Two highly homologous members of the ClC chloride channel family in both rat and human kidney

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ABSTRACT We have cloned two closely related putative Cl⁻ channels from both rat kidney (designated rClC-K1 and rClC-K2) and human kidney (hClC-Ka and hClC-Kb) by sequence homology to the ClC family of voltage-gated Clchannels. While rClC-K1 is nearly identical to ClC-K1, a channel recently isolated by a similar strategy, rClC-K2 is 80% identical to rClC-K1 and is encoded by a different gene. hClC-Ka and hClC-Kb show ~90% identity, while being ≈80% identical to the rat proteins. All CIC-K gene products are expressed predominantly in the kidney. While rClC-K1 is expressed strongly in the cortical thick ascending limb and the distal convoluted tubule, with minor expression in the S3 segment of the proximal tubule and the cortical collecting tubule, rClC-K2 is expressed in all segments of the nephron examined, including the glomerulus. Since they are related more closely to each other than to the rat proteins, hClC-Ka and hClC-Kb cannot be regarded as strict homologs of rClC-K1 or rClC-K2. After injection of ClC-K cRNAs into oocytes, corresponding proteins were made and glycosylated, though no additional Cl⁻ currents were detectable. Glycosylation occurs between domains D8 and D9, leading to a revision of the transmembrane topology model for CIC channels.

The bulk of Cl⁻ filtered into the proximal tubule across the glomerular membrane must be reabsorbed subsequently along the nephron. Mechanisms such as $Na^+/K^+/2$ Cl⁻ cotransport, K⁺/Cl⁻ cotransport, various anion exchangers, and Cl⁻ channels (1) are important in this transpithelial transport as well as in cellular homeostatis.

Recently, our knowledge of Cl^- channels has been extended dramatically by molecular biological tools (2–5). At least two different structural classes of anion channels are found in the kidney: the cystic fibrosis transmembrane conductance regulator (CFTR; refs. 4 and 6) and several members of the ClC family of voltage-gated Cl⁻-channels (7–9).

We have used a combined reverse transcriptasepolymerase chain reaction (RT-PCR) and hybridization approach to clone members of the ClC Cl⁻-channel family from rat and human kidney (rClC-K and hClC-K, respectively).[§] rClC-K1 is nearly identical to the previously cloned ClC-K1 from rat kidney (9) and has limited intrarenal expression, while the closely related rClC-K2 is expressed in every nephron segment examined.

In humans there are two closely related ClC proteins that have 90% identity to each other (hClC-Ka and hClC-Kb). The high intraspecies identity precludes a conclusive correlation of rat channels with human counterparts. In our hands, these putative channels could not be expressed functionally, in contrast to reports for ClC-K1 (9). ClC-K proteins can be synthesized both in oocytes and *in vitro* and are glycosylated in the presence of pancreatic microsomes. Identification of the glycosylation sites leads to a revision of the working model for transmembrane topology of these channels (5, 10).

MATERIALS AND METHODS

Cloning of Renal Members of the CIC CI⁻ Channel Family. Degenerate oligonucleotides against segments conserved in ClC Cl⁻ channels were used as primers for PCR. Rat kidney poly(A)⁺ RNA was reverse-transcribed and used as a template for PCR using 60 cycles consisting of 92°C for 1 min. 45°C for 1.5 min, and 72°C for 2 min. The primers amplifying the rClC-K1 fragment had the following sequences: AA-GAATTCTCTGGAATCCCIGARMTGAA, where R = G or A and M = C or A, and AATCTAGASGTGASCTCIATGC-TGAA, where S = G or C. The cloned and sequenced PCR fragment then was used to screen cDNA libraries of rat kidney (in λ gt11, provided by Mike M. Mueckler, St. Louis, and in λ Uni-Zap, Stratagene no. 937503) and human kidney (in λ gt10, Clontech HL 1123a) under high stringency. Complete cDNAs were assembled from the resultant partial clones (rClC-K2, hClC-Ka, and hClC-Kb). Since no rClC-K1 clone could be obtained from two different cDNA libraries, we used RT-PCR with primers directed against the published sequence (9) to clone rClC-K1. Amino acids in rClC-K1 differing from the published ClC-K1 sequence (except for a stretch containing frameshift mistakes in ref. 9; see the legend to Fig. 1) were changed to construct a cDNA equivalent to CIC-K1.

Site-Directed Mutagenesis. Point mutations, as well as the *Torpedo 5'* untranslated sequence, were introduced to the CIC-K cDNAs by recombinant PCR (10) with Pfu DNA polymerase (Stratagene). Stretches amplified by PCR were sequenced fully.

RNA (Northern) Blots. A nylon membrane [described previously (7, 8)] blotted with 10 μ g of poly(A)⁺ RNA from different tissues was reprobed under high stringency with cDNA probes labeled with ³²P by random priming.

