Cloning and localization of KCC4 in rabbit kidney: expression in distal convoluted tubule

Heino Velázquez and Teresa Silva

Veterans Affairs Connecticut Healthcare System, West Haven, Connecticut 06516

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Velázquez, Heino and Teresa Silva. Cloning and localization of KCC4 in rabbit kidney: expression in distal convoluted tubule. Am J Physiol Renal Physiol 285: F49–F58, 2003; 10.1152/ajprenal.00389.2002.—Cl-dependent K secretion is a feature of renal distal tubules and collecting ducts. Recent cloning and identification of K-Cl cotransporter proteins led us to search for additional novel KCC isoforms expressed in the renal distal nephron. A human expressed sequence tag (EST) with high homology to KCC1 was identified. The rabbit isoform was cloned by homology using degenerate primers and rapid amplification of cDNA ends (RACE). Our isoform is the rabbit homologue of mouse and human KCC4 published previously. The 4.35-kb rabbit KCC4 cDNA encodes a protein of 1,106 amino acids. Antibodies were generated to both NH2-terminal and COOH-terminal fusion proteins. Northern and Western blot analyses showed widespread mRNA and protein expression in many rabbit organs, in renal cortex, outer medulla, and inner medulla but not in skeletal muscle. Immunohistochemical localization of KCC4 showed expression exclusively along the basolateral membrane in many nephron segments. The distal convoluted tubule and connecting tubule exhibited the highest level of KCC4 immunoreactivity, followed by the medullary thick ascending limb. A low level of immunoreactivity was detected in the proximal tubule and collecting ducts. We postulate that KCC4 mediates potassium and chloride exit from the cell and may play an important role in salt absorption by the distal convoluted tubule. [abstract]

In the kidney, the control of overall K excretion is achieved primarily through regulated secretion of K by cells of the renal distal tubule (31). The predominant mechanism for K secretion is diffusion via K channels located in the apical membrane. However, other secretory K transport pathways also appear to be present in distal tubule apical membranes in parallel to these channels. We have previously demonstrated Cl-dependent K secretion in rat renal distal tubules (6, 23, 32) and postulated the presence of a K-Cl cotransporter in the apical membrane of the initial collecting duct and the distal convoluted tubule (DCT) (25). This pathway is stimulated when luminal fluid Cl concentration falls to levels below 10 mM.

In addition to being involved in K secretion, renal distal cells are also involved in active Na and Cl absorption. In the DCT, a high rate of Na absorption predominantly occurs via a thiazide-sensitive Na-Cl cotransport mechanism (3, 7, 26). To sustain this high rate of Na and Cl absorption by the DCT, we have hypothesized, based on preliminary work in our laboratory, that K taken up in exchange for Na by the Na-K-ATPase exits across the basolateral membrane via a K-Cl cotransporter mechanism (24). The presence of this transporter would also provide a pathway for Cl from cell to blood and would sustain continued apical NaCl entry into the DCT cell. Thus, in our working model of a DCT cell, we propose that two distinct forms of a K-Cl cotransporter are expressed, one in the apical membrane and another in the basolateral membrane.

The transport of K coupled with Cl via electroneutral K-Cl cotransport has been recognized in many tissues for some time (4, 12, 18, 33, 35, 36). In 1996, two cDNA clones corresponding to K-Cl cotransporter proteins (KCC1 and KCC2) were identified (9, 17). KCCs are members of the larger cation Cl cotransporter gene family that includes the thiazide-sensitive Na-Cl cotransporter and the furosemide-sensitive Na-K-2Cl co-transporter. They possess 12 putative transmembrane segments and cytoplasmic NH2 and COOH termini. Although KCC2 is a neuronal-specific isoform, KCC1 is found in many organs, including the kidney, and is believed to be involved in volume regulation (15). KCC1 may be a candidate for the K-Cl cotransporter we postulate for the basolateral membrane of the DCT; however, we reasoned that additional isoforms encoding K-Cl cotransporters may exist, including ones that may be expressed at the apical membrane. We attempted to identify novel K-Cl cotransporter genes in the kidney. A search of the expressed sequence tag (EST) database identified ESTs that encoded proteins with a high homology to KCC1 and KCC2. One of these was derived from a novel K-Cl cotransporter that we named KCC4 (29). Simultaneously, KCC3 and KCC4 cDNAs were also cloned in mice and humans (10, 16). Here, we report the cloning of rabbit KCC4 and the intrarenal distribution of rabbit KCC4 mRNA and protein.

