrane proteins tested for the presence of KCC2 with the commercial anti-KCC2 antibody used in Figs. 3A, B. Fig. 4 reveals the membrane presence of a ~95 kDa molecular mass protein detected by the commercially available anti-KCC2 antibody which disappeared when blocking peptides were used (c.f. Fig. 3B). A faint band was also detected at >200 kDa (not visible in Fig. 4, were the arrow indicates the position in the actual gel). This may represent KCC2 dimers of either 95 kDa bands or of the fully N-glycosylated KCC2a molecule.

Immunofluorescence of KCC2 expression

In order to test for the presence of KCC2a by immuno-fluorescence, a rb anti-rt KCC2 antibody was employed which successfully localized KCC2 in brain slices [26]. This antibody is directed against a sequence within the KCC2 extracellular loop 2 (ECL2) which is downstream from the extra-40 amino acid sequence of

Fig. 5. Immunofluorescence in FHL-124 cells at 400 X magnification. A. Membrane IF-staining in the absence of saponin (-) for KCC2 with rb anti-rtKCC2 IgG 1:200 as primary antibody from reference [26], and secondary antibody Cy3-conjugated dk anti-rb IgG at 1:250 dilution. B. General cytosolic and membrane IF staining in the same cell batch as in A, but in the presence of saponin (+). C and D: IF-positive staining for KCC2 in vesicular distribution (same primary antibody as in A&B, but secondary FITC-conjugated dk anti-rb IgG at 1:200) after saponin permeabilization. E and F: IF-positive staining for both α A crystallin (E) and α B crystalline (F) using commercial mouse (ms) anti- α A and α B crystallin antibodies at 1:250 dilutions. G: Cytoplasmic IF-positive staining of neuron-specific enolase (NSE) in cytoplasmic vesicular distribution with primary commercial ms anti-NSE antibody (1:100) and secondary CY3-labeled rb anti-ms IgG (1:250). H. Plasma membrane localization for rb anti-h α 1 subunit Na.K ATPase (1:50) and secondary CY3-labeled dk anti-rb IgG (1:150).

KCC2a, toward the C-terminus, and thus should detect both KCC2 variants. Consequently, we first fixed the cells in the absence and presence of saponin (Fig. 5A) to

compare presumably exclusive membrane from membrane plus cytosol (Fig. 5B) staining of bound rb anti-rtKCC2 with dk anti-rb C3-labeled IgG. Fig. 5A shows one cell with apparent cytosolic staining and a second cell, covered by a DAPI-positive nucleus, with clear membrane immunofluorescence suggesting not only cytosolic but obvious presence of the ECL2 motif in the FHL124 cell membrane. Fig. 5B shows intensive staining throughout the entire cells commensurate with saponin-generated holes in the plasma membrane. Figs. 5C, D shows fine punctate green fluorescence staining, apparently mostly within distinct small vesicles and detected by FITC-tagged secondary dk anti-rb IgG antibodies. Secondary anti-rb IgG did not stain these cells (not shown) arguing against the possibility of non-specific staining. A second anti-KCC2 (B22) which immunoblotted for a 100-135 kDa protein in Fig. 4, only faintly stained for KCC2 even at dilutions of 1/100 which was a lower dilution than the one used by Williams et al. [27] suggesting that this antibody is better for Western blots than for immunofluorescence at least for FHL124 cells. It is possible that in our cell line, the 112 terminal amino acids are buried within the cells' particulate matter and hence inaccessible to this antibody. Alternatively, the KCC2 in these cells could have experienced either programmed or proteolytic removal of this C-terminal protein segment, which also would explain the somewhat lower MWs noted in the gels of Figs. 3, 4.

As positive controls for our IF experiments, we selected α A and α B crystallins, two major proteins expressed in the lens [28]. These α A and α B crystallins, although diffusely, stained positive in the FHL-124 cells (Figs. 5E, F, respectively). Because of the presence of KCC2 in these cells, we decided to also test for neuron-specific enolase (NSE). NSE, which is found only in cells of the central and peripheral nervous system (CNS and PNS, respectively), and in neuroendocrine cells [29]. In FHL124 cells, the NSE protein was found in a comparable punctate labeling pattern as KCC2 (Fig. 5G). In order to contrast the vesicular staining of KCC2, FHL-124 cells were also stained for the α 1 subunit isoform of the Na/K ATPase. Fig. 5H clearly shows distinct membrane fluorescence due to the presence of the Na/K ATPase α 1 subunit.