RT/PCR for CIC-K Distribution and Analysis. Total RNA was prepared (11, 12) from different tissues and microdissected renal segments. Five glomeruli, two to five segments (0.5–1.5 mm) from the mid-convoluted (S2) and straight (S3) portions of the proximal tubule, the cortical thick ascending limb (cTAL), the distal convoluted tubule (DCT), and the cortical collecting tubule (CCT) were isolated from adult rat kidney as described (13). The RNAs were reverse-transcribed (14) into cDNAs that were used as templates in PCR. PCR was performed in a final volume of 100 μ l of buffer (50 mM KCl/4.5 mM MgCl₂/0.1 mg of gelatin per ml/10 mM

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Abbreviations: rClC-K and hClC-K, Cl^- channels from rat and human kidney, respectively; RT-PCR, reverse transcriptase–PCR; DCT, distal convoluted tubule; CCT, cortical collecting tubule; cTAL, cortical thick ascending limb.

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[§]The sequences reported in this paper have been deposited in the GenBank data base (accession nos. Z30663, Z30643, and Z30644 for rCIC-K2, hCIC-Ka, and hCIC-Kb, respectively).

Tris·HCl, pH 8.3) containing 10% (vol/vol) dimethyl sulfoxide, 40 μ M dNTP, 1 μ Ci (37 kBq) of [α -³²P]dCTP, 1 unit of *Taq* polymerase, 100–200 ng of each oligonucleotide primer, and 50 ng of β -actin primers (internal control). We used the following primers: rClC-K1, sense AGACCATGCTGTCCG-GTGTGG and antisense GAAGGGGGCTGCGAAAACT; rClC-K2, AAACCATCTTGACAGGTGTGA and GATTGG-GGCGGCGAAGACC; and β -actin, CGTGGGCCGCCCTA-GGCACCA and TTGGCCTTAGGGTTCAGGGGGGG. Amplification reactions were subjected to 20–35 cycles of the following thermal cycling program: 94°C for 30 sec, 54°C for 30 sec, and 72°C for 1 min. Amplification products were run on a 4% polyacrylamide gel and autoradiographed.

Expression of kidney CIC Gene Products in Oocytes and *in Vitro.* Full-length cDNAs of all four CIC-Ks were assembled and cloned into pBluescript plasmid. To boost expression, we replaced their 5' untranslated sequence with that of CIC-0 (5), a previously successful strategy (7, 8). We also inserted rCIC-K2, hCIC-Ka, and hCIC-Kb cDNAs into the pSP64T vector containing the 5' β -globin untranslated sequence (15). Capped cRNA was transcribed *in vitro* and injected into *Xenopus* oocytes (5–50 ng per oocyte). After 1–5 days oocytes were analyzed using the two-electrode voltage clamp. cRNAs having the CIC-0 5' end were also translated *in vitro* by using rabbit reticulocyte lysate with or without dog pancreatic microsomes (Promega) in the presence of [³⁵S]methionine. Analysis was by SDS/PAGE and autoradiography, in some experiments preceded by immunoprecipitation.

Antibodies and Immunoprecipitation. A peptide representing the C terminus of rClC-K2 (Cys-Lys-Lys-Ala-Ile-Ser-Thr-Leu-Thr-Asn-Pro-Pro-Ala-Pro-Lys) was synthesized, coupled via its cysteine to keyhole limpet hemocyanin, and used to raise polyclonal antibodies in rabbits. rClC-K proteins were solubilized in buffer containing 0.5% Nonidet P-40 and immunoprecipitated by using this antiserum and protein A-sepharose and were analyzed by SDS/PAGE and autoradiography.

RESULTS

Cloning of Putative Rat and Human Kidney Chloride Channels. We used degenerate primers directed against conserved regions within the ClC family of voltage-gated Cl⁻ channels to amplify sequences from rat kidney mRNA by RT-PCR. A novel, homologous fragment was cloned and used to screen rat kidney cDNA libraries under high stringency. All cDNAs isolated were only 80% homologous to the PCR fragment used for screening, indicating that they originated from a different ClC gene. While this work was in progress, Uchida et al. (9) published the sequence of a rat kidney channel that they named ClC-K1. Our original PCR fragment is identical in sequence to a portion of this cDNA. We named the putative channel corresponding to our PCR fragment rClC-K1, and the clones isolated by library screening rClC-K2. Within the coding region, sequence identity is 85% at the nucleic acid level and 83% at the protein level. A full-length rClC-K1 cDNA was cloned by RT-PCR. After accounting for obvious mistakes in the published sequence (9) (Fig. 1), its sequence differs at nine amino acid positions from the published ClC-K1 sequence (see Fig. 1). At most, three of these represent PCR artifacts in our clone, since in the remaining cases they conform to other ClC-K sequences. Eleven independent clones isolated from a human kidney cDNA library also fell into two closely related classes. They are related more to each other (94% and 91% identity at the RNA and protein level, respectively) than to the rat genes. Since no unambiguous cross-species correlation is possible, we named them hClC-Ka and hClC-Kb.

Structural Features of CIC-K Proteins. Like other members of this gene family, CIC-K proteins also have 13 conserved

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hydrophobic domains (D1 through D13). ClC-K proteins are the smallest known members of the ClC family (687 amino acids, calculated molecular mass = 75 kDa). One distinctive feature of ClC-K channels is the insertion of two additional amino acids in putative transmembrane domain D3. There also are six more amino acids in the stretch linking D8 and D9, introducing another consensus site for N-linked glycosylation nine residues downstream of a highly conserved one. Further potential glycosylation sites are found between D4 and D5 for rClC-K2, hClC-Ka, hClC-Kb, and ClC-2. In all ClC-K proteins, further sites are present after D12. However, the current model for ClC channels (5, 10) localizes all these sites to the cytoplasm, suggesting that they may not be used.