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METHODS

Cloning of KCC4 in rabbits. The human EST database was screened for sequences homologous to KCC1. One clone was identified (aa 297597) with high homology to KCC1 and KCC2. Two sets of primers were selected. A set of three antisense primers was selected from the EST clone (kc3c, kc3f, and kc3g). A second set of three sense primers was identified by aligning the KCC1 and KCC2 sequence upstream of the location of the EST clone and selecting exactly matched primers to regions of nucleotide identity in regions conserved between KCC1 and KCC2 at the protein level (5kc1, 5kc2, and 5kc3). Subsequent PCR (XL DNA polymerase, PerkinElmer1) in rabbit cDNA, with all possible combinations of these primers, yielded cDNA bands in some reactions. The product of 5kc3 and kc3g was cloned and sequenced (kc3.12) and identified as novel. Two specific primers were selected from the upstream region of kc3.12 (kc3c– and kc3n–) to obtain the 5' end of the cDNA by rapid amplification of cDNA ends (RACE). 5' RACE yielded two cDNA clones with different 5' ends (kc3G4 and kc3G5). Although 3'-RACE was attempted with primers in the 3'-end of kc3.12, these XL-generated cDNA products could not be cloned into a vector. The 3'-end of the coding sequence was obtained by PCR using a degenerate antisense primer that matched the aligned COOH-terminal protein sequences of KCC1 and KCC2 (kc310– and a sense primer selected from the downstream part of kc3.12 (kc3N1)). A second attempt at 3'-RACE using a primer very close to the 3'-end of the coding sequence was successful and produced a single sequence (kc3KE).

Rabbit organ Northern blot analysis. Total RNA was isolated from up to 1-g samples of rabbit tissue using TRIzol reagent (GIBCO BRL). Organs harvested were kidney, heart, lung, liver, pancreas, salivary gland, spleen, brain, adrenal gland, bladder, small intestine, large intestine, skeletal muscle, and stomach. Twenty-five micrograms of total RNA were loaded per lane and transferred to a nylon membrane (GeneScreen Plus, NEN Life Sciences Products). Each blot was prehybridized for at least 4 h in Church-Gilbert solution [0.5 M Na phosphate (pH 7.2), 1 mM EDTA, 7% SDS, 1% bovine serum albumin, 100 μg/ml salmon sperm DNA] at 68°C and then hybridized (2 × 106 cpm/ml, where cpm is counts/min) overnight using a random prime-labeled probe (Life Technologies). The blot was rinsed once and then washed for 15–30 min in 2× SSC (SSC composition: 0.15 M NaCl, 0.15 sodium citrate, pH 7) and 0.5% SDS at room temperature. A final wash at 68°C was done for 15–30 min in 0.5× SSC and 0.1 SDS, with periodic monitoring by a Geiger counter for low background counts. The autoradiograph of the blot was obtained electronically (Instant Scanner, Packard) or on film.

Dissection of nephron segments for RT-PCR analysis. New Zealand White rabbits (1–2.5 kg) were anesthetized with ketamine (50 mg/kg im) and pentobarbital sodium (50 mg/kg iv), and their kidneys were removed via a flank incision and chilled in ice-cold PBS. The rabbits were euthanized by overdose of pentobarbital sodium. RNAase-free media, beakers, and instruments were used. Transverse slices (1 mm) of the kidney were transferred to a dissection dish that was placed on the stage of a dissecting microscope (transmitted light, model SZH, Olympus) fitted with a water-chilled chamber.

In preliminary experiments, several DNA polymerases were used to amplify KCC4 template. The XL enzyme was the only one that consistently generated a product.

The cortex, outer medulla, and inner medulla were separated. Tissue from each region was incubated in a collage-nase solution according to Schafer et al. (20), and tubules were dissected by hand from each sample. The dissecting solution was MEM or medium 199 (GIBCO). Guidelines for dissection in the heterogeneous distal portion of the nephron were taken from Morel et al. (13). Additional details for the identification of individual nephron segments have been published previously (28, 30). To minimize contamination of specific nephron samples with other cells, specific nephron segments were transferred to a new dish for final rinsing in fresh medium. For single-nephron RT-PCR experiments, a total of 4 mm of tubule (2–12 nephron fragments) was transferred directly into a 0.5-ml microtube containing 10 μl of lysis solution (2% Triton X-100, 5 mM dithiothreitol, 2.2 U/μl RNAsin, diethylypyrocarbonate-treated water). Tubule fragments were transferred by adsorbing them to glass beads (0.5-mm diameter) held by forceps. All samples were frozen at −70°C until the time of the experiment. Nephron segments collected were glomerulus, proximal convoluted tubule, proximal straight tubule, medullary thick ascending limb of Henle's loop (mTAL), cortical thick ascending limb of Henle's loop (cTAL), DCT, connecting tubule (CNT), cortical collecting duct (CCD), outer medullary collecting duct (OMCD), and inner medullary collecting duct (IMCD).