An extra-neuronal KCC2 isoform was first seen in VSMCs by Di Fulvio et al. [20] and first reported as KCC2a by Uvarov et al. [25] in mouse brain stem and spinal cord. Here, KCC2a was identified for the first time in FHL124 at the levels of transcription by RT-PCR, and of translation by Western blots and IF staining.

Discussion

Earlier we reported the presence of hKCC2 mRNAs in FHL124 cells [11] in addition to that for KCC1, 3 and 4 transcripts [9, 10]. Figs. 1, 2 further clarify the nature of the hKCC2 mRNA by demonstrating the presence in the FHL124 cell line of only one of the two main hKCC2 variants, *i.e.*, hKCC2a but not hKCC2b. The presence of extra-neuronal hKCC2a is in fact intriguing. *In silico* analysis of published expression databases in the form of microarrays and analysis of the normal mammalian transcriptome show that the hKCC2 gene can express extra-neuronal transcripts. As an example, hKCC2 transcripts have been detected in gene expression profiling of physiologically normal human tissues obtained from various sources [30] (data record found under accession number GDS596 in GeoProfiles, www.ncbi.nlm.nih.gov/geo). Moreover, our findings corroborate a recent oligonucleotide microarray hybridization analysis of matched human LEC and cortical LFC showing several LEC and LFC arrays positive for SLC12A5 (hKCC2) transcripts (Geo access #GSM41472-7 see [31]). Notably, either hKCC2a, hKCC2b, or both, have been recently detected in cancer cells where this protein plays a role in cell migration [32]. Therefore, the expression pattern of hKCC2 might be more widespread than originally thought. An extra layer of complexity is further added by the fact that more than two promoters appear to exist in the hKCC2 gene and that hKCC2b exists as several spliced variants, all of them cloned from neuronal tissues (Fig 2). As indicated above, the GenBank database of nucleotide sequences holds several hKCC2b transcripts. For instance, hKCC2b-s1 (AK294059), cloned from human cerebellum, and hKCC2b-s2 (AK295096) and hKCC2b-s3 (AK098371), both cloned from human brain. These hKCC2b variants appear to be alternative splicing of the main hKCC2b variant [KCC2b (AF208159)] originally cloned from human brain [33]. These findings suggest that transcription of the human KCC2 gene via election of the second putative promoter *i.e.*, the one located immediately upstream exon 2 (exon b) has the potential to produce a hKCC2b transcript that can be further post-transcriptionally modified by alternative splicing in neurons, but not in FHL124 cells. Taken together, the presence of hKCC2a, but not hKCC2b transcripts in cell lines raises the possibility that the hKCC2 gene promoter commanding transcription of hKCC2a mRNAs may be operative or less restricted in extra-neuronal tissues.

Western blots (Figs. 3A-C) yielded immuno-reactive proteins of molecular mass between 95 and 135 kDa, using two different antibodies. The first commercially available antibody against a proprietary epitope showed a KCC2-specific band that was removed when the antibody was absorbed with the peptide against which it was made (*c.f.* Fig. 3A, B). The peroxidase-positive band of ~95 kDa was also attenuated when the antibody was absorbed with its peptide. Furthermore, a broad band of peptides of molecular weight between 95 and 135 kDa was seen with the second antibody directed against the B22 C-terminal peptide [27], increasing the certainty that FHL124 cells indeed express KCC2 proteins. In support of this conclusion, the biotinylation experiments showed presence of a 95 kDa peptide staining positively with the commercially available KCC2 antibody (Fig. 4). The smaller than expected molecular weight of the anti-KCC2-positive protein may be due to either some unavoidable degradation of the 135 kDa KCC2 protein, or perhaps due to expression of shorter protein chains commensurate with the possibility that shorter KCC2a DNA reading frames cannot be excluded. Such fragments were definitely not of KCC2b nature since an independent experiment including a positive control for KCC2b was found negative (Fig. 2). Since, according to the RTPCR data, the antibodies used do not differentiate between the KCC2a and KCC2b variants, these findings constitute the first report of KCC2a expression outside the central nervous system.