Tissue Distribution of CIC-K Messages. Northern analysis of rat tissues demonstrates that CIC-K expression is highly specific for the kidney (Fig. 2A). Rabbit kidney showed expression both in the medulla and in cortex, while no signal was detected in the MDCK, LLC-PK1, BSC-1, COS, and HEK293 kidney cell lines (data not shown).

Because of their high degree of identity, Northern analysis is unsuitable for differentiation between ClC-K isoforms. We synthesized primers designed to amplify specifically rClC-K1 and rClC-K2 fragments. Both primer pairs amplified correctly sized products from rat kidney RNA (Fig. 2B). Analytical digests confirmed their identities. Amplification of β -actin was used as an internal control. Slight amounts of correctly sized amplification products also were obtained in brain (ClC-K1 and ClC-K2) and muscle (ClC-K2).

To assess intranephronal ClC-K distribution, we used RT-PCR on microdissected tubules (Fig. 3). ClC-K1 was found predominantly in cTAL and DCT, and to a lesser degree in the S3 segment of the proximal tubule and the CCT. ClC-K2 was expressed in all nephron segments investigated, which includes the S2 segment and the glomerulus.

Expression and Glycosylation of CIC-K Gene Products. Since we could not observe the expression of additional currents in oocytes injected with any of the native CIC-K cRNAs, we replaced their 5' untranslated region with that of CIC-0, a previously successful strategy (7, 8). Additionally, we inserted the cDNAs into the pSP64T vector, which uses β -globin 5' and 3' untranslated regions to stimulate expression in *Xenopus* oocytes (15). We also analyzed rCIC-K1 after mutating it to conform to the published sequence (9).

While we easily could detect Cl^- currents in oocytes expressing ClC-0 or ClC-1 cRNAs, we were unable to detect new Cl⁻ currents in the same batches of oocytes injected with ClC-K cRNAs. Since ClC channels are multimers (16) and rClC-2, rClC-K1, and rClC-K2 are coexpressed in some segments of the nephron, they may form functional heterooligomers. However, ClC-K cRNAs remained nonfunctional even when coexpressed in several different combinations.

Immunoprecipitation experiments of injected oocytes demonstrated the synthesis of correctly sized ClC-K proteins (Fig. 4A). When translated in vitro, all CIC-K proteins, as well as CIC-0, CIC-1, and CIC-2, were shifted to higher molecular weights when core glycosylation was made available by adding microsomes (Fig. 4 B and C). With rClC-K1, this shift appears larger than with rClC-K2. This challenges the working model of CIC channels (5, 10), which places all consensus sites for N-linked glycosylation into the cytoplasm. The first site between D8 and D9 is the only one conserved in all CIC members; CIC-K proteins have a second site nearby. Using site-directed mutagenesis, we eliminated both sites in rClC-K1 and rClC-K2 and reexamined glycosylation (Fig. 4C). In each case, no shift was observed upon the addition of microsomes, suggesting that this segment is extracellular.