Single-nephron RT-PCR analysis. The single-nephron RT-PCR technique (14) was modified as described previously (30). Briefly, total RNA was first extracted from individual nephron samples (RNAqueous, Ambion) after the addition of 20 μg Escherichia coli rRNA (Boehringer). All RNA was precipitated with Na acetate in 70% ethanol. The pellet was resuspended in 10 μl of diethylypyrocarbonate-treated water, and the entire amount was used as a template for cDNA synthesis (Superscript II, GIBCO) using oligo(dT) or random hexamer primers in a final volume of 20 μl. In each experiment, up to 10 nephron segments were tested. In addition, two control tubes were added: a reagent blank consisted of 10 μl of lysis solution, but no kidney tissue, and all other kit reagents; an RT-PCR control to determine the quality of the kit reagents consisted of 50 ng of rabbit kidney total RNA. A volume of 2–4 μl of cDNA was used for each subsequent PCR reaction.

In each experiment, all tubes were assayed for multiple genes. One set of cDNA aliquots was used to determine whether any cDNA was synthesized (positive control): primers were used to amplify a segment of the gene responsible for the oculocerebrorenal syndrome of Lowe (8). This gene product is expressed in all nephron segments examined, from the glomerulus to the collecting duct (8), and serves as a positive control for cDNA synthesis during the RT step.

Rabbit-specific primer pairs were selected from two regions of the cloned KCC4 cDNA. Midgene primers were GAGGCCCCGTGACGGGGAGACCTTCGTCACA (kc3Ri+) and GGT-GTCGGGACCAGGTGTCGAGAAC (kc3Rg2–). 3'-End primers were GAACCGGCAGGGAGACAAGACAATCAGAAG (kc3N3+) and ACACGCACTGGCATGCGCCACGAT (kc3N3–).

In separate pilot experiments, a lack of PCR amplification from the genomic template was confirmed by omitting the RT step in tubes containing tubules or 50 ng total RNA. Samples were electrophoresed on a 1% gel and photographed to record ethidium bromide staining.

Generation of KCC4 NH2-terminal and COOH-terminal fusion proteins. Fusion protein constructs were generated with maltose binding protein (pMAL; New England Biolabs) to make antigens for antibody production. Both NH2-terminal (87UM, 51LW) and COOH-terminal (B-6, B-16) polypeptides were selected from putative cytoplasmic portions of KCC4 for
fusion. 87UM encompassed the first 141 amino acids, and B-6 encompassed the last 284 amino acids. The 51LW polypeptide has a deletion of amino acids 31–66, and the B-16 polypeptide has a deletion of amino acids 903–288. These two deletions were encountered during the cloning of rabbit KCC4. The sense oligonucleotide primer began with the first amino acid of the polypeptide, whereas the antisense oligonucleotide contained a stop codon and a HindIII restriction site. cDNA fragments were generated by PCR from cDNA isolated from rabbit DCT segments and cloned into the PCR- II-TOPO vector (Invitrogen). After the correct sequence was confirmed, the KCC4 sequence was cut out with EcoRI and HindIII and cloned into the pMAL vector. Selected pMAL fusions were sequenced from the ends to confirm in-frame insertion into the vector. Fusion proteins were generated from these constructs in the presence of protease inhibitors (Roche Molecular Biochemicals, 1697498) according to the kit manufacturer’s instructions and purified on an amylose column.

Antibody production. Three guinea pigs were immunized per antigen (87UM, 51LW, B-6, and B-16). All animal immunizations were performed by the Yale Animal Resource Center. One hundred micrograms of fusion protein were injected per guinea pig in complete Freund’s adjuvant, and subsequent boosts were done with 100 μg of antigen in incomplete Freund’s adjuvant.

Initial screens of early and late bleeds of all guinea pigs identified those animals with the strongest response. The intensity of immunofluorescent (IF) and Western blot signals was generally weak but qualitatively similar in all guinea pigs. One animal (guinea pig 5; 51LW, NH2-terminal antigen) had a notably stronger response by Western blot analysis, and two animals (guinea pig 5 and guinea pig 11; B-16, COOH-terminal antigen) had a notably stronger IF response than any other animal. Antiserum from guinea pig 5 (GP5) was selected for further study because of its suitability for both Western blot and IF analysis. Antibodies were partially purified by employing a protein A affinity column (Pierce) or by affinity purification against the immunizing antigen coupled to an Affigel column (Bio-Rad).

