Cloning and Functional Expression of Rat CLC-5, a Chloride Channel Related to Kidney Disease*

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We have cloned a novel member of the CLC chloride channel family from rat brain, rCLC-5. The cDNA predicts a 83-kDa protein belonging to the branch including CLC-3 and CLC-4, with which it shares ~80% identity. Expression of rCLC-5 in *Xenopus* oocytes elicits novel anion currents. They are strongly outwardly rectifying and have a conductivity sequence of $NO_3^- > Cl^- > Br^- > I^- \gg glutamate^-$. Although CLC-5 has consensus sites for phosphorylation by protein kinase A, raising the intracellular cAMP concentration had no effect on these currents. Currents were also unchanged when rCLC-5 was coexpressed with rCLC-3 and rCLC-4, either singly or in combination. rCLC-5 is expressed predominantly in kidney and also in brain, lung, and liver. Along the nephron, rCLC-5 message is detectable in all tubule segments investigated, but expression in the glomerulus and the S2 segment of the proximal tubule is low.

CLC[1] chloride channels form an expanding gene family with members identified in bacteria (1), yeast (2), fish (3), and mammals. The expression cloning of the first CLC chloride channel, CLC-0, from the marine ray *Torpedo marmorata* (3) led to the discovery so far of seven mammalian *CLC* genes in a single mammalian species (4–10). The gene family has several branches that, in some cases, show no more than 25% identity at the amino acid level (11). Expression patterns range from completely ubiquitous to highly tissue-specific. CLC-1 provides the major chloride conductance of skeletal muscle (4), where it is necessary for the stabilization of the plasma membrane potential. CLC-2 mRNA has been found in any tissue examined (5), and functional expression in *Xenopus* oocytes suggests that it may play a role in cell volume regulation (12). CLC-3 and CLC-4 are expressed in several tissues (8, 9, 11, 13), whereas CLC-K1 and CLC-K2 are highly kidney-specific (6, 7). Functional expression of CLC-K channels and rCLC-3 is controver-

sial (8, 11, 13). Thus, their respective physiological roles await understanding.

Recently, analysis of a pedigree with Dent's disease, an X-linked hereditary nephrolithiasis, led to the isolation of a partial cDNA sequence of a novel *CLC* gene, *CLC-5* (10). It was identified as a kidney-specific transcript encoded in a region on the X chromosome, which was at least partially deleted in affected members of that pedigree. With mutations in human *CLC-1* causing dominant and recessive forms of myotonia (14, 15), this is the second member of the *CLC* gene family known to be affected in human hereditary disease.

By homology to other CLC proteins, we have independently cloned full-length CLC-5 from rat brain and have determined its expression pattern. Since it is predominantly expressed in kidney and since human CLC-5 may be defective in a hereditary kidney disease, special emphasis was laid on its intrarenal distribution. Furthermore, we demonstrate that rCLC-5 elicits novel chloride currents when expressed in *Xenopus* oocytes, strongly suggesting that it encodes a chloride channel.

MATERIALS AND METHODS

Cloning of rCLC-5 cDNA—A first 200-bp fragment of rCLC-5 was obtained using an anchor RT-PCR approach. cDNA was reverse-transcribed from rat brain polyadenylated RNA using a bipartite synthetic primer; one-half of the primer provided the anchor for subsequent PCRs, and the other half was a random heptamer to prime cDNA synthesis. PCR was performed with the anchor primer and two nested oligonucleotides directed against conserved regions of CLC proteins. The successful pair of overlapping nested degenerate primers were directed against the highly conserved amino acid sequence GKEG-PLVH after domain D3 and allowed for all possible codons. They incorporated 5′-*Eco*RI sites for subcloning. PCR was performed on ~100 ng of rat brain cDNA using 40 cycles for 30 s at 94 °C, 30 s at 53 °C, and 45 s at 72 °C. Amplification products were cloned into pBluescript KS⁺ and sequenced. From 29 clones analyzed, one was CLC-2, one was CLC-4, and one was a novel homologue closely related to CLC-3 and CLC-4 and subsequently named CLC-5. The 200-bp PCR fragment was used to screen a rat brain cDNA library in λZAP (a gift from Wolfgang Meyer-hof) under high stringency conditions. From 31 clones isolated, most encoded rCLC-3 and rCLC-4, and only one clone (found five times) coded for rCLC-5. This 3-kilobase clone lacked 648 bp of coding region at the 5′-end. This region was obtained using 5′-RACE PCR with rat brain 5′-RACE-Ready cDNA (CLONTECH). A full-length cDNA clone was assembled from two RACE fragments and the cDNA clone; a recombinant PCR fragment was ligated to the *Bsm*I site of the cDNA clone, and the whole construct was cloned into the *Nco*I and *Kpn*I sites of the expression vector pTLN.[2] The cDNA was fully sequenced on both strands using the chain termination method.