1					_	D		_		
hClC-Ka	MEELVGLREG	FSGDPVTLQE	LWGPCPHIRR	A TOGGLEWILK	OVUPPI OPDW	VEL MEL CUT M	AT WOWN MOTOR		I VID D T OD OUT	
ncic-kb	MEEFVGLREG	SSGNPVTLOE	LWGPCPLIRR	GIRGGI EWI K	OKLERIGEDW	VPI MTT CVI M	ALVCCAMDUA	VIDONIND & HOLD	T VDBTODOW	1 0111 01000000
rClC-K2	MEEIVGLREG	SPRKPVPLOE	LWRPCPRIRR	NIQGSLEWLK	ERLFRVGEDW	YFLVALGVLM	ALISYAMNEA	IGRUVRAHKW	LVRPTODONL.	LEVICONVVD
C1C-K	MEE-VGLREG	SPRKPVPLOE	LW-PCP-IRR	LEWLK	FR-GEDW	-FLLGVLM	AL-S-AMA	VVRAH-W	LYRE-CD-HL	L.RVI.CWTVVD
rClC-2 -	-RLOGPEPWKG	SPSARATPEL	LEYGQSRCAR	CRICSVRCHK	FLVSRVGEDW	IFLVLLGLLM	ALVSWAMDYA	IAVCLOADOW	MSRGLATINTI.	LOVIAWUTVP
101	- D2			-		D3		D4 -		-
hClC-Ka	VALVSFSSGF	SOSITPSSOG	SGIPELKTML	AGVILEDYLD	IKNFGAKVVG	LSCTLATGST	LFLGKVGPFV	HLSVMIAAYL	GRVRTTTIGE	PENKSKONEM
rClC-K1	VALLSPSSUP	SUSISPESGA	SGLPELKINL	SGVVLEDVLD	T KNEGA KVVC	LCCTT ATCCT	TRICKNODER	117 MIRITON 199	CONTRACTOR & MARCHINE CONTRACTOR	COMPANY OF A DESCRIPTION OF A
CIC-K	VALUEF BAGE	SOSITPSSOG	SGIPBYNTIL	TGVILEDYLD	IKNFGAKVVG	LSCTLATGST	IFLOKLOPFV	HLSVMIAAYL	GRVRTKTVGE	PENKTKEMEL
C10-N		SQSI-P-SOG	11 11 11 1		IKNEGAKVVG	LSCTLA-GST	-FLGK-GPFV	HLSVMAYL	GRVRT-GE	-ENK-KE-
rClC-2	VVI.ITESAGE	TOILAPOAVG	SGTPEMKTTI.	ROVINEVIA	LETEVARUTO	LTCNL CEO	MPI OKRODET			11 1
					DATE VARVIG	DICKD030	MELIGKEGPE V	ILASHCAALL	SKFLSLFGGI	YENESRNTEM
201		D5			D6 •					D7
hClC-Ka	LVAAAAVGVA	TVFAAPFSGV	LFSIEVMSSH	FSVRDYWRGF	FAATCGAFIF	RLLAVFNSEO	ETITSLYKTS	FRVDVPFDLP	BT BBBBD I OO	TOOTH CONVE
ncic-kb	LVAAAAVGVA	TVFGAPFSGV	LFSIEVMSSH	PSVWDVWDQP	FAATCCAPMP	PLI.IVPNCPO	PTTTCI.VVTC	PD17N/7DPD/ D	BT DDDD DD OO	1 0011 001 10
rcic-ki	LSAAAAVGVA	TVFAAPFSGV	LFSIEVMSSH	FSVWNYWRGF	FAATCGAFMF	RLLCVFNSEO	FTTTSvVKMD	PRUNUDPHI.D	PTPPPMATOP	TOOTT CONVI
rcic-k2	LAAGAAVGVA	TVFAAPISGV	LFSIEVMSSH	FSVWDYWRGF	FAATCGAFMF	HLLAVENSEO	RTTTCTVKTC	PDUDT OPDT D	PTPPPNATAN	TOOTI COOVI
C1C- K	L-A-AAVGVA	TVF-AP-SGV	LFSIEVMSSM	FSVYWRGF	FAATCGAF-F	-LL-VFNSEO	ETITS-YKT-	F-VD-PFDI.P	RTPPPV-10-	-CG-LY-
rClC-2								1 1 1111		1
ICIC-2	LAAACAVGVG	CCFAAPIGGV	LESIEVISIE	FAVENIWRGF	FAATFSAFIF	RVLAVWNRDE	BTITALFKTR	FRLDFPFDLQ	ELPAFAVIGI	ASGFGGALFV
301				D8				•		
hClC-Ka	FCORTFLSFI	KTNRYSSKLL	ATSKPVYSAL	ATLLIASTTY	PPOVCHET.AS	RUSHKOHLDS	L.PDHHSWATM	TORCODWDP		WUDDERTES
DCIC-KD	FCORIFFGFI	RNNRFSSKLL	ATSKPVYSAL	ATT.VI.ACTTV	DDCACDFT.AC	DI.CMKOULDC	T.PINUCWATW	MORE CONUNE	BT DDAILT LEAD	
rClC-K1	FCORTFLRFI	KTNRYTSRLL	ATSKPSYAAL	VALVLASITY	PPGVGREMAS	RISMANHINS	T.PDMNGWATM	TOMCCODWDN	ODDONT MI D	WOUDDBOTTOO
rcic-K2	YCORTSLFFL	KSNGFTSKLL	ATSKPLYSAL	AAVVI.ASTTV	DDCVCDFMAC	RI.CMCRVI.PT	T.PDMNGWATM	THREEDOWCA	DDDDDDDT LIT I	LIGHT DOLUTE TOO
C1C-K								T-MSSPPW	B-DPO-LW-E	W-HPT-FG
						11 1	1111 1			1 1 1 1
rClC-2	YLNRKIVQVM	RKOKTINRFL	MKKRLLFPAL	VTLLISTLTF	PPGFGQFMAG	QLSQKETLVT	LFDMRTWV	RQGLVE	DLGAPSTSQA	WSPPRANVFL
401	D9 =		-	D10				D11 -		
hClC-Ka	TLAFFLVMKF	WMI.TLATTIP	MPAGYFMPIF	TIGANTORIJ.	GRALAVARDR	GIVIGGVINP	TMDCQVALAC	A A A POONTMU	TOTOTAL TANK	I MOOTUNAL D