Western blot analysis. A crude membrane prep was generated from rabbit kidney. Two grams of tissue in 6 ml of buffer (100 mM K acetate) containing 1% Tween 20 (Blotto-T). Antiserum was diluted in Blotto-T rinsed in PBS, and blocked for 20 min in 5% Blotto containing 0.25% Triton X-100 (P8340, Sigma) were homogenized with 100 μg of antigen in incomplete Freund’s adjuvant.

Membranes were soaked in methanol, protein/lane (samples were not heated to prevent aggregation) for 10 min to pellet debris and nuclei. The supernate was discarded, and the pellet was resuspended in PBS containing protease inhibitors. Additional membranes were run on an 8% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride (Immobilon-P, Millipore) membrane using a semidry transfer apparatus (Multiphor II, Pharmacia Biotech). Membranes were soaked in methanol, rinsed in PBS, and blocked for 20 min in 5% Blotto containing 1% Tween 20 (Blotto-T). Antiserum was diluted in Blotto-T and applied to blots for 1 h. Strips were rinsed twice and then washed 4 × 5 min in Blotto-T, followed by incubation with the secondary antibody (horseradish peroxidase-conjugated rabbit anti-guinea pig, Zymed) for 1 h. Blots were rinsed twice and then washed 2 × 5 min in Blotto-T, followed by 2 × 5 min in PBS and incubation in chemiluminescence reagent (PerkinElmer Life Sciences) for 1 min, and then exposed to film.

Immunocytochemistry. New Zealand White rabbits (1–2.5 kg) were anesthetized with ketamine (50 mg/kg im) and pentobarbital sodium (50 mg/kg iv) given gradually to achieve deep anesthesia. The kidneys were perfusion-fixed by retrograde perfusion from the abdominal aorta (PLP fixative; 2.5% paraformaldehyde, 10 mM NaIO4, 75 mM lysine, 0.175 M Na phosphate buffer; final pH 6.2) after first being flushed free of blood with heparinized PBS. Kidneys were cut into slices with different orientations and fixed for 4 h in PLP at room temperature. Tissue was subsequently placed in PBS-30% sucrose overnight at 4°C and frozen in optimal cutting tissue compound at –180°C. Five-micrometer sections were cut at –25°C and placed on gelatin-coated slides. All sections were pretreated with 1% SDS (2) because this pretreatment intensified the KCC4 signal. Immunocytochemical characterization of GP5 was performed by incubating the tissue sections in the primary antibody diluted in 25% goat serum +0.25% Triton X-100 in PBS for 1 h and then washing them for 2 × 5 min in PBS containing high salt (2% NaCl, 1% PBS-HS) or 2 × 5 min in PBS. The secondary antibody (goat anti-guinea pig, Alexa 568, Molecular Probes) was diluted 1:1,000 in 25% goat serum +0.25% Triton X-100 in PBS and incubated for 1 h. The washes were done as for the primary antibody. Additional antibodies used were mouse anti-rabbit high-affinity thiazide-diuretic receptor (JM5; Dr. David H. Ellison) and goat anti-mouse coupled to FITC (Zymed). The section was mounted using crystal mount or an antifade medium (ProLong, Molecular Probes) and visualized using a fluorescence microscope.

RESULTS

Cloning of rabbit KCC4. The full-length cDNA for rabbit KCC4 was obtained by PCR of a middle cDNA fragment and 5’- and 3’-RACE ends. These fragments were sequenced and assembled by computer to yield the full-length consensus sequence. Sequences for KCC isoforms 3 and 4 in humans and mice have been published in the interim (10, 16). Our sequence is the rabbit homologue of the KCC4 isoform. The complete protein sequence for rabbit KCC4 is given in Fig. 1 (GenBank accession no. AF538347). The full-length

Fig. 1. Rabbit K-Cl cotransporter isoform (KCC4) protein amino acid sequence. Underlined amino acids are two deletions encountered during sequencing. Italics denote regions used to generate antibodies in this study. Truncated form of KCC4 is missing first 92 amino acids. See text for details.
KCC4 cDNA sequence is ~4.35 kb and has a 66% GC content. Even though multiple attempts were made to clone the full-length PCR product (XL DNA polymerase; see METHODS) into a vector, no transformants were obtained. Rabbit KCC4 encodes a protein of 1,106 amino acids. Two 5’-RACE clones were obtained with the same nucleotide sequence at their 3’-ends but a differing sequence at their 5’-ends. The open reading frame of one of these two 5’-variants results in a truncation of the KCC4 protein by 92 amino acids. Percent identity (DNA Star, Clustal method, gap penalty of 10 for multiple alignments and PAM250 residue weight table) to published KCC1, 2, or 3 proteins (9–11, 16, 34) ranges from 64 to 69%. Percent identity to mouse and human KCC4 proteins (16) is 87 and 85%, respectively.