Northern Blots—Polyadenylated RNA was prepared from 12 different cell lines, and 5 μg/cell line was resolved by denaturing formaldehyde electrophoresis and blotted on a nylon membrane. This blot, a rat multiple-tissue Northern blot with 2 μg of poly(A)⁺ RNA/tissue (CLONTECH), and a mouse developmental Northern blot (CLONTECH) were

[1] The abbreviations used are: CLC, chloride channel of the *CLC* gene family; rCLC-*X*, member *X* of the *Clc* gene family from rat; bp, base pair(s); RT-PCR, reverse transcription-polymerase chain reaction; RACE, rapid amplification of cDNA ends; CFTR, cystic fibrosis transmembrane conductance regulator; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid.

[2] C. Lorenz, M. Pusch, and T. J. Jentsch, manuscript in preparation.

hybridized under high stringency conditions with a ^{32}P-labeled cDNA insert encompassing the entire open reading frame of rCLC-5 using standard conditions. Autoradiography was performed with either a PhosphorImager (Fuji BAS1500) or conventional films.

RT-PCR for rCLC-5 Intrarenal Distribution—Total RNA was prepared from rat kidney cortex and medulla and from microdissected segments. One-month-old rats fed a standard diet were anesthetized with Nembutal, the abdominal cavity was opened, and the kidneys were removed. Glomeruli and tubule segments were microdissected as described previously (7, 16). Briefly, thin pyramid sections from the renal cortex and medulla were incubated for 1 h at 37 °C in sterile phosphate-buffered saline supplemented with 1 mM $CaCl_2$ and 0.1% (w/v) collagenase (Boehringer Mannheim). Slices were then rinsed in fresh phosphate-buffered saline devoid of collagenase. Glomeruli, S2 and S3 segments from the proximal tubules, thin descending limbs of Henle, medullary and cortical ascending limbs of Henle, distal convoluted tubules, and cortical and medullary collecting tubules were isolated using fine sterile needles under a stereomicroscope at room temperature. Pools of five glomeruli and tubule segments (0.5–2 mm) were then rinsed in sterile phosphate-buffered saline, and samples (in 20 μl) were transferred to Eppendorf tubes and stored at -80 °C before use. Total RNA was extracted from tissues and isolated tubular segments as described and treated with RNase-free DNase I (Boehringer Mannheim) at 37 °C for 30 min. For tissue samples, the RNA concentration was evaluated by spectrophotometry. The integrity of RNA preparations stained with ethidium bromide was analyzed by gel electrophoresis on agarose-formaldehyde minigels. The RNAs were reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL, Eragny, France) at 42 °C for 45 min, and cDNAs were amplified in a 100-μl total volume containing 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 40 μM dNTP, 4.5 mM $MgCl_2$, 1 μCi of [^{32}P]dCTP, 1 unit of *Taq* polymerase, 37 pmol of rCLC-5 primers, and 0.4 pmol of β-actin primers, used as internal standard. The two rCLC-5 primers were 5′-CTGTGCCACTGCTTCAAC and 5′-AGTGTTGAAGTGGTTCTC. The β-actin primers (5′-CGTGGGCCGCCCTAGGCACCA and 5′-GGGGGGACTTGGGATTCCGGTT) were the same as described previously (17). Amplification reactions were subjected to 27–35 cycles of the following thermal program: 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s. Amplification products were run on a 4% polyacrylamide gel and autoradiographed. The correct size of the amplified products was controlled by digestion with *Nco*I. This restriction site is not present in the closely related rCLC-3 and rCLC-4 sequences. As expected, the 556-bp PCR product was cut into two fragments of 292 and 264 bp.

Functional Expression in Xenopus Oocytes—Using PCR-based mutagenesis, a *Nco*I site was introduced at the initiator ATG codon in rCLC-5. The cDNA was then cloned into the *Nco*I site of the expression vector pTLN,[2] which places the start site for translation immediately downstream of the *Xenopus* β-globin 5′-untranslated region (18). Human CFTR (a gift of Jack Riordan) was cloned into the same vector. Using Sp6 RNA polymerase, capped cRNA was prepared from this construct after linearization. 10–25 ng of cRNA was injected into *Xenopus* oocytes prepared and handled as described (3). Oocytes were kept in modified Barth's solution (88 mM NaCl, 1.0 mM KCl, 1.0 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$, 10 mM Hepes, pH 7.6). After 2–5 days at 18 °C, they were investigated by two-electrode voltage clamping using a Dagan amplifier and pCLAMP software (Axon Instruments, Inc.). Recordings were performed in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.4). In ion substitution experiments, 80 mM chloride was replaced by equal concentrations of bromide, glutamate, nitrate, or iodide.