hClC-Kb	TLAFFLVMKF	WMLILATTIP	MPAGYFMPIF	VYGAAIGRLF	GETLSFIFPE	GIVAGGITNP	IMPGGYALAG	AAAFSGAVIN	TETALLAPE	LIGUIVIALP
rClC-K1	TLAFFLVMKF	WMLILATTIP	MPAGYFMPIF	IIGAAIGRLL	GEALSVAFPE	GIVAGREVNP	IMPGGYALAG	AAAPSCAVTH	TTOTALLAPP	LTCOTVINI, D
rClC-K2	TLVFFLVMKF	WMLILATTIP	IPAGYFLPIF	VYGAAIGRIF	GEVI.SLAFPE	GIVAGGKVSP	TMPGAVALAG	AAAPCCAUPU	TT OTAT I APP	VICCOTVILLE
C1C-K	TIFFI.VMKF	WMLTLATTIP	-PAGYF-PIF	GAATGRI	CR-L FDR	GTV-GP	TMDC-VALAC	A A A PCOAUNU	CONTINES	COTINIALD
	11 1 111	11 11111		111 111	11 11	11	1 1 11 1		1 111 11	111 1 11
rClC-2	TLVIFILMKF	WMSALATTIP	VPCGAFMPVF	VIGAAFGRLV	GESMAAWFPD	GIHTDSSTYR	IVPGGYAVVG	AAALAGAVTH	TVSTAVIVFE	LTGQIAHILP
F.A.4										
	D12			VI DD TT ODI						
hcic-Ka	VLMAVLAANA	IAQSCOPSFY	DOWNTWEEP	ILPRILGENI	GSHHVRVEHF	MINHSITTLAK	DIPLEEVVKV	VISTOVIEYP	LVESTESQIL	VGIVQRAQLV
rclc-KD	V LEWAY LAANA	IAQSCOPSFY ISONCOPSFY	DOLLAR WYKER	VI.Pr TROPOT	GONKVKVEHF	MACHTAN	DIPLEEVVKV	VISIDVAEYP	LVESTESQIL	VGIVRRAQLV
TOTO-VI			201 TRUNULE	TOLI TUOKOI	GOIFVIVERF	PEVCHUIILAN	DIFLEEVVKV	VISTEVSQYP	LVEIKESQTL	VGIVERTHLV
rClC-K2	VLMAVLAANA VI.MAVI.AANA	TCOSYOPSEY	DGTIIVKKI.P	YLPWIRGRET						
rClC-K2	VLMAVLAANA	ICOSYOPSFY	DGTIIVKKLP	YLPWIRGRKI YLP-I-GR-T	GSHLVTVGHF GSV-V-HP	MNTTLAK	D-PLEEVIKV	VISTOVTOYP	LVETTESOVL	VGIVARIALV
rC1C-K2 <i>C1C-K</i>	VLMAVLAANA VLMAVLAANA	ICOSYOPSFY I-QQPSFY	DGTIIVKKLP DGTKKLP	YLP-I-GR-I	GSV-V-HF	MNTTLAK	D-PLEEV-KV	VISTOVIOYP V-ST-VYP	LVETTESOVL LVEESQ-L	VGIV-RLV
rClC-K2	VLMAVLAANA VLMAVLAANA	ICOSYOPSFY	DGTIIVKKLP DGTKKLP	YLP-I-GR-I	GSV-V-HF	MNTTLAK	D-PLEEV-KV	V-ST-VYP	LVEESQ-L	VGIV-RLV
rC1C-K2 C1C-K	VLMAVLAANA VLMAVLAANA	ICOSYOPSFY I-QQPSFY	DGTIIVKKLP DGTKKLP	YLP-I-GR-I	GSV-V-HF	MNTTLAK	D-PLEEV-KV	V-ST-VYP	LVEESQ-L	VGIV-RLV
rClC-K2 ClC-K rClC-2	VLMAVLAANA VLMAVLAANA	ICOSYOPSFY I-QQPSFY	DGTIIVKKLP DGTKKLP	YLP-I-GR-I	GSV-V-HF QQYRVRVEDI	MNTTLAK MVRDVPHVAL	D-PLEEV-KV SCTFRDL	V-ST-VYP	LVEESQ-L .3 linker /	VGIV-RLV
rClC-K2 ClC-K rClC-2 601	VLMAVLAANA VLMAVLAANA VMIAVILANA	ICOSYOPSFY I-QQPSFY I III I VAQSLQPSLY	DGTIIVKKLP DGTKKLP DSIIRIKKLP	YLP-I-GR-I YLPELGWGRH	GSV-V-HF	MNTTLAK I I MVRDVPHVAL D13	D-PLEEV-KV SCTFRDL	V-ST-VYP	LVEESQ-L .3 linker / 687	VGIV-RLV
rClC-K2 ClC-K rClC-2 601 hClC-Ka	VLMAVLAANA VLMAVLAANA II II III VMIAVILANA QALQAEPPSR	ICOSYOPSFY I-QQPSFY I III I VAQSLQPSLY	DGTIIVKKLP DGTKKLP DSIIRIKKLP ILARGCPTEP	YLP-I-GR-I YLPELGWGRH VTLTLFSETT	GSV-V-HF QQYRVRVEDI LHQAQNLFKL	MNTTLAK MVRDVPHVAL D13 LNLQSLFVTS	D-PLEEV-KV SCTFRDL RGRAVGCVSW	V-ST-V-YP	LVEESQ-L .3 linker / 687 TNPPAPK	VGIV-RLV
rClC-K2 ClC-K rClC-2 601 hClC-Ka hClC-Kb	VLMAVLAANA VLMAVLAANA VMIAVILANA QALQAEPPSR QALKAEPPSW	ICOSYOPSFY I-QQPSFY IIIII VAQSLQPSLY APGHQQCLQD	DGTIIVKKLP DGTKKLP DSIIRIKKLP ILARGCPTEP ILAAGCPTEP	YLP-I-GR-I YLPELGWGRH VTLTLFSETT VTLKLSPETS	GSV-V-HF QQYRVRVEDI LHQAQNLFKL LHEAHNLFEL	MNTTLAK MVRDVPHVAL D1: LNLQSLFVTS LNLHSLFVTS	D-PLEEV-KV SCTFRDL RGRAVGCVSW RGRAVGCVSW	V-ST-V-YP / D12-D1 VEMKKAISML VEMKKAISML	LVEESQ-L .3 linker / 687 TNPPAPK TNPPAPK	VGIV-RLV