**Distribution of KCC4 in the rabbit (Northern blot analysis).** KCC4 is widely distributed in rabbit tissues. Figure 2 shows a composite of representative Northern blots of all rabbit organs tested. Twenty-five micrograms of total RNA were loaded onto each lane and confirmed by ethidium bromide staining (data not shown). Blots were probed with cDNAs corresponding to amino acids 112–849 (clone kc3.12) or 822–1106 (clone B-6) and gave similar results. A band of ~4.4 kb was detected in most organs except brain, which in addition expressed a higher molecular weight band at ~6.4 kb.

**Characterization of KCC4 antibodies.** GP5 antiserum (immunized with the NH2-terminal fusion protein 51LW) detected a broad band at ~150 kDa by Western blot analysis in rabbit kidney crude membranes (Fig. 4). Preimmune serum did not reveal a band of this size. The signal was specifically blocked by preadsorption with NH2-terminal fusion proteins (51LW or 87UM). Preadsorption with an unrelated fusion protein (B-6) did not affect the signal. Qualitatively similar results were obtained by Western blot analysis of rabbit kidney with antisera from other guinea pigs immunized with 51LW and with antiserum...
much lower yield from the affinity purification process, the protein A-purified antiserum was used in all subsequent experiments. The nonspecific background staining with preimmune serum was low and is shown in Fig. 7, a1, a4, and a7. Figure 7a2 is a low-power view of the kidney cortex stained with GP5. A small number of tubule profiles in the cortex stand out, suggesting primarily a distal distribution of rabbit KCC4 protein. A faint signal is apparent in proximal tubules. The signal is specific because 1) it was eliminated when the antiserum was preadsorbed with 51LW fusion protein (Fig. 7a3) and 2) the signal was not affected by preadsorbing antiserum with an unrelated fusion protein (B-6) (data not shown).

In a low-power view of the outer medulla (Fig. 7a5), staining of many tubule profiles is apparent in contrast to the cortex. This signal is blocked (Fig. 7a6) by preadsorption with 51LW fusion protein. A low-power view of the inner medulla reveals faint general staining that is not readily discernible at this power (Fig. 7a8). This low-level signal is reduced (Fig. 7a9) by preadsorption with 51LW fusion protein.

At higher magnification, a prominent basolateral signal is detected in some distal tubule profiles (Fig. 7, b–d). Co-localization experiments with an antibody (JM5) to the high-affinity thiazide-diuretic receptor (5) were performed. Figure 7, b1, c2, d2, and e2, shows that JM5 intensely labels the apical membrane of all DCT cells and more weakly labels most cells of the TAL, CNT, and collecting duct (5). Immunolocalization with GP5 antibody indicated that KCC4 was most prominent along the basolateral aspect of cells lining the DCT and the downstream CNT (Fig. 7, b1, c1, and d1). Intermediate levels of KCC4 expression were observed in the CCD. Faint staining of the basolateral aspect of proximal tubules was apparent. Intense staining of the basolateral membrane stops abruptly at the junction of the DCT with the cTAL. The KCC4 signal in the cTAL was weak or undetectable. No signal was detected in cells of the glomerulus.

In the outer medulla (Fig. 7e), in contrast to the cortex, the TAL basolateral membrane is positive for KCC4. Staining of the basal membrane of proximal tubules extends to the S3 segment in the outer medulla and appears to increase slightly in intensity compared with the cortical proximal tubule. Intermediate levels of staining extend to the medullary portion of the collecting duct. In the inner medulla (Fig. 7f), low-level

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**Fig. 4.** Western blot analysis of rabbit kidney crude membranes with protein A-purified guinea pig 5 antiserum (GP5 antibody) showing specificity for NH2-terminal antigen. GP5 antiserum was used at a dilution of 1:400. Lane 1, protein A-purified GP5 preimmune serum at 1:400 dilution; lane 2, GP5 antiserum; lane 3, GP5 antiserum preadsorbed with unrelated fusion protein B-6; lane 4, antiserum preadsorbed with fusion protein 51LW. Fusion protein 87UM was equally effective in blocking signal. Right, size marker (GIBCO Benchmark protein unstained ladder).