In the immunofluorescence studies, the rb anti-rt KCC2-ECL2 should primarily detect an epitope presumably located on the ECL2 of KCC2 [26]. Indeed, it Fig. 5A shows membrane staining in the absence of saponin known to permeabilize membranes. In the presence of saponin (Fig. 5B), the antibody detected the N-terminal ECL2 epitope on KCC2 molecules that both were still processed within the cytosol, and already assembled in the plasma membrane. However, one should bear in mind, that cell fixation with p-formaldehyde or glutaraldehyde does not exclude formation of membrane breaches through which even antibodies that are supposed to label only external membrane epitopes can pass. Tissue cross sections are clearly better suited to distinguish external plasma membrane from cytosolic labeling as we have previously shown in rat brain slices [26].

Our results are perhaps not surprising because, ontogenetically, like the neural crest, all lenses derive from the ectoderm. However, the lens being an independent inward fold of the ectoderm, in contrast to the ciliary body,

is not expected to contain a neurogenic component. Does it mean then that KCC2a may simply be expressed in fetal LECs, and becomes inactivated later in life? Or is it perhaps that only senescent cells no longer possess the KCC2a? There are several possibilities that could explain these data:

1) The cell culture received would contain a neurogenic cellular contamination. However, based on the gene chip analysis of this cell line provided to us by the late Professor G. Duncan, its make-up is more than 90% identical to human LECs from other sources containing various characteristic LEC markers such as crystallins, paired box gene 6 (PAX6), FOXE3, tumor growth factor alpha (TGF α) receptors, phospholipid synthesis, growth response to thrombin and other factors [23, 24, 34]. In addition, the illumine bead array of FHL124 cells showed presence of mRNA from SLC12A5, which is the KCC2 gene (personal communication by Dr. Michael Wormstone, University of East Anglia).

2) A laboratory-introduced contamination at our facilities is equally unlikely, since we have seen over a long time the same RT-PCR, and IF data in numerous batches and passages (>14 experiments), respectively.

3) The fact that NSE was also present immunologically in apparently small vesicles, suggests an expression of a second neuronal-specific marker, commensurate with the possibility that this LEC line behaves de-repressed *vis a vis* at least these two neuronal markers, the KCC2a and NSE. Regarding the KCC2, it was suggested that its expression, and presumably that of KCC2b, in non-neuronal cells and tissues may be under the repressive control of a 21 bp sequence, within intron 1, with 80% identity to the consensus site for neuronal-restrictive silencing factor binding proteins [35]. Nuclear proteins from a mouse neural progenitor cell line interact with this 21 bp element and thus repress both the reporter gene and KCC2 expression in non-neuronal cells [35]. It would be of interest to see whether the putative KCC2 repressor is actually functional in FHL124 cells. In another, yet SV-transformed LEC line, the B3 cell, we could not detect molecular or immunological evidence for KCC2 expression [10]. Since p53 and other tumor repressors may be absent in these cells, future studies should test the possibility that the lack of KCC2 expression is a related issue, i.e. whether the KCC2 silencing factor [35] actually is also under the control of these tumor repressors. In this context it should be noted that a recent report by Ellory's group [32] has shown that KCC2

expression and function indeed is not necessarily linked, and restricted only to neurons as a KCC2 isoform independent of transporting ions was found in human cervical cancer cell lines.

Conclusions

This study shows for the first time the existence of the KCC2-spliced variant, KCC2a, outside the central nervous system, and in a cell line that is of ectodermal origin where KCC2 expression silencer genes are predicted to be at work. A recent report that LEC growth and differentiation into LFCs may be under the fluctuating control of "a fundamental set of regulatory factors.... also governing neurogenesis" [36], further corroborates our observation of an up regulation of KCC2, even if as a transient phenomenon also occurring in cultured human LECs. Although earlier work revealed absence of KCC2 in phaco-emulsification-extracted human lens material [10], it cannot be excluded at this time that KCC2a plays an important role in the early development of the fetal lens and thus might impact lens growth with aging [37], or in age-dependent or diabetic cataract development [38]. We have previously shown for the first time that these FHL124 cells contain NCC (SLC12A3), the Na⁺-Cl⁻ cotransporter [14]. Together with the now established KCC2a expression, these findings make the study of human lens epithelial cell lines an even more attractive model system.

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