rClC-K2 ClC-K rClC-2 601 hClC-Ka hClC-Kb rClC-K1	VLMAVLAANA VLAVLAANA IIIIIII VMIAVILANA QALQAEPPSR QALKAEPPSR QALCTOPPTPR	ICOSYOPSFY I-QQPSFY I III I VAQSLQPSLY APGHQQCLQD APGHQQCLQD APGQERFLQD	DGTIIVKKLP DGTKKLP DSIIRIKKLP ILARGCPTEP ILARGCPTEP ILAGCCPTOP	YLP-I-GR-I YLPELGWGRH VTLTLFSETT VTLKLSPETS VTLQLSPETS	GSV-V-HF QQYRVRVEDI LHQAQNLFKL LHEAHNLFEL LYQAHSLFER	MNTTLAK i i MVRDVPHVAL D13 LNLQSLFVTS LNLHSLFVTS LTLQSLFVTS	D-PLEEV-KV SCTFRDL RGRAVGCVSW RGRAVGCVSW RGRAVGSVSW	V-ST-VYP / D12-D1 VEMKKAISHL VEMKKAISHL AELKKAISTL	LVEESQ-L 3 linker / 687 TNPPAPK INPPAPK INPPAPK	VGIV-RLV
rClC-K2 ClC-K rClC-2 601 hClC-Ka hClC-Kb	VLMAVLAANA VLMAVLAANA II II III VMIAVILAANA QALQAEPPSR QALQAEPPSR QALQTQPtpW OSLHTDSASW	ICOSYOPSFY I-QQPSFY I IIII VAQSLQPSLY APGHQQCLQD APGRQCLQD APGQERFLQD APGQOPCLQD	DGTIIVKKLP DGTKKLP I III DSIIRIKKLP ILARGCPTEP ILAAGCPTEP ILAAGCPTOP	YLP-I-GR-I III YLPELGWGRH VTLTLFSETT VTLKLSPETS VTLQLSPETS VTLQLSTETS	GSV-V-HF QQYRVRVEDI LHQAQNLFKL LHANNLFEL LYQAHSLFER LHETHNLFEL	MNTILAK MVRDVPHVAL D1: LNLQSLFVTS LNLQSLFVTS LTLQSLFVTS LNLQSLFVTS	D-PLEEV-KV SCTFRDL RGRAVGCVSW RGRAVGCVSW RGKAVGSVSW RGRAVGSVSW	V-ST-VYP / D12-D1 VEMKKAISML VEMKKAISML AELKKAISTL VELKKAISTL	LVEESQ-L 3 linker / 687 TNPPAPK TNPPAPK INPPAPK TNPPAPK	VGIV-RLV
rClC-K2 ClC-K rClC-2 601 hClC-Ka hClC-Kb rClC-K1 rClC-K2 ClC-K	VLMAVLAANA VLMAVLAANA II II III VMIAVILANA QALQAEPPSR QALKAEPPSR QALKAEPPSR QALQTOPEPR QSLHTDSASR Q-L	ICOSYOPSFY I-QQPSFY I III I VAQSLQPSLY APGHQQCLQD APGQERFLQD APGQERFLQD APGQPCLOD APGLQD	DGTIIVKKLP DGTKKLP DSIIRIKKLP ILAGCPTEP ILAGCPTEP ILAGCPTOP ILA-GCPT-P	YLP-I-GR-I III YLPELGWGRH VTLTLFSETT VTLKLSPETS VTLQLSPETS VTLQLSTETS VTL0LSTETS III	GS-V-V-HF QQYRVRVEDI LHQAQNLFKL LHEAHNLFEL LYQAHSLFER LHETHNLFEL LLF	MNTILAK MVRDVPHVAL D1: LNLQSLFVTS LNLHSLFVTS LTLQSLFVTS LNLQLFVTS L-LLFVTS 	D-PLEEV-KV SCTFRDL RGRAVGCVSW RGRAVGCVSW RGRAVGSVSW RGRAVGSVSW RGRAVGSVSW RGRAVGSVSW	V-ST-VYP / D12-D1 VEMKKAISHL VEMKKAISHL AELKKAISTL VELKKAISTL -E-KKAIS-L 	LVEESQ-L 687 TNPPAPK TNPPAPK TNPPAPK TNPPAPK -NPPAPK	VGIV-RLV
rClC-K2 ClC-K rClC-2 601 hClC-Ka hClC-Kb rClC-K1 rClC-K2 ClC-K	VLMAVLAANA VLMAVLAANA II II III VMIAVILANA QALQAEPPSR QALKAEPPSR QALKAEPPSR QALQTOPEPR QSLHTDSASR Q-L	ICOSYOPSFY I-QQPSFY I IIII VAQSLQPSLY APGHQQCLQD APGRQCLQD APGQERFLQD APGQOPCLQD	DGTIIVKKLP DGTKKLP DSIIRIKKLP ILAGCPTEP ILAGCPTEP ILAGCPTOP ILA-GCPT-P	YLP-I-GR-I III YLPELGWGRH VTLTLFSETT VTLKLSPETS VTLQLSPETS VTLQLSTETS VTL0LSTETS III	GS-V-V-HF QQYRVRVEDI LHQAQNLFKL LHEAHNLFEL LYQAHSLFER LHETHNLFEL LLF	MNTILAK MVRDVPHVAL D1: LNLQSLFVTS LNLHSLFVTS LTLQSLFVTS LNLQLFVTS L-LLFVTS 	D-PLEEV-KV SCTFRDL RGRAVGCVSW RGRAVGCVSW RGRAVGSVSW RGRAVGSVSW RGRAVGSVSW RGRAVGSVSW	V-ST-VYP / D12-D1 VEMKKAISHL VEMKKAISHL AELKKAISTL VELKKAISTL -E-KKAIS-L 	LVEESQ-L 687 TNPPAPK TNPPAPK TNPPAPK TNPPAPK -NPPAPK	VGIV-RLV