RESULTS

Analysis of clones obtained from a homology screen in rat brain for new CLC family members revealed a 200-bp fragment very homologous but not identical to rCLC-4. When this fragment was used to screen a rat brain library under high stringency conditions, many clones encoding rCLC-3 and rCLC-4 were found in addition to one clone encoding a novel member of the CLC-3/4 subfamily, rCLC-5. While this work was in progress, a partial sequence of human CLC-5 was published, which was identified as a strong candidate gene for Dent's disease, an X-linked hereditary nephrolithiasis (10). It is 98% identical at the amino acid level in the overlapping region. The coding region of the new rat *Clc* gene was extended using 5′-RACE PCR. The initiator codon was assigned to the first

ATG codon in frame. It is preceded by in-frame stop codons. The open reading frame of 2238 bp predicts a protein of 746 amino acids with a molecular mass of 83 kDa. Homology to rCLC-3 and rCLC-4 is evenly distributed over the protein (Fig. 1). Compared with rCLC-5 and rCLC-4, rCLC-3 has 13 additional amino-terminal amino acids. Hydropathy analysis of rCLC-5 gives a pattern similar to that of most other CLC proteins. The current topology model for CLC proteins places N and C termini intracellularly and assumes that the poorly conserved domain D4 does not cross the membrane (19). The weakly hydrophobic region D13 is conserved between different CLC proteins and was suggested to be intracellular (12). The amino acid sequence of rCLC-5 contains two potential *N*-glycosylation sites, one before D1 and the other one between D8 and D9. While the former site is predicted to be cytoplasmic and therefore cannot be used, the latter site is highly conserved within the *CLC* gene family and was shown to be used *in vitro* (7). Biochemical analysis of native CLC-0 arrived at the same conclusion (20). The intracellular loop between D7 and D8 contains two overlapping consensus sites for cAMP-dependent phosphorylation (Fig. 1). Several possible sites of protein kinase C action are found throughout the sequence: before D1, in D6, and in the loop between D8 and D9 as well as in D12 and three sites between D12 and D13. Only these latter three sites and the one before D1 could be expected to be used according to the topology model, and only one of them is conserved between CLC-3, CLC-4, and CLC-5.

Tissue distribution of rCLC-5 was determined by Northern analysis. On two blots, containing polyadenylated RNA from eight different rat tissues or from 12 cell lines of diverse origin, respectively, the rCLC-5 probe recognized a $\approx$9.5-kilobase message (Fig. 2, *A* and *B*). Sizes of the highly related rCLC-3 and rCLC-4 messages are 3.0 and 5.5 kilobases (8) and 5 and 7.5 kilobases (11), respectively. Hence, hybridization conditions were specific for rCLC-5. When compared with signals obtained with a rCLC-4 probe on the same blot (data not shown), expression of rCLC-5 seems to be low. rCLC-5 is most highly expressed in kidney, but mRNA is also detectable in brain and liver and, to a lesser extent, in lung and testis (Fig. 2*A*). Among the cell lines investigated, strong CLC-5 expression was observed for Neuro-2a (mouse neuroblastoma), PC-12 (rat adrenal pheochromocytoma), LLC-PK1 (pig kidney), HIT-T15 (Syrian hamster pancreatic beta-cell), and 3T3 (mouse fibroblast) cell lines (Fig. 2*B*). When normalized to the control hybridization signal obtained with β-actin, expression of CLC-5 is especially high in the mTAL cell line (mouse thick ascending limb of the loop of Henle) (21). CLC-5 expression was also detected in other kidney cell lines (M1 (mouse cortical collecting tubule) (22), Madin-Darby canine kidney, and HEK293 (human embryonic kidney)) as well as in other epithelial cells (T84 (human colon carcinoma) and human cystic fibrosis pancreatic adenocarcinoma). Only Chinese hamster ovary cells lacked CLC-5 expression at this level of sensitivity. A developmental Northern with poly(A)$^+$ RNA from total mouse embryos from days 7 to 17 showed a marked increase in CLC-5 message from days 7 to 11 (Fig. 2*C*).

Since CLC-5 is predominantly expressed in kidney and as inactivation of its gene probably leads to hereditary hypercalciuric nephrolithiasis (10), we were interested in its expression along the rat nephron. A rough picture was obtained by RT-PCR with CLC-5-specific primers performed on microdissected tubules (Fig. 3). The mRNA seems to be almost absent from glomeruli and S2 segments of the proximal tubule (Fig. 3*C*), but expression in all other segments investigated is significant. Cortical collecting tubules, the S3 segment of the proximal

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                                                                          D1
RCLC-5 ..........  ...MDFLEEP IPGVGTYDDF NTIDWVREKS RDRDRHREIT NKSKESTWAL IHSVSDAFSG WLLMLLIGLL SGSLAGLIDI SAHWMTDLKE  87
RCLC-3 MTNGGSINSS THLLDLLDEP IPGVGTYDDF HTIDWVREKC KDRERHRRIN SKKKESAWEM TKSLYDAWSG WLVVTLTGLA SGALAGLIDI AADWMTDLKE 100
RCLC-4 ..........  ...MDFLDEP FPDVGTYEDF HTIDWLREKS RDTDRHRKIT SKSKESIWEF IKSLLDAWSG WVVMLLIGLL AGTLAGVIDL AVDWMTDLKE  87
       ----------  ----D-L-EP -P-VGTY-DF -TIDW-REK- -D--RHR-I- -K-KES-W-- --S--DA-SG W----L-GL- -G-LAG-ID- ---WMTDLKE