**Fig. 5.** Western blot analysis of rabbit organ protein with GP5. From guinea pigs immunized with the other fusion proteins, although the signals were generally much less intense. The antisera from some guinea pigs were positive except for skeletal muscle. All organs tested. Figure 5 shows the Western blot analysis of whole kidney, kidney cortex, kidney outer medulla, kidney inner medulla, lung, heart, colon, small intestine, stomach, liver, and skeletal muscle. All organs were positive except for skeletal muscle.

KCC4 protein was also detected in most rabbit organs tested. Figure 5 shows the Western blot analysis of whole kidney, kidney cortex, kidney outer medulla, kidney inner medulla, lung, heart, colon, small intestine, stomach, liver, and skeletal muscle. All organs were positive except for skeletal muscle.

GP5 antiserum did not recognize any protein in rat or mouse kidney membrane preparations (Fig. 6), suggesting that this antibody does not cross-react with KCC4 in other species and also does not cross-react with KCC1 or KCC3 in other species. GP5 cross-reactivity was also tested specifically against rabbit KCC1 (protein a gift of Dr. B. Forbush) overexpressed in HEK 293 cells. GP5 does not cross-react with rabbit KCC1.

**Immunocytochemical localization of KCC4 in rabbit kidney.** Distribution of KCC4 in rabbit kidney was studied by immunocytochemistry. Kidney tissue sections were stained with partially purified GP5 (protein A) antiserum (Fig. 7). The signal with 51LW affinity-purified antibody was indistinguishable from that with protein A partially purified antibody. Because of the

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**Fig. 7.** Western blot analysis of rabbit kidney crude membranes with protein A partially purified GP5 antibody was used at a dilution of 1:400. WK, whole kidney; C, kidney cortex; OM, kidney outer medulla; IM, kidney inner medulla; Lu, lung; Ht, heart; Co, colon; Sl, small intestine; St, stomach; Li, liver; SM, skeletal muscle. All organs except skeletal muscle were positive.
staining is observed in most structures, including the IMCD.

Qualitatively similar staining is observed with both NH₂-terminal (GP5) and COOH-terminal (data not shown) antibodies. The NH₂-terminal cytoplasmic portion of KCC4 is highly divergent across species, whereas the COOH-terminal cytoplasmic portion of KCC4 has significant homology across species. Our results are consistent with both NH₂-terminal and COOH-terminal antibodies, being specific for KCC4 protein because the IF and Western blotting pattern of staining of all antibodies is similar. Furthermore, GP5 only recognizes protein (Western blotting and IF) in the rabbit and not in the rat or mouse, consistent with its specificity for the unique rabbit NH₂-terminal KCC4 sequence.

DISCUSSION

We report the cloning of the rabbit homologue of KCC4. This K-Cl cotransporter is widely distributed in the rabbit and highly expressed in kidney, heart, and lung. In the kidney, KCC4 is expressed at high levels in the basolateral membrane of DCT and CNT cells in the cortex, with weak staining of the proximal tubule basal membrane. KCC4 is also expressed in the mTAL, collecting ducts, and inner medullary structures.

There are at least two variants of this protein expressed in the kidney, a full-length form and a truncated form with an NH₂ terminus 92 amino acids shorter. The first 92 amino acids are highly divergent from KCC4 of other species and from other KCC isoforms. In contrast, the remaining amino acids in the putative NH₂-terminal cytoplasmic portion (amino acids 93–141) are highly conserved across KCC4 of different species and different isoforms. The significance of these and two additional variants exhibiting deletions in the NH₂ and COOH termini is unknown.

Northern blot analysis of KCC4 revealed high levels of mRNA expression in the kidney, colon, heart, gallbladder, and lung. This widespread distribution of KCC4 in the rabbit is similar to the pattern of distribution of the other members of the K-Cl cotransporter family except KCC2, which is restricted to the brain. Of note were skeletal muscle and the salivary gland, in which KCC4 message was undetectable in the rabbit. These results support previous data showing that the salivary gland expresses KCC1 or KCC3 as its primary KCC isoform (19). Within the kidney, mRNA levels were similar in whole kidney compared with cortex, outer medulla, or inner medulla, suggesting it is widely distributed in structures throughout the entire organ.