FIG. 1. Sequence comparison of ClC-K putative Cl⁻ channels and ClC-2. Amino acid sequences for the two human isoforms (hClC-Ka and hClC-Kb) and the rat isoforms (rClC-K1 and rClC-K2) are shown. ClC-K sequence gives the residues conserved in all four kidney proteins and is compared to rClC-2 (8) (below). Conserved hydrophobic domains of ClC channels (5) are indicated above the sequences (D1 through D13). Consensus sites for N-linked glycosylation are printed in boldface letters; the site conserved within the ClC-K branch is indicated by a star, and the site conserved in all ClC channels is indicated by a boldface star. Lowercase letters in the rClC-K1 sequence indicate positions where differences to ClC-K1 (9) were found: $R27 \rightarrow P$, $V266 \rightarrow I$, $E356 \rightarrow Q$, $E381 \rightarrow D$, $P382 \rightarrow A$, $L389 \rightarrow P$, $R534 \rightarrow W$, $T608 \rightarrow A$, $P609 \rightarrow S$ (last amino acid as in ref. 9). The ClC-K1 sequence in ref. 9 lacks three cytosines at positions 1311, 1344, and 1403 (initiator A=1), leading to three frameshifts in the D10 region.

DISCUSSION

Both the rat and the human kidney express two highly homologous members of the ClC Cl⁻-channel family, respectively named rClC-K1 and -K2 and hClC-Ka and -Kb. They stem from different genes judging from their differences in sequence and their differential intrarenal distribution. Their surprisingly high degree of homology (83% in rat and 91% in human) suggests comparatively recent evolutionary divergence. The degree of intraspecies homology makes it impossible to deduce from sequences alone which rat protein would be the direct functional homologue of a given human protein.

A functional correspondence between the rat and the human putative channels may only be established after either the transcriptional regulation (promoter structure), intrarenal distribution, or channel properties of all four proteins are known. Dehydration increases CIC-K1 transcription in the rat kidney (9), but such studies are unfeasible in the human. Difficulties in working with human kidney precluded investigation of the intrarenal distribution of hCIC-K messages. rCIC-K mRNAs show a different distribution along the rat nephron. Among the segments examined, rClC-K1 is predominantly expressed in the cTAL and DCT, while rClC-K2 is expressed in every nephron segment we examined. For ClC-K1, Uchida *et al.* (9) reported high expression in the medullary thin ascending limb, a segment that we did not investigate. ClC-K message was not detected in any kidney cell line we studied, suggesting highly differentiation dependent expression. Identification of low levels of message in muscle, brain, and intestine (Fig. 3A) by RT-PCR may represent illegitimate transcription without functional significance.

The intranephronal distribution suggests different functions for both isoforms. These may result from differences in biophysical properties, polarized expression, or control of gene expression. CIC channels are probably multimers with more than three identical subunits (16). The high homology within the CIC-K subfamily may allow the formation of heterooligomers with novel functional properties. The ubiquitous CIC-2 channel is also coexpressed with CIC-K gene products, but functional heterooligomers between these more divergent proteins (\approx 45% homology) seem less likely.

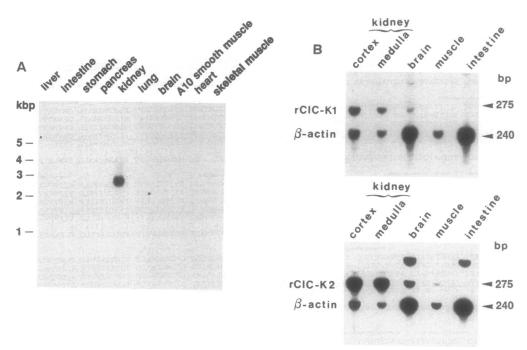


FIG. 2. Tissue distribution of rClC-K messages. (A) Northern analysis with rClC-K2 as a probe, which also detects the highly homologous rClC-K1. (B) RT-PCR analysis of rClC-K1 and rClC-K2 expression in different rat tissues. RNAs from several tissues were submitted to RT-PCR [35 cycles for rClC-K1 (B Upper) and 30 cycles for rClC-K2 (B Lower)]. A β -actin fragment was coamplified as internal standard. With ClC-K2 primers we observed products of aberrant size in rat brain and intestine. Sequencing of the large brain product revealed that it represented unspliced rClC-K2 message, possibly because of contaminating genomic DNA. Identities of the correctly sized bands were verified by restriction digests with BssHII and Fsp I, which cut only the rClC-K1 or the rClC-K2 fragment, respectively (data not shown).