                                                                          D2
RCLC-5 GICTGGFWFN HEHCCWNSEH VTFEDRDKCP EWNSWSQLII STDQGAFAYI VNYFMYVLWA LLFFAFLAVSL VKAFAPYACG SGIPEIKTIL SGFIIRGYLG 187
RCLC-3 GICLSALWYN HEQCCWSGNE TTFEERDKCP QWKTWAELII GQAEGPGSYI MNYIMYIFWA LSFAFLAVSL VKVFAPYACG SGIPEIKTIL SGFIIRGYLG 200
RCLC-4 GVCLSAFWYS HEQCCWTSNE TTFEDRDKCP LWQKWSELLL SQSEGASAYI LNYLMYILWA LLFFAFLAVSL V--FAPYACG SGIPEIKTIL SGFIIRGYLG 187
       G-C----W-- HE-CCW-S-- -TFE-RDKCP -W--W---L-- ----G---YI -NY-MY--WA L-FAFLAVSL V--FAPYACG SGIPEIKTIL SGFIIRGYLG

          D3                            (D4)                                              D5
RCLC-5 KWTLVIKTIT LVLAVSSGLS LGKEGPLVHV ACCCGNILCH CFNKYRKNEA KRREVLSAAA AAGVSVAFGA PIGGVLFSLE EVSYYFPLKT LWRSFFAALV 287
RCLC-3 KWTLMIKTIT LVLAVASGLS LGKEGPLVHV ACCCGNIFSY LFPKYSTNEA KKREVLSAAA AAGVSVAFGA PIGGVLFSLE EVSYYFPLKT LWRSFFAALV 300
RCLC-4 KWTLLIKTVT LVLVVSSGLS LGKEGPLVHV ACCCGNFFSS LFSKYSKNEG KRREVLSAAA AAGVSVAFGA PIGGVLFSLE EVSYYFPLKT LWRSFFAALV 287
       KWTL-IKT-T LVL-V-SGLS LGKEGPLVHV ACCCGN---- -F-KY--NE- K-REVLSAA- AAGVSVAFGA PIGGVLFSLE EVSYYFPLKT LWRSFFAALV

                                                                           D7                                   D8
RCLC-5 AAFTLRSINP FGNSRLVLFY VEFHTPWHLF ELVPFIVLGI FGGLWGALFI RTNIAWCRKR KTTQLGKYPV VEVLIVTAIT AILAFPNEYT RMSTSELISE 387
RCLC-3 AAFVLRSINP FGNSRLVLFY VEYHTPWYLF ELFPFILLGV FGGLWGAFFI RANIAWCRRR LEVIIVAAIT AVIAPNPYT RLNTSELIKE 400
RCLC-4 AAFTLRSINP FGNSRLVLFY VEYHTPWYMA ELFPFILLGV FGGLWGTVFT RCNIAWCRRR KTTRLGKYPV LEVIVVTAIT AIIAYPNPYT RQSTSELISE 387
       AAF-LRSINP FGNSRLVLFY VE-HTPW--- EL-PFI-LG- FGGLWG--F- R-NIAWCR-R K-T--GKYPV -EV--V-AIT A--A-PN-YT R--TSELI-E

                                  *                                              D9/D10
RCLC-5 LFNDCGLLDS SKLCDYENHF NTSK.GGELP DRPAGVGVYS AMWQLALTLI LKIVITIFTF GMKIPSGLFI PSMAVGAIAG RLLGVGMEQL AYYHHDWGIF 486
RCLC-3 LFTDCGPLES SSLCDYRNDM NASKIVDDIP DRPAGVGVYS AIWQLCLALI FKIIMTVFTF GIKVPSGLFI PSMAIGAIAG RIVGIAVEQL AYYHHDWFIF 500
RCLC-4 LFNDCGALES SQLCDYINDP NMTRPVDDIP DRPAGVGVYT AMWQLALALI FKIVITIFTF GMKIPSGLFI PSMAVGAMAG RMVGIGVEQL AYYHHHDWIIF 487
       LF-DCG-L-S S-LCDY-N-- N--------P DRPAGVGVY- A-WQL-L-LI -KI--T-FTF G-K-PSGLFI PSMA-GA-AG R--G---EQL AY-HHDW-IF

                                                         D11/D12
RCLC-5 NSWCSQGADC ITPGLYAMVG AAACLGGVTR MTVSLVVIMF ELTGGLEYIV PLMAAAMTSK WVADALGREG IYDAHIRLNG YPFLEAKEEF AHKTLAMDVM 586
RCLC-3 KEWCEVGADC ITPGLYAMVG AAACLGGVTR MTVSLVVIVF ELTGGLEYIV PLMAAVMTSK WVGDAFGREG IYEAHIRLNG YPFLDAKEEF THTTLAADVM 600
RCLC-4 RNWCRPGADC VTPGLYAMVG AAACLGGVTR MTVSLVVIMF ELTGGLEYIV PLMAAAVTSK WVADAFGKEG IYEAHIHLNG YPFLDVKDEF THRTLATDVM 587
       --WC--GADC -TPGLYAMVG AAACLGGVTR MTVSLVVI-F ELTGGLEYIV PLMAA--TSK WV-DA-G-EG IY-AHI-LNG YPFL--K-EF -H-TLA-DVM

RCLC-5 KPRRNDPLLT VLTQDSMTVE DVETIISETT YSGFPVVVSR ESQRLVGFVL RRDLIISIEN ARKKQDGVVS TSIIYFTEHS PPMPPYTPPT LKLRNILDLS 686
RCLC-3 RPRRSDPPLA VLTQDNMTVD DIENMINETS YNGFPVIMSK ESQRLVGFAL RRDLTIAIES ARKKQEGVVG SSRVCFAQHT PSLPAESPRP LKLRSILDMS 700
RCLC-4 RPRRGEPPLS VLTQDSMTVE DVETLIKETD YNGFPVVVSR DSERLIGFAQ RRELILAIKN ARQRQEGIVS NSIMYFTEEP PELPANSPHP LKLRRILNLS 687
       -PRR--P-L- VLTQD-MTV- D-E--I-ET- Y-GFPV--S- -S-RL-GF-- RR-L---I-- AR--Q-G-V- -S---F---- P--P---P-- LKLR-IL--S

           (D13)
RCLC-5 PFTVTDLTPM EIVVDIFRKL GLRQCLVTHN GRLLGIITKK DVLKHIAQMA NQDPESILFN Stop   746
RCLC-3 PFTVTDHTPM EIVVDVFRKL GLRQCLVTHN GRLLGIITKK DILRHMAQTA NQDPASIMFN Stop   760
RCLC-4 PFTVTDHTPM ETVVDIFRKL GLRQCLVTRS GRLLGIITKK DVLRHMAQMA NQDPESIIFN Stop   747
       PFTVTD-TPM E-VVD-FRKL GLRQCLVT-- GRLLGIITKK D-L-H-AQ-A NQDP-SI-FN
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FIG. 1. **Comparison of the rCLC-5 amino acid sequence with those of the closely related rCLC-3 and rCLC-4.** The consensus sequence below gives identical amino acid residues for the three proteins. Conserved domains of CLC channels are indicated above the sequences (D1–D13). Except for D4 and D13, these domains are proposed to be transmembrane domains. *Closed inverted triangles* mark potential protein kinase A consensus sites conserved within the three protein sequences. A conserved *N*-linked glycosylation site is indicated (*).

tubule, and the medullary thick ascending limb gave strong signals.

The possible function of rCLC-5 as a chloride channel was addressed by electrophysiological analysis of *Xenopus* oocytes injected with rCLC-5 cRNA transcribed *in vitro*. Two days after injection, a strongly outwardly rectifying chloride current was observed reproducibly (Fig. 4*A*). These currents could reach up to 18 μA 3 days after injection and were >10 times larger than currents in uninjected control oocytes (Fig. 4*B*, *inset*). Within the time resolution of these measurements, hardly any gating relaxations could be observed for CLC-5 (Fig. 4*A*, *inset*). An activating component at the beginning of a pulse to positive test potentials was seen, but deactivation seemed almost instantaneous, with the limit of time resolution being ~1 ms (step to −90 mV; data not shown). Partial replacement of extracellular chloride by other anions revealed a conductivity sequence of $NO_3^- > Cl^- > Br^- > I^- \gg$ glutamate$^-$ (Fig. 4*B*). At the most positive potential tested (+100 mV), the current observed in 80 mM NO_3^- was ~1.4 times the size of the current seen in chloride. Total replacement of external chloride by glutamate indicates that this organic anion is impermeable (data not shown). The conductivity sequence of CLC-5-associated currents differs from the much smaller endogenous oocyte current of control oocytes (Fig. 4*B*, *inset*). Most notably, the endogenous current has an $I^- > Cl^-$ selectivity. We tested several known inhibitors on the currents elicited by rCLC-5 (DIDS (1 mM), anthracene-9-carboxylic acid (1 mM), 5-nitro-2-(3-phenylpropylamino)benzoic acid (0.5 mM), diphenylamine-2-carboxylic acid (1 mM), and niflumic acid (1 mM)). None of these had significant effects.

Physiological studies have revealed the presence of a cAMP-activated chloride channel in the thick ascending limb of Henle's loop (23, 24). Since CLC-5 has consensus sites for cAMP-dependent phosphorylation at a presumably intracellular position, we tested the effect of raising intracellular cAMP concentrations. As a positive control, we expressed CFTR, a cAMP-activated chloride channel, in oocytes of the same batch. A mixture containing 200 μM chlorophenylthio-cAMP, 12 μM forskolin, and 500 μM 3-isobutyl-1-methylxanthine elicited currents up to 40 μA in CFTR-injected oocytes. However, no change in currents was observed with rCLC-5-injected oocytes under the same conditions.

Since CLC-1 functions as a homomultimer (most likely as a tetramer) (15, 25), we investigated the possible formation of hetero-oligomers between rCLC-5 and the highly related rCLC-3 and rCLC-4 gene products by coexpressing all three cRNAs in *Xenopus* oocytes. However, currents observed in these experiments were indistinguishable from those seen in oocytes injected with rCLC-5 cRNA alone.

DISCUSSION

Using homology screening, we have identified a novel rat *Clc* gene (*rClc-5*) that gives rise to outwardly rectifying anion currents when expressed in *Xenopus* oocytes. It shows high amino acid identity to the previously cloned rCLC-3 (8) and rCLC-4 (11) gene products. Sequence comparison with a partial human cDNA reveals that it is the species homologue of a human candidate gene for Dent's disease (10). These three members of the *CLC* gene family form a distinct subbranch with ~80% amino acid identity between them, but only ~30% amino acid identity to other members of the gene family.
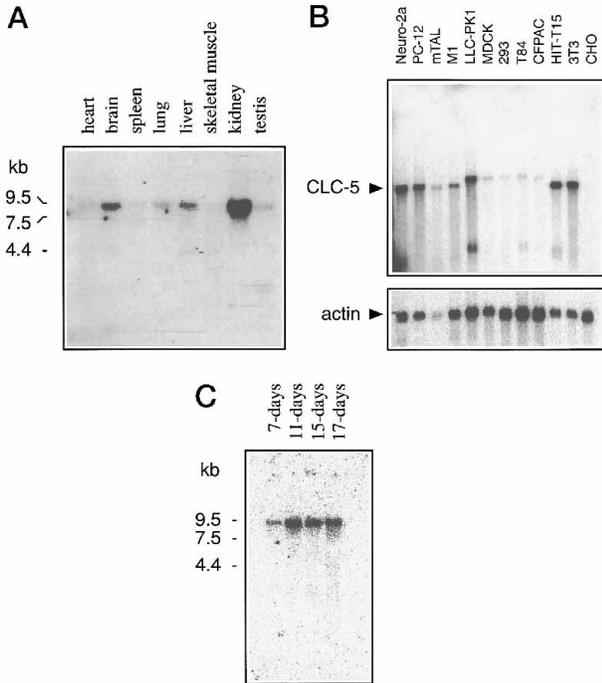
In contrast to rCLC-3 and rCLC-4, which are strongly ex-

FIG. 2. **Tissue distribution and developmental induction of chloride channel CLC-5.** Northern blots containing poly(A)$^+$ RNA from different rat tissues (*A*), from different cell lines of diverse origin (*B*), and from mouse embryos at different developmental stages (*C*) were probed with a CLC-5-specific cDNA. Lanes in *A* and *C* contained 2 μg of poly(A)$^+$ RNA, and lanes in *B* contained 5 μg. The *lower panel* of *B* shows hybridization with a human β-actin cDNA of the same blot to control for equal loading and absence of degradation. *kb*, kilobases; *MDCK*, Madin-Darby canine kidney; *CFPAC*, cystic fibrosis pancreatic adenocarcinoma; *CHO*, Chinese hamster ovary.
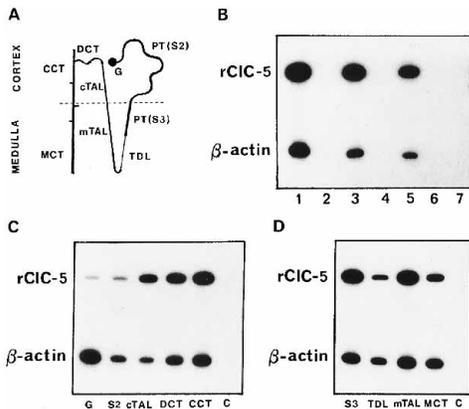

FIG. 3. **RT-PCR analysis of the intrarenal distribution of the rCLC-5 message.** *A*, schematic organization of the nephron. *G*, glomerulus; *PT*, proximal tubule (S2 and S3 segments); *TDL*, thin descending limbs of Henle; *mTAL* and *cTAL*, medullary and cortical ascending limbs of Henle, respectively; *DCT*, distal convoluted tubule; *CCT* and *MCT*, cortical and medullary collecting tubules, respectively. The *dashed line* represents the border between the cortex and medulla. *B*, cDNA and nonreverse-transcribed RNA extracted from whole kidney (*lanes 1* and *2*, respectively), kidney cortex (*lanes 3* and *4*, respectively), and kidney medulla (*lanes 5* and *6*, respectively) were submitted to RT-PCR. Each sample was amplified (30 cycles) with two sets of primers specific for rCLC-5 and β-actin, used as internal standard. *C* and *D*, cortical and outer medullary distribution of rCLC-5, respectively. The larger product (556 bp) represents rCLC-5, while the shorter fragment (250 bp) represents β-actin, which was amplified as a control. No amplified products were detected when cDNA was omitted as a negative control (*B, lane 7*; and *C* and *D, lane C*).

pressed in several tissues like brain, lung, liver, and kidney, rCLC-5 is predominantly expressed in one tissue, namely kidney. However, messages are also present in brain, liver, lung,
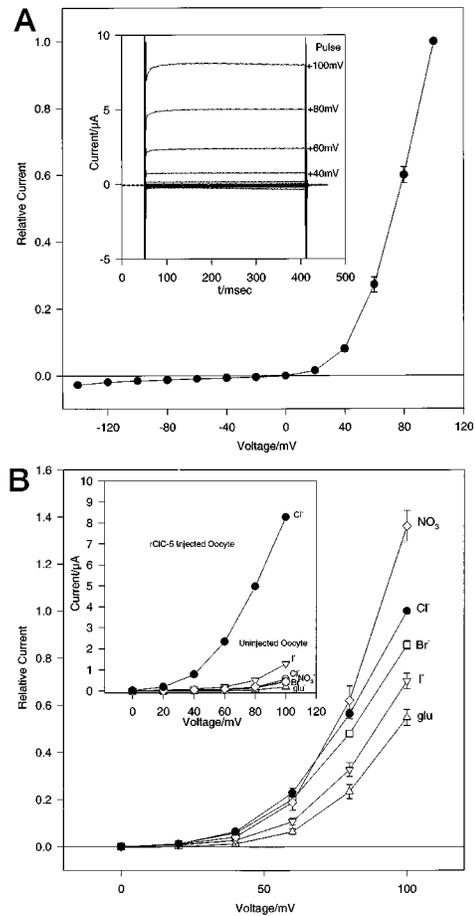

FIG. 4. **Functional expression of rCLC-5 in *Xenopus* oocytes.** *A*, I–V relationship of CLC-5. Oocytes were subjected to a pulse protocol that stepped the membrane potential from holding potential to test pulses from +100 to −140mV and back to holding potential. Currents at given test potentials were normalized to current at +100 mV for individual oocytes and averaged for five oocytes from three different batches (*bars* indicate standard errors). The *inset* shows one of the original traces. All measurements were performed in ND96 buffer. *B*, conductivity of rCLC-5 in the presence of different anions. Oocytes were subjected to the same pulse protocol as described for *A*. Chloride (80 mM) was replaced with a 80 mM concentration of the indicated anion ($\bullet$, Cl$^-$; $\square$, Br$^-$; $\triangle$, glutamate$^-$; ∇, I$^-$; $\diamond$, NO$_3^-$). Currents at given test potentials and ion concentrations were normalized to the current at +100 mV in ND96 buffer for individual oocytes. Data were averaged for five oocytes from three different batches (*bars* indicate standard errors). The *inset* shows absolute I–V relationships for an rCLC-5-injected oocyte in ND96 buffer and an uninjected oocyte in the different anion replacement medium.

and testis. This is in contrast to human CLC-5, which is almost exclusively expressed in kidney (10). Species-dependent differences in tissue distribution have also been observed with CLC-3 and CLC-4 (8, 9, 11, 13). Species differences may also explain in part the differences in CLC-5 expression in different cell lines (Fig. 2*B*).

Since inactivation of the *CLC-5* gene probably leads to nephrolithiasis and proteinuria in humans, the determination of the intrarenal expression pattern is important for unraveling the mechanism by which CLC-5 inactivation may lead to hypercalciuria and proteinuria. In the absence of good CLC-5 antibodies, we examined rCLC-5 mRNA levels in microdissected rat kidney tubules by RT-PCR. This revealed a rather broad intrarenal expression pattern. Glomeruli and S2 segments of proximal tubules being the only exception, substantial expression of rCLC-5 was observed throughout the nephron segments examined. Strong expression was observed in the cortical collecting tubule, the S3 segment of the proximal tubule, and the thick

ascending limb. As the expression pattern of rCLC-3 and rCLC-4 along the nephron is unknown, it is an open question whether members of this subfamily are coexpressed in any nephron segment. *In situ* hybridization data for the developing mouse show that murine CLC-3 appears in the cortical region of the mesonephros around day 14 (13). Here we have shown that rCLC-5 is present in whole mouse embryos as early as day 7. Thus, both genes may be coexpressed in embryonic kidney.

Given the high structural similarity between rCLC-3, rCLC-4, and rCLC-5, we were surprised that only rCLC-5 cRNA induced chloride currents in *Xenopus* oocytes. Both results, the absence of significant novel currents with rCLC-3- or rCLC-4-injected oocytes as well as the appearance of strongly rectifying chloride currents with rCLC-5 expression, were highly reproducible. We tested at least 20 oocyte batches for rCLC-3 and rCLC-4 and almost 50 batches for rCLC-5. Our negative results with rCLC-3 are in contrast to the work of Kawasaki *et al.* (8). These authors reported the appearance of slightly outwardly rectifying, rather small (≈ 1 μA) chloride currents with rCLC-3 expression in *Xenopus* oocytes (compare Refs. 8 and 13).

The strong outward rectification of CLC-5 currents could reflect open channel rectification or the voltage dependence of a gate with fast kinetics. At present, we cannot distinguish between these possibilities. The conductivity sequence of rCLC-5 currents was typical for CLC channels (Cl$^-$ > Br$^-$ > I$^-$). Glutamate seems to be impermeable. In contrast to CLC-0, however, medium containing 80 mM nitrate gave current amplitudes $\sim$1.4 times larger (at +100 mV) than those with chloride saline. For CLC-0, where nitrate conducts less well than chloride, an anomalous mole fraction behavior of Cl$^-$/NO$_3^-$ mixtures was found for both conductance and gating (19). No such effect was observed with rCLC-5.

We cannot strictly exclude that the observed currents are endogenous oocyte currents that are activated by rCLC-5 expression. Such problems have been described with other proteins initially thought to be chloride channels (26, 27). However, we favor the role of CLC-5 as a chloride channel. First, other members of this gene family (CLC-0, CLC-1, and CLC-2) unambiguously qualify as chloride channels (26). Second, the Cl$^-$ > Br$^-$ > I$^-$ selectivity of the rCLC-5-induced currents is typical for CLC channels, but differs markedly from all known endogenous oocyte chloride currents, which have an I$^-$ > Cl$^-$ selectivity. Most of these endogenous currents also differ markedly in their rectification. Final proof that CLC-5 is an anion channel can only be obtained by site-directed mutagenesis or by reconstitution of the purified protein.

Several investigators have described basolateral chloride currents in the thick ascending limb of Henle's loop (23, 24, 28). The underlying chloride channel was reported to have a linear I–V relationship and a selectivity sequence of Cl$^-$ > Br$^-$ > NO$_3^-$ > I$^-$ and was found to be blocked by 10 μM 5-nitro-2-(3-phenylpropylamino)benzoic acid. Some authors report that it is activated by cAMP. Each of these details argues against a molecular identity to rCLC-5.

In *Xenopus* oocytes, rCLC-5 elicited chloride currents only at voltages more positive than about +20 mV. Most cells will never reach such inside positive plasma membrane voltages. However, for the heavily transporting epithelium from the urinary bladder of *Necturus*, inside positive potentials of up to +60 mV have been reported (29). We are not aware of kidney epithelia having such positive potentials *in vivo*. On the other hand, we may have missed another subunit (homologous or not) that may interact with CLC-5. The resulting hypothetical heteromultimer could have a different voltage dependence.

One obvious hypothesis is that rCLC-3, rCLC-4, and rCLC-5

could be subunits of a heteromeric channel. This is, for example, the case with cyclic nucleotide-gated channels, where some homologous subunits contribute to heteromultimers, but do not yield currents when expressed alone (30–32). The strongly overlapping yet distinct expression patterns of these genes make it unlikely that their products have to form a heterotrimeric complex in order to function properly. Nevertheless, coexpression in the same tissue, *e.g.* the presence of both rCLC-3 and rCLC-5 in kidney and of all three homologues in brain, renders interactions between these highly homologous proteins a real possibility. However, electrophysiological analysis of oocytes coexpressing these CLC proteins in various combinations did not reveal any signs of functional heteromultimers with novel properties.

We may not yet have identified the true physiological stimulus that activates native rCLC-5 chloride channels, *e.g.* a certain second messenger pathway. We could not establish any effect of cAMP on rCLC-5 currents, but many other effectors remain to be investigated. Another possibility is that some or all CLC proteins from this branch may be localized to intracellular compartments. In this scenario, rCLC-5 currents observed in oocytes could result from "spill-over expression," where vesicles normally targeted to an intracellular compartment reach the plasma membrane due to overexpression, or because control elements are lacking in the oocyte. The seemingly "unphysiological" voltage dependence of CLC-5 may make sense in an intracellular membrane having a different membrane voltage. The iron-sensitive *petite* phenotype of a yeast mutant lacking a CLC homologue (scCLC-a) (2) may suggest an intracellular localization. Indeed, a plausible hypothesis to explain this phenotype places scCLC-a into the vacuolar membrane, where it would facilitate the acidification of this organelle (which is important for iron storage) by vacuolar proton pumps. The CLC-3/4/5 branch is most closely related to scCLC-a among mammalian *CLC* genes.

Of the symptoms found in patients with Dent's disease, low molecular weight proteinuria is a constant feature and is also found in carrier females (33). Proteins passing the filter of the glomerulus are reabsorbed in the proximal tubule. They are taken up by endocytosis (34) and subsequently degraded in a lysosomal compartment. Acidification of the endosomes may be important for this uptake (35). Although rCLC-5 expression was low in the S2 segment, S3 segments of the proximal tubule significantly expressed this channel. In this scenario, CLC-5 could provide an endosomal chloride conductance necessary for efficient acidification of this compartment and hence for endocytotic protein uptake. Among others, this will be one hypothesis to be tested in further studies aimed at understanding CLC-5 function.

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