Single-nephron RT-PCR experiments showed expression of this gene in all nephron segments tested (Fig. 3). Because the results from this type of experiment are not quantitative, it cannot be determined whether there are differences in expression levels among the different nephron segments. Taken together, it appears that KCC4 message is expressed in each of the three major regions of the kidney and can be detected in all nephron segments and structures tested. Because amplification of cDNA by PCR is extremely sensitive, in the present study (see METHODS) it is not possible to evaluate whether biologically significant levels of mRNA are expressed in each tubule segment. These experiments also cannot establish whether significant amounts of protein are made in each nephron segment.

To be able to detect KCC4 protein, antibodies were generated. Fusion proteins to both the NH₂-terminal and COOH-terminal portions of KCC4 were used because previous attempts at generating antibodies to unique KCC4-specific synthetic peptides (amino acids 30–45 and 980–997) had failed. By Western blot analysis, a broad band of ~150 kDa was consistently ob-
Fig. 7. Immunohistochemical detection of KCC4 in rabbit kidney. a: Low-power micrographs of rabbit kidney cortex (a1, a2, a3), outer medulla (a4, a5, a6), and inner medulla (a7, a8, a9) stained with GP5 preimmune serum (a1, a4, a7), GP5 antiserum (a2, a5, a8), or with GP5 antiserum preadsorbed to the immunizing antigen 51LW (a3, a6, a9). Protein A partially purified preimmune serum and GP5 antiserum were used at a dilution of 1:100. 51LW fusion protein was used at 5 μg/ml for preadsorption. All images were acquired at the same exposure setting. b: High-power (×400) micrograph of cortex with GP5 (b1) and mouse anti-rabbit high-affinity thiazide-diuretic receptor (JM5; b2). JM5 labels the apical membrane of all DCT cells intensely and the apical membrane of most TAL, CNT, and CD cells more weakly. GP5 labels the basolateral aspect of the DCT (D) intensely and PCT (P) faintly (arrow) along the basal membrane. GP5 staining of the cTAL (T) is faint or below the detection threshold. The CCD exhibits an intermediate level of staining. c: High-power micrograph in cortex showing DCT (D)-to-CNT (CN) transition. KCC4 immunoreactivity remains high throughout the transition to CNT, demarcated by the decrease in intensity of JM5 signal. d: High-power micrograph in cortex showing intense KCC4 staining in DCT and CNT, whereas glomeruli are negative. e: High-power micrograph in outer medulla. Medullary thick ascending limb (mT) profiles are positive as well as the medullary S3 segment of the proximal tubule (mP) and the OMCD. f: High-power micrograph in inner medulla shows relatively weak KCC4 staining of the IMCD as well as other unidentified structures.
erved in whole kidney. Strong signals were detected in kidney cortex, outer medulla, and inner medulla, confirming the results from the single-nephron RT-PCR experiments and suggesting that many of the nephron segments synthesize KCC4 protein. In addition to being expressed in kidney, high levels of KCC4 protein are detected in many other rabbit organs. At least one exception to this widespread distribution is of note because no signal could be detected in skeletal muscle. These data correlate well with the Northern blotting results, where also no signal could be detected in this organ. Thus it appears that KCC4 protein is not an essential component for skeletal muscle function. Nevertheless, K-Cl cotransport likely does play a role in skeletal muscle function because message for KCC1 and KCC3 can be detected by Northern blot analysis (9, 16).

Our antibody appears to be specific for rabbit KCC4 and does not cross-react with KCC4 in other species. The antibodies directed against the NH2-terminal fusion proteins should be specific for KCC4 because of the low homology of the amino acid sequence of this region compared with KCC1–3. In addition, there is very little homology between the NH2 termini of rabbit, mouse, and human KCC4. This prediction is confirmed by the results in Fig. 6. In rabbit kidney, a broad band is detected at ~150 kDa; however, no signal can be detected in either mouse kidney or rat kidney. Also, GP5 does not cross-react with rabbit KCC1 protein expressed in vitro. These data suggest that GP5 antibodies are directed specifically against rabbit KCC4 and are in agreement with sequence data that there is little similarity between the NH2 termini of KCC4 of different species, including the rat. Although a portion of 51LW fusion protein against which GP5 was raised (amino acids 93–141) does have high homology with KCC1–4, preadsorbing GP5 with a fusion protein of amino acids 93–141 did not block the signal, suggesting that the active epitope resides within the first 92 amino acids of rabbit KCC4.

By IF, intense basolateral staining is observed in a minority population of nephron segments in the cortex with only a faint signal detectable along the basal aspect of proximal tubules. The weak signal in the rabbit proximal tubule is a significant divergence from results in the mouse that have been published previously (1, 22). One possible reason for this is that there are significant species differences in the distribution of KCC4 within the kidney and that the rabbit proximal tubule expresses much lower levels than in the mouse. Another possibility is that because the antibodies in the three studies were raised against different antigens on KCC4, they are recognizing different variants of this protein. Also, it is possible that secondary and tertiary conformational differences may contribute to partial concealment of our epitope in the rabbit proximal tubule. Although we found that using antigen retrieval methods such as pretreatment with SDS (2) increased the intensity of the signal several-fold, we did not detect any significant change in the relative distribution of the signal using this method.

Colocalization experiments using JM5 (5) (which recognizes an epitope of the DCT and also of the TAL, CNT, and collecting duct apical membrane) identify intense basolateral staining beginning at the transition from the cTAL to the DCT and continuing throughout the DCT into the CNT, and possibly into initial portions of the CCD. Although our results from the single-nephron RT-PCR experiments suggested that the cTAL expresses KCC4, the high sensitivity of this method may amplify a low abundance mRNA transcript that is not biologically important. This contrasts with the mTAL, which stains intensely with GP5 along the basolateral membrane. One can speculate that a higher expression of KCC4 in the mTAL than in the cTAL may be related to the higher load of salt that is delivered to it from the inner medulla. It may be that basolateral KCl exit is rate limiting, and, to absorb the large load of K, Cl, and Na efficiently, greater amounts of KCC4 in the mTAL are required to facilitate this salt recovery.

All DCT cells stain at the apical membrane with JM5 and at the basolateral membrane with GP5. These results conflict somewhat with recent studies of KCC4 expression in the mouse. In one study, the level of KCC4 expression in the DCT was very low or undetectable (1), whereas in another preliminary study, also in the mouse, significant staining was observed along the basolateral membrane of the DCT (22). The reasons for the discrepancy in mouse DCT staining are not clear but could be related to the different antigens used to generate the antibodies in these two studies. Specific DCT staining (and the pattern of staining of the other nephron segments) was also evident with antisera from guinea pigs of each of the other antigen groups (87UM, B-6, B-16; data not shown; see Results). Taken together, these data suggest that KCC4 protein is expressed along the basolateral membrane of the DCT.

Strong basolateral KCC4 staining extends beyond the DCT into the CNT and dissipates gradually toward the transition to the CCD. Other structures in the inner medulla, likely thin ascending or thin descending limbs of the loop of Henle, or possibly vasa recta, are also KCC4 positive. Although the role of KCC4 expression in these cells remains to be determined, it may be related to adjustment of their volume in response to changes in inner medullary interstitial osmolality.

Mice in which the KCC4 gene is knocked out are deaf and exhibit renal tubular acidosis (1). The α-intercalated cell of the distal tubule and collecting duct plays a central role in regulating acid secretion by the kidney and expresses KCC4 in the mouse. Basolateral KCC4 appears to be critical for the intercalated cell of the collecting duct to secrete acid effectively by extruding the Cl that is taken up via basolateral Cl/HCO3 exchange. The intercalated cell in the rabbit also expresses KCC4; however, it remains to be determined whether in this species KCl exit mediated by this protein plays a critical role in intercalated cell function and acid secretion.

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K secretion by the renal distal tubule is conductive and occurs primarily via K channels in the connecting tubule and initial collecting duct. Although a small amount of K is secreted by the DCT (21, 23), this likely does not occur via K channels because no significant conductances to ions can be detected in DCT apical membrane (27, 30). The DCT absorbs Na and Cl avidly (7), however, and must exchange Na for K continuously across the basolateral membrane to do so. We postulated that the DCT expresses a K efflux pathway across the basolateral membrane that counterbalances the uptake of K into the cell via the Na-K-ATPase pump. The K efflux pathway is not likely a channel because in preliminary studies, we showed that the intracellular K concentration is near or below electrochemical equilibrium across the basolateral membrane of the DCT cell (24). Thus, even though the basolateral membrane possesses a dominant K conductance, K exit via this channel likely does not occur because of a low or adverse electrochemical driving force. We proposed that an alternate possibility for K exit is an electroneutral K-Cl cotransporter. The results of the present study show that KCC4 is expressed at the basolateral membrane of the rabbit DCT and may be involved in mediating electroneutral efflux of K from the DCT cell. Further studies will be necessary to determine whether KCC4 plays a role in NaCl absorption or in K secretion by the DCT.

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