In contrast to Uchida *et al.* (9), we were unable to functionally express any of the putative ClC-K channels, while other ClC channels were easily expressible. Occasionally we observed time-independent, slightly outwardly rectifying Cl⁻ currents. Since they could also be observed in negative controls, we attributed these to endogenous oocyte Cl⁻ channels. Oocytes contain several different Cl⁻ channels that can be activated by second messengers, during maturation, or by injection of RNA coding for nonrelated proteins (16– 20), making analysis of newly expressed Cl⁻ currents difficult. We also exchanged the untranslated regions of our cDNAs, mutated rClC-K1, to conform to the published sequence of ClC-K1 (9), and coinjected ClC-K complementary RNAs with other ClC cDNAs. Nonetheless, since by sequence ClC-K proteins are members of an established Cl⁻-channel family, we favor the view that they are anion channels or are subunits in such channels. ClC-K channels may serve roles in transpithelial transport or be involved in housekeeping functions such as cell-volume regulation. Indeed, ClC-2 is activated by hypotonicity (10), and volume regulation may be of special importance for epithelial cells transporting large amounts of solute and water (21).

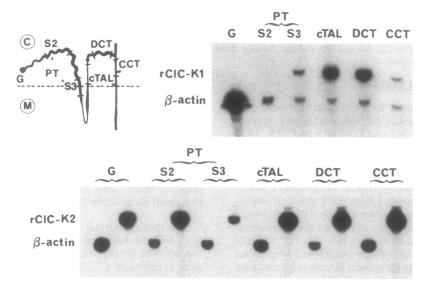


FIG. 3. Intrarenal distribution of rClC-K1 and rClC-K2 measured by RT-PCR on microdissected tubules. (*Upper Left*) Schematic of nephron segments. G, glomerulus; PT, proximal tubule (S2 and S3 segments). Dashes represent the border between kidney cortex (C) and medulla (M). (*Upper Right*) Intrarenal distribution of amplification products for rClC-K1 and β -actin (internal standard). (*Lower*) Intrarenal distribution of amplification products for rClC-K1 and β -actin (internal standard). (*Lower*) Intrarenal distribution of amplification products for rClC-K2 and β -actin expression from the same cDNA initially divided in two equal parts. Number of amplification cycles was 25 for ClC-K1 and 35 for ClC-K2.

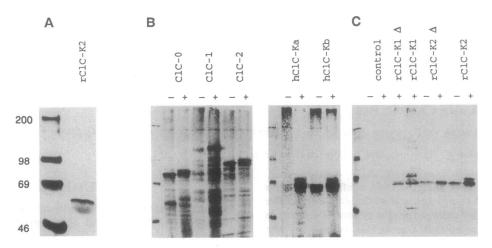


FIG. 4. Expression and glycosylation of different members of the ClC Cl⁻ channel family. (A) Immunoprecipitation of rClC-K2 protein expressed in *Xenopus* oocytes. Oocytes were injected with rClC-K2 message and labeled with [³⁵S]methionine. A solubilized membrane fraction was immunoprecipitated by using an antiserum directed against a C-terminal ClC-K peptide. (B and C) in vitro translation of ClC proteins in the absence (lanes –) or presence (lanes +) of dog pancreatic microsomes. In C, the products were immunoprecipitated with the anti-ClC-K antiserum before SDS/PAGE. For rClC-K1 and rClC-K2, we also analyzed mutants in which both consensus glycosylation sites between domains D8 and D9 were eliminated by replacing the asparagine residues by glutamine (N364 \rightarrow Q, N373 \rightarrow Q) (indicated by Δ in construct name).

Several possibilities may explain a lack of functional expression. CIC-K proteins may not function as Cl^- channels but perhaps transport other substances. We may miss another essential subunit, or the expression system may be inappropriate. Alternatively, the channel may be regulated by second messengers, but preliminary experiments testing several candidates did not reveal any novel currents. Finally, the channel may serve an intracellular role, even though other ClC proteins can be functionally expressed in the plasma membrane of different cells (5, 7, 8, 22, 23).

Whereas we cannot distinguish between these possibilities, we have shown that proteins of the correct size are synthesized both in oocytes and *in vitro* and that these are inserted into membranes and glycosylated. Though all ClC proteins contain consensus sites for N-linked glycosylation, their use seemed unlikely as they were thought to be cytoplasmic (5, 10). Our present observations indicate that this model is incorrect. Elimination of consensus sites between D8 and D9 abolished glycosylation with ClC-K proteins. This is probably not due to protein misfolding, since the equivalent mutant of ClC-1 is still functional in the oocyte (C. Lorenz, K. Steinmeyer, and M. Pusch, personal communication). Thus, the D8-D9 linker cannot be cytoplasmic, and one of the less likely candidates for transmembrane spans (such as D3, D4, or D8) may not cross the membrane. Alternatively, an additional, less hydrophobic segment on the N-terminal side of D8 may span the lipid bilayer.

In summary, we have shown that kidney of both rat and human each express highly homologous members of the ClC channel family. Differential expression along the nephron suggests specialized functions for each of these isoforms. Glycosylation of ClC-K proteins at sites previously thought to be cytoplasmic necessitates a revision of the topology model that is applicable to all members of this gene family.

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