Cloning and functional expression of a ClC Cl\textsuperscript{-} channel from the renal cell line A6

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Lindenthal, Sabine, Sandra Schmieder, J Ordi Ehrenfeld, and Nancy K. Wills. Cloning and functional expression of a ClC Cl\textsuperscript{-} channel from the renal cell line A6. Am. J. Physiol. 273 (Cell Physiol. 42): C1176–C1185, 1997.—Cl\textsuperscript{-} channels are important for ion transport and cell volume regulation in A6 renal cells. In the present study, we used reverse transcriptase (RT)-polymerase chain reaction (PCR) and rapid amplification of cDNA ends (RACE) to identify proteins homologous to ClC Cl\textsuperscript{-} channel proteins in A6 cells. Using degenerate primers designed on consensus sequences for members of the ClC family, we amplified an RT-PCR product that had significant homology to the ClC sequences. RACE-PCR was then used to isolate several full-length clones that had total lengths from 2,764 to 3,016 base pairs. Although the coding regions were identical, sequence differences occurred in the 5’ noncoding regions. The amino acid sequences of the clones had high homologies to rat and an outwardly rectifying Cl\textsuperscript{2-} channel that may provide a route for Cl\textsuperscript{2-} in differentiated monolayers that have properties similar to the apical 8-pS channel described by Marunaka and Eaton (19). The purpose of the present work was to determine whether members of another ClC channel family, ClC channels, were present in this cell line. This rapidly growing group of voltage-gated Cl\textsuperscript{2-} channels includes several members that are expressed preferentially in renal epithelial cells (9). At least one ClC member, ClC-2, is activated by hypotonicity (11, 27).

The cultured renal epithelial cell line A6 forms well-differentiated monolayers that have properties similar to renal distal tubule epithelia. Although A6 cells have been most frequently used as a model of transepithelial Na\textsuperscript{+} transport, these cells are also advantageous for investigations of Cl\textsuperscript{-} channels. Several types of Cl\textsuperscript{-} channels that have been implicated in either transepithelial Cl\textsuperscript{-} transport or cellular homeostasis have been described in the apical and basolateral membranes of polarized A6 monolayers. For example, Marunaka and Eaton (19) reported 3-pS and 8-pS Cl\textsuperscript{-} channels in the apical membrane that may provide a route for Cl\textsuperscript{-} secretion following hormonal stimulation mediated by adenosine 3’5’-cyclic monophosphate (cAMP) (14, 32). Cl\textsuperscript{-} channels are also involved in the transepithelial Cl\textsuperscript{-} reabsorption that occurs in parallel with Na\textsuperscript{+} transport and is stimulated by cell swelling (6a). In the basolateral membrane, patch-clamp studies by Banderali and Ehrenfeld (2) have identified four different types of Cl\textsuperscript{-} channels. Three present linear current-voltage (I-V) relationships with unitary conductances of 12 pS, 30 pS, and 42 pS, whereas the fourth shows an outwardly rectifying I-V relationship with inward and outward conductances of 16 pS and 57 pS, respectively. All of these channels were activated by hyposmotic bathing solutions.

Little is known about the molecular identity of the A6 Cl\textsuperscript{2-} channels, although recently the cystic fibrosis transmembrane conductance regulator has been cloned from A6 cells by Price et al. (21). This channel has properties similar to the apical 8-pS channel described by Marunaka and Eaton (19). The purpose of the present work was to determine whether members of another Cl\textsuperscript{-} channel family, ClC channels, were present in this cell line. This rapidly growing group of voltage-gated Cl\textsuperscript{2-} channels includes several members that are expressed preferentially in renal epithelial cells (9). At least one ClC member, ClC-2, is activated by hypotonicity (11, 27).

In the present study, we used a polymerase chain reaction (PCR) cloning strategy to isolate proteins homologous to members of the ClC family in A6 Xenopus laevis renal cells. We now report a novel ClC sequence, Xenopus ClC-5 (xCIC-5), that has strong homology to mammalian ClC-5. The xCIC-5 gene is most abundantly expressed in kidney, intestine, and oocytes. Nonetheless, injection of Xenopus oocytes with xCIC-5 cRNA leads to the expression of a novel Cl\textsuperscript{2-} conductance, not observed in control (water-injected) oocytes, that is blocked by 4,4’-diisothiocyanostilbene-2,2’-disulfonic acid (DIDS) and has a selectivity sequence of I\textsuperscript{−} > Cl\textsuperscript{−} >> gluconate.

MATERIALS AND METHODS

A6 cell culture. A6 cells from X. laevis kidney (American Type Culture Collection) were grown according to the method of Willis et al. (30, 31). Briefly, cells from passages 74–79 were grown in specific culture medium (GIBCO 84–5022 supplemented with antibiotics and 10% fetal calf serum; HyClone Laboratories, Logan, UT) on plastic flasks or on permeable filter supports (Transwell 3419; Costar) at 28°C in an atmosphere of 1% CO\textsubscript{2} in air. Isolation of mRNA. Total RNA was prepared from A6 cells or X. laevis tissues following the method of Chomczynski and Sacchi (6). Organs were removed from anesthetized animals and either extensively washed or perfused with saline buffer to remove most of remaining blood (heart, liver, muscle, brain, kidney, oocytes) and contents (stomach, urinary bladder) or further dissected to prepare different epithelial layers (intestine, skin). For A6 cells, poly(A)\textsuperscript{+} RNA was selected on...
oligo(dT) cell culture columns from the total RNA preparation (1) and analyzed by agarose gel electrophoresis. The average final yield was 15 µg/10⁶ cells for the total RNA and 130–180 ng/10⁶ cells for poly(A)+ RNA.

Reverse transcriptase-PCR. Degenerate sense (U) and antisense (D) oligonucleotide primers were designed from consen- sus sequences of several ClC-channel sequences of members of the ClC family (ClC-0, -1, -2, and -3) as follows: U: GT/C6 CNT TT(C/T) AG(C/T) AT(A/T/C) GA(G/A) GTN A; and D: AT(C/T) TGN CCN GTN A(G/A)(C/T) TC(G/A) AA. For first-strand synthesis, 0.5 µg of A6 poly(A)+ RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (RT) H- (Stratagene, La Jolla, CA) at 37°C for 20 min. After incubation at 70°C for 15 min, ribonuclease (RNase) H was added and the reactions were kept again at 37°C for 20 min. The RT reaction (1-3 µl) was used directly for amplification. The PCR solution was assembled and heated at 94°C for 3 min before Taq DNA polymerase (Stratagene) was added. Subsequently, PCR was performed using the following profile: 55°C for 1 min, 72°C for 2 min, and 94°C for 45 s, for 40 cycles. The last cycle was terminated after an elongation time of 8 min. The RT-PCR products were gel purified, cloned into the PCR II vector (Invitrogen), and partially sequenced.

Rapid amplification of cDNA ends. The PCR template used for rapid amplification of cDNA ends (RACE) was generated through a cDNA amplification kit (Marathon, Clontech, Palo Alto, CA) according to the supplier’s recommendations. Briefly, poly(A)+ RNA was converted into double strand cDNA, ligated to a DNA adaptor, diluted 100-fold, and denatured by heating at 95°C for 5 min. Aliquots were stored at –20°C. After 5 microliter at a time were used for a PCR reaction of 50 µl final volume. Amplification was carried out using a polymerase mix containing thermostable Taq and Pwo DNA polymerases (Expand Long Template PCR System, Boehringer Mannheim, Mannheim, Germany). Both the 5’ and the 3’ RACE reactions were primed with an internal gene-specific primer and the Marathon adaptor primer. The PCR reactions were performed under the following conditions: denaturation at 94°C for 45 s, annealing at 60°C for 30 s, and elongation at 68°C for typically 1–2 min, depending on the expected size of the PCR product (1–3 kb or base, kb1) as follows: U, GT/C6 (C/T) AG(C/T) AT(A/T/C) GA(G/A) GTN A; D, AT(C/T) TGN CCN GTN A(G/A)(C/T) TC(G/A) AA. For first-strand synthesis, 0.5 µg of A6 poly(A)+ RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (RT) H- (Stratagene, La Jolla, CA) at 37°C for 20 min. After incubation at 70°C for 15 min, ribonuclease (RNase) H was added and the reactions were kept again at 37°C for 20 min. The RT reaction (1-3 µl) was used directly for amplification. The PCR solution was assembled and heated at 94°C for 3 min before Taq DNA polymerase (Stratagene) was added. Subsequently, PCR was performed using the following profile: 55°C for 1 min, 72°C for 2 min, and 94°C for 45 s, for 40 cycles. The last cycle was terminated after an elongation time of 8 min. The RT-PCR products were gel purified, cloned into the PCR II vector (Invitrogen), and partially sequenced.

To acquire the maximum sequence information for the 5’ end, two independent PCR were performed and the 5’ ends of seven separate clones of the 5’ RACE products were sequenced. Full-length cDNAs were obtained by standard long-distance PCR with gene-specific primers from the 5’ and the 3’ ends, using the adaptor-ligated A6 cDNA.

cDNA sequencing. In general, cDNA was sequenced by the chain termination method of Sanger et al. (25). Selected RACE products as well as full-length cDNAs were sequenced in both directions, using an Applied Biosystems model 373A sequencing unit with synthetic primers (Ribocontgent DNA Laboratory, University of Texas Medical Branch). The BLASTN database search program (National Center for Biotechnology Information) was used to identify homologies of the obtained sequences to published sequences. Putative transmembrane domains in the sequence data were predicted using the algorithm of Kyte and Doolittle (16).

Northern blot analysis. A6 poly(A)+ RNA (10 µg) was resolved on a 1.2% agarose gel under denaturing conditions and transferred overnight in 10× saline sodium citrate (SSC) onto a nylon membrane (Hybond N+, Amersham, Buckingham- hamshire, UK). After the RNA was ultraviolet-cross-linked to the membrane, the blots were hybridized for 3 h at 65°C in rapid-hybridization buffer (Amersham) to a xClC-5 cDNA probe labeled with [32P]dCTP (Redprimelabeling kit, Amersham). The membranes were washed with a final stringency of 0.1% SSC-0.1% sodium dodecyl sulfate at 65°C. Hybridiza- tion was visualized by autoradiography.

Antisense RNA probe synthesis. The plasmid containing the full-length xClC-5 cDNA was digested with Sst I, gel purified, and religated. The resulting construct was linearized by digestion with Pvu II. From the linearized plasmid, a radiolabe- led antisense RNA fragment with a length of 257 bases, 34 bases of the polyliner region and 223 bases complementary to the xClC-5 sequence, was produced. For in vitro transcription, SP6 polymerase (10 units) and 50 µCi [α-32P]UTP (10 mCi/ml, 800 Ci/mmol) were used (MAXScript, Ambion, Aus- tin, TX), following the protocol given by the manufacturer. The DNA template was removed by digestion with RNase-free deoxyribonuclease (4 units) at 37°C for at least 20 min. After addition of an equal volume of gel-loading buffer, the solution was heated at 95°C for 3 min and purified by electrophoresis on a 5% polyacrylamide gel containing 8 M urea. The radiolabeled RNA runoff was localized by autoradiogra- phy, excised, and eluted overnight at room temperature in 350 µl of elution buffer. This solution has been stored at –20°C without removal of the gel fragment.

To generate an RNA probe that can be used as an internal control, a 751-base pair (bp) fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified by PCR from A6 cDNA and cloned. After digestion with Apa I, the above-described procedure was carried out. The labeled full- length antisense RNA probe was 244 bases long, with 169 bases complementary to the cloned GAPDH.

RNase protection assay. For the RNase protection assay (RPA), an RPA II kit (Ambion) was used. The experimental procedure followed the recommendations of the supplier. The xClC-5 and GAPDH antisense RNA probes (0.3–1.0 × 10⁵ counts/min) were incubated with 10 µg of total RNA from various X. laevis tissues. The hybridization mixture was heated at 95°C for 3 min and incubated at 44°C overnight. After hybridization, single-stranded RNA was digested with 0.13 units of RNase A and 5.3 units of RNase T1 at 37°C for 40 min. Protected RNA was precipitated, resuspended, and electrophoresed through a 5% polyacrylamide-8 M urea gel. The migration was visualized by autoradiography. The sizes of the protected fragments were determined with respect to 32P-labeled RNA molecular mass markers (Sigma, St. Louis, MO).

Functional expression in Xenopus oocytes. With use of T7 RNA polymerase, capped cRNA was prepared from the pGEM- 5Zf(+) cloning vector containing the xClC-5 sequence and stored at –80°C in diethyl pyrocarbonate-treated water at a final estimated concentration of 50 ng/µl. cRNA (2.5–5 ng) was injected into X. laevis oocytes prepared and handled as previously described (22). After 3–4 days, at 18°C, the oocytes were investigated by two-microelectrode voltage clamping using a TEV 200 amplifier (Dagan, Minneapolis, MN) moni- tored by computer through a Digidata 1200 analog-to-digital converter and pCLAMP software (Axon Instruments, Foster City, CA). Microelectrodes were pulled using a Zeitz puller (Augsburg, Germany), were filled with a 3 M KCl solution, and had resistances of 1.5–2.5 MΩ. Oocytes were voltage clamped at a holding potential of –50 mV, and 800-ms voltage steps from –100 to +80 mV in 20-mV increments were applied. All experiments were performed at room temperature. Oocytes were perfused with an experimental medium...
that contained (in mM) 95 NaCl, 2 KCl, 2.4 NaHCO₃, 0.82 MgSO₄, 0.33 Ca(NO₃)₂, 0.41 CaCl₂, 5 N-2-hydroxyethylpipera-
zine-N’-2-ethanesulfonic acid, and 3 NaOH (pH 7.4). In ion substitu-
tion experiments, 80 mM Cl⁻ was replaced with an equal con-
centration of gluconate or I⁻, and 3 M KCl agar-agar bridges were used to minimize junction potentials (6-8 mV).

The relative permeability of I⁻ or gluconate vs. Cl⁻ was estimated using the following expression derived from the
Goldman-Hodgkin-Katz equation

\[ V_c - V_e = 58 \log \left( \frac{P_\text{X} / [\text{Cl}^-]}{P_\text{Cl} / [\text{X}^-]} \right) \]

where \( V_c \) is the membrane potential in the control solution, \( V_e \) is the membrane potential in the experimental solution (containing anion \( \text{X} \)), \([\text{Cl}^-]_c \) is the Cl⁻ concentration in the control solution, \([\text{Cl}^-]_e \) is the Cl⁻ concentration in the experi-
mental solution, \([\text{X}^-]_c \) is the substituted anion concentration, and \( P_\text{Cl} \) and \( P_\text{X} \) are the permeabilities for Cl⁻ and the substituted anions, respectively.

RESULTS

Cloning of an amphibian renal ClC homologue. RT-
PCR was performed using A6 cell mRNA and degener-
ate oligonucleotide primers designed from consensus
sequences of several ClC channel sequences of members
of the CIC channel family (CIC-0, -1, -2, and -K; see
materials and methods). A PCR product with an ex-
pected length of 830 bp was obtained. Sequence analy-
sis revealed that the amplified cDNA had significant
homology to the sequences of ClC-2 and ClC-K. The
expected length of 830 bp was obtained. Sequence analy-
sis revealed that the amplified cDNA had significant
homology to the sequences of ClC-2 and ClC-K. The

amplification reactions were sequenced completely in
clones (5A2, 5A6, 5B1) from two separately performed
amplification reactions were sequenced completely in
clones (5A2, 5A6, 5B1). As shown in Fig. 1, the
longest of the obtained cDNAs consisted of 3,016 bp
with an open reading frame of 2,424 bp that was
preceded by a 435-bp 5’ noncoding region and followed
by a 157-bp 3’ noncoding region. The initiation Met was
assigned to the first ATG codon in-frame that was
preceded by an in-frame stop codon. cDNAs of three
clones (5A2, 5A6, 5B1) from two separately performed
amplification reactions were sequenced completely
in one direction, and one of the clones (5A2) was
sequenced in both directions. Comparison of the three
coding sequences showed that clone 5A2 has an aden-
osine residue at position 585 instead of a guanosine
residue (as in clones 5A6 and 5B1), which does not
result in an amino acid difference. Only clone 5A6 had a
nucleotide difference that resulted in an amino acid
substitution. Specifically, there was a T/A exchange at
position 1276, such that amino acid 426 was Ser
instead of Thr in the other two sequences. Whether
these differences were due to the existence of two
different DNA sequences or to misincorporations of
bases during cDNA synthesis or during the amplifica-
tion reaction has not been studied. However, these
results clearly show the high fidelity of the Pwo DNA
polymerase used in the PCR reactions and the advan-
tages of the RACE cloning strategy.

Interestingly, we found that, although the clones
possessed identical coding and 3’ noncoding sequences,
there were several differences in their 5’ noncoding
regions. Compared with the sequence of clone 5A2 (Fig.
2), the 5’ untranslated sequences of clone 5A6 and 5B1
are not only shorter at their 5’ extremities but show
specific deletions within their sequences [from nucleo-
tides -121 to -268 (5A6) and -217 to -269 (5B1)].

Because the length and composition of the 5’ noncoding
region are known to be important for the translation
efficiency of mRNA in vivo (10), we injected cRNAs of all
three clones into Xenopus oocytes. Three batches of
oocytes were injected with cRNA for clone 5A2 (n = 10),
and one batch of oocytes was injected with cRNA for
clones 5B2 and 5B1 (n = 2 and n = 5, respectively). No
qualitative differences in expression were detected
among the clones. The experiments reported below
were performed using clone 5B1.

The open reading frame predicts an 808-amino acid
translation product with a molecular mass of 90 kDa.

Figure 3 shows a hydrophobicity analysis of the pre-
dicted amino acid sequence using the method of Kyte
and Doolittle (16). Like that of other members of the
CIC Cl⁻ channel family, the hydrophobicity profile of
this channel shows 10-12 hydrophobic regions, indi-
cated as numbered lines above the sequence data in
Fig. 4. Three potential N-glycosylation sites have been
found at positions Asn-17, Asn-169, and Asn-470. The
latter is located between hydrophobic regions D8 and
D9 and represents a highly conserved glycosylation
motif among all CIC channels except for CIC-7. In vitro
translation experiments by Kiefert et al. (15) showed
that the corresponding segments of CIC-0, -1, -2, and -K1
are glycosylated. Consequently, it has been suggested
that this segment is positioned at the extracellular side
of the cell membrane. Likewise, Asn-169 is located in a
predicted extracellular loop between hydrophobic
regions D1 and D2. Because Asn-17 is located close to
the amino terminus, and therefore the cytoplasmic part
of the protein, it is not very likely that this amino acid
is glycosylated in vivo [see Ref. 17 for a putative trans-
membrane model of human (h) CIC-5]. Consensus
sequences for cAMP- and guanosine 3’,5’-cyclic mono-
phosphate-dependent protein kinase phosphorylation
are present at positions Ser-10, Thr-411, and Thr-412.

Protein kinase C consensus sites were found at posi-
tions Thr-353, Thr-411, and Ser-459, Thr-606, Ser-639, Ser-
690, Ser-708, Thr-719, Ser-738, and Thr-786. As in
other CIC proteins (except CIC-7), the Glu at position
234 and Pro at position 513 were conserved.

The CIC family has rapidly grown to eight members,
at present, which can be classified into three sub-
families as follows: 1) CIC-0, -1, -2, -K1, and -K2; 2) CIC-3, -4, and -5; and 3) CIC-6 and -7. Analysis of the
full-length sequence of xClC-5 revealed its high homol-
gy to the more recently isolated group of CIC Cl⁻
channels, the outwardly rectifying Cl⁻ channel (ORCC)
subbranch comprising CIC-3, -4, -5. The protein
sequence of xClC-5 has a 69% homology to hCIC-3 (3),
73% to hCIC-4 (29), and 77% to hCIC-5 (Ref. 7; see Fig.
4). The homologies to other mammalian CIC proteins
are 72% to rat (r) CIC-3 (13), 74% to rCIC-4 (T. J.
entsch, W. Günther, M. Pusch, and B. Schwappach,
GenBank no. Z36944; direct submission), and 77% to
rCIC-5 (24, 26). In contrast, the homology was only
19.8% to rClC-2 (27) and 14.5% to hClC-6 (4). If Met-65 (i.e., the 5th Met of the open reading frame) is assigned as the initiation ATG, the homology to hClC-5 increases to 84% (and for rClC-5 to 85%). Because of the high homology to the hClC-5 and rClC-5 sequences, the identified clone has been named xClC-5.

In Northern blot analysis (Fig. 5), a full-length xClC-5 cDNA probe hybridized to a band at 3.4 kb. As Fig. 1. Nucleotide sequence of Xenopus (x) ClC-5. Deduced amino acids are shown below sequence. Dots, potential N-linked glycosylation sites; asterisks, potential protein kinase A phosphorylation sites; c, sites for potential phosphorylation by protein kinase C; lines above sequence, polymerase chain reaction primer sites. xClC-5 sequence data have been deposited in GenBank/European Molecular Biology Laboratories data library, no. Y09940.
clones 5A2, 5A6, and 5B1. Asterisks, identical nucleotides.

... variation in GAPDH mRNA levels included in each sample, serving as internal control for antisense probe for GAPDH mRNA detection was included. An no was discovered in stomach, muscle, and skin. Lower amounts were found in liver, blood, brain, heart, and urinary bladder. Very low but detectable levels were mostly present in oocytes, kidney, and intestine. xClC-5 is a broadly expressed gene. mRNA was computed using algorithm of Kyte and Doolittle with a window size of 21 residues. Large positive numbers indicate the most hydrophobic regions and negative numbers the least hydrophobic regions.

Fig. 3. Hydrophobicity profile of xClC-5. Mean hydrophobicity index was calculated using Eq. 1 with a window size of 21 residues. Large positive numbers indicate the most hydrophobic regions and negative numbers the least hydrophobic regions.

Electrophysiological characteristics of expressed xClC-5. The membrane potential (V_m) of control oocytes had a mean value of −66 ± 6 mV (n = 10). As illustrated in Fig. 7C (water-injected oocytes), the currents applied to clamp the oocyte membrane were low over the measured potential range (<300 nA at +80 mV and less than −50 nA at −100 mV). The V_m of xClC-5 cRNA-injected oocytes (4 days after the cRNA injection) were significantly (P < 0.0001) depolarized compared with controls, with a mean V_m of −23 ± 1 mV (n = 29; 4 frogs). As shown in the example presented in Fig. 7, B and C, cRNA-injected oocytes showed a large outwardly directed current at positive holding potentials. Partial substitution of Cl− with gluconate led to a decrease of the current amplitude in the positive voltage range, as expected for a relatively large Cl−-to-gluconate permeability ratio (Fig. 8A). The resting potential shifted from −21 ± 2 mV to −7 ± 2 mV [change in V_m (ΔV_m) = −14 ± 3 mV; n = 4; P < 0.05] following partial substitution of Cl− with gluconate and from −23 ± 3 mV to −30 ± 5 mV (ΔV_m = 8 ± 1 mV; n = 4; P < 0.025) after partial substitution of Cl− with I−. The permeability ratios calculated using Eq. 1 were P_{gluconate}/P_{Cl} = 0.5 ± 0.2 and P_I/P_{Cl} = 1.4 ± 0.1. Consequently, the anion selectivity sequence was P_I > P_{Cl} > P_{gluconate}.

Application of the Cl− channel blocker DIDS (500 µM) to cRNA-injected oocytes held at +80 mV reduced the clamping current from 11,095 ± 2,381 nA to 2,864 ± 662 nA (Fig. 8B; n = 5). The inhibitory effect was voltage dependent, since it was stronger at positive potentials (72.2 ± 7.3% at +80 mV) than at negative potentials (31.9 ± 8.1% at −100 mV). The inhibition was not reversible after a 6-min perfusion period.

DISCUSSION

The present study describes the first cloning and functional expression of a CIC Cl− channel isolated from the renal epithelial cell line A6. The longest complete cDNA had 3,016 nucleotides and contained a 5′ untranslated sequence of 435 nucleotides, a 3′ untranslated sequence of 157 nucleotides, and an open reading frame of 2,424 nucleotides coding for an 808-amino acid protein. The first initiation Met is preceded by an in-frame stop codon. The amino acid sequence shows a high homology to rClC-5 and hClC-5, members of this family of mammalian cationic Cl− channels.
of the CIC family that is associated with the genetic disorder Dent's disease (7, 8, 17). The homology to both of these clones increases to 85 and 84%, respectively, if the first amino acid of the sequence is assigned to Met-65. Relatively poor homology was found to other ClC Cl\textsubscript{2} channels except for ClC-3 and ClC-4. Therefore, this new channel, named xClC-5, can be considered a member of the ORCC subbranch of the ClC Cl\textsubscript{2} channel family.

**Structural properties of xClC-5.** An alignment of the xClC-5 amino acid sequence with the hClC-5 sequence (Ref. 8; Fig. 4) revealed several structural features that are common to all members of the ClC family. The N\textsubscript{-}glycosylation motif between D8 and D9 was highly conserved, as were the sites for putative phosphorylation (see Fig. 1). An additional putative glycosylation site was found in a predicted extracellular loop at Asn-169, and an additional putative protein kinase A phosphorylation site is present at Ser-10. As in ClC-2 (11), domains necessary for channel activation could be present in the amino terminus of the sequence, where Ser-10 could offer a target sequence for regulatory mechanisms. As in other ClC members, the hydrophobic domain D13 was also found. It is remarkable that

![Fig. 4. Comparison of xClC-5 with human CIC-5 (hClC-5) amino acid sequence. Asterisks, identical amino acids; dots, similar amino acids. Putative transmembrane domains (1–13) are highlighted by solid lines according to topology proposed by Jentsch et al. (12).](image-url)

![Fig. 5. Northern blot analysis of A6 RNA; 10 μg poly(A)\textsuperscript{+} RNA was separated on a 1.2% agarose formaldehyde gel, blotted on a nylon membrane, and hybridized with a \textsuperscript{32}P-labeled full-length probe of xClC-5. Probe hybridized to a transcript of \textsim 3.4 kilobases.](image-url)
the sequence homology for the 53 amino acids of the cytosolic carboxy terminus (which includes region D13) was 100% for the xClC-5 and the hClC-5 sequences. This finding may indicate the importance of this segment for channel function or regulation. Amino acids 293–373, a fragment of 80 amino acids that includes hydrophobic domains D5 and D6, constituted another entirely conserved part of the amphibian sequence compared with the human sequence. A high homology (97%) was also found for a segment of 63 amino acids from position 553 to position 616, comprising D11 and D12.

The size of the xClC-5 mRNA obtained by Northern blot analysis was ~3.4 kb. This value was consistent with the length of the obtained clones (2.7–3.0 kb), assuming that an additional portion is created by polyadenylation. However it was notably smaller than that found for the mammalian transcripts of ClC-5. hClC-5 and rClC-5 probes both recognized messages of 9.5 kb (8, 24, 26), but the open reading frames encoding the respective proteins were confined to a portion of only 2,238 bp. Similar findings have been obtained for hClC-4 (29), in which an open reading frame of ~2.2 kb was found at the 3' end of a 7.5-kb message. Consequently, these authors suggested the existence of large 5' noncoding regions in the mammalian sequences. Apparently, the amphibian clone is lacking these large 5' untranslated regions.

Previous studies using Northern blot analysis have shown that rClC-5 and hClC-5 were predominantly expressed in kidney (7, 24, 26), although mRNA has also been detected in rat brain, liver, lung, and intestine. In the present study, RPA showed that xClC-5 is a rather broadly expressed gene that is most highly transcribed in oocytes, kidney, and intestine. xClC-5 mRNA is also present in liver, blood, brain, heart, and urinary bladder. Significant, lower but detectable amounts were found in stomach, muscle, and skin. Considering the high sensitivity of the RPA technique, a contamination caused by mRNA of erythrocytes remaining in capillary blood vessels of the analyzed tissues cannot be excluded in muscle, heart, kidney, and liver, where complete perfusion was difficult to obtain. To our knowledge, there is no previous report of the presence of any member of the ClC family in erythrocytes. It will be interesting to determine whether xClC-5 is present only in nucleated erythrocytes like those from X. laevis or whether this protein can be found as well in mammalian erythrocytes that lack a nucleus.

Functional properties of xClC-5. Injection of xClC-5 cRNA in *Xenopus* oocytes induced the appearance of outwardly rectifying Cl\textsuperscript{−} currents that were not found in control oocytes. This current had a selectivity sequence of I_{5} > Cl\textsuperscript{−} >> gluconate and was blocked by DIDS. Outward rectification has been described for hClC-5 and rClC-5, also expressed in *Xenopus* oocytes (8, 26), and has been interpreted as an open channel rectification or a voltage dependence of a gate with fast kinetics (26). The currents induced by rClC-5 (26) were time independent, similar to our results. The permeability ratio (based on changes in reversal potential) was not given for the rat study (26); however, the reported (conductance) selectivity sequence was NO\textsubscript{3} > Cl\textsuperscript{−} > I\textsuperscript{−} and, therefore, differs from our study. Cl\textsuperscript{−} channel blockers such as DIDS, 5-nitro-2-(3-phenylpropylamino)benzoic acid, or diphenylamine-2-carboxylic acid had no effect (26). In contrast, Sakamoto et al. (24), who used rClC-5 cDNA in stably transfected Chinese hamster ovary (CHO) cells, observed time-dependent activation of Cl\textsuperscript{−} currents that showed moderate outward rectification, were blocked by DIDS, and had a selectivity sequence of I\textsuperscript{−} > Cl\textsuperscript{−}. It is unclear whether the time-dependent activation of the currents observed by Sakamoto et al. (24) for rClC-5 expressed in CHO cells is related to the use of a different expression system. We note that xClC-5 expressed in oocytes showed features
common to mammalian CIC-5 proteins expressed both in Xenopus oocytes (i.e., strong outward rectification, time independence) and in CHO cells (i.e., similar selectivity sequence and DIDS sensitivity).

RPA analysis results showed the presence of significant amounts of endogenous xClC-5 message in Xenopus oocytes. Nonetheless, injection of xClC-5 cRNA unequivocally led to a 10- to 20-fold increase of the total oocyte conductance that was due to the development of an anion pathway. Consequently, it is reasonable to assume that this current was due to overexpression of xClC-5 protein, although we cannot discern whether xClC-5 is truly a channel of a channel regulator. Furthermore, we cannot exclude that the observed current was mediated by activation of an endogenous channel due to overexpression of a structurally unrelated protein (5). Further studies are needed to assess the single-channel properties of this protein in other expression systems. In addition, we must note that coexpression of different members of the CIC family has been shown to produce Cl⁻ currents that are different from those of the same clones expressed individually (18). Lorenz and co-workers (18) concluded that this phenomenon was due to the formation of functional heteroligomeric channels with novel properties. Given the high homology of several CIC channels between even poorly related species (for example, 84% homology between xClC-5 and hClC-5), such interactions could also occur between expressed proteins and endogenous proteins in Xenopus oocytes. However, these interactions could affect the expressed Cl⁻ current only if the quantity of endogenous protein is sufficient to allow the inclusion of at least one endogenous protein per heteroligomeric unit.

The role of xClC-5 in epithelial ion transport, and in the maintenance of Vₐₖ or cell volume, and the factors that regulate this protein remain to be investigated.

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**Fig. 7.** Electrophysiological properties of xClC-5 expressed in Xenopus oocytes. A: voltage-clamp protocol. Oocytes were sequentially clamped from a holding potential of -50 mV to voltages between -100 and +80 mV for 800 ms in steps of 20 mV. B: representative typical trace of whole cell current (I) 4 days after cRNA xClC-5 injection. C: mean current-voltage (I-V) relationships of water-injected oocytes (n = 10) and cRNA-injected oocytes (n = 29).

**Fig. 8.** A: effect of anion substitution on current of cRNA xClC-5 injected oocytes. Current ratio of substituting anion to Cl⁻ measured at a clamping potential of +80 mV is given by ordinate (values given above columns). Strongly outwardly rectifying anion current is reduced by partial (80 mM) replacement of Cl⁻ with gluconate (n = 12) and increased by replacement with I⁻ (n = 14). B: effect of DIDS (500 µM) application (6 min) on I-V relationship of xClC-5 cRNA-injected oocytes (n = 5).
Such an attempt was performed by Uchida et al. (28), who reported that the abundance of mRNA for the kidney-specific ClC channel, ClC-K1, was increased in renal cells following dehydration in rats. An advantage of the A6 cell line is that it allows one to precisely regulate expression of xClC-5 by environmental factors or hormones or during differentiation. In addition, this system may be a valuable model for elucidating whether xClC-5 is expressed in a polarized manner in epithelial cells.

In summary, the A6 cell line expresses a ClC channel that has high homology to hClC-5. The use of cultured A6 epithelial monolayers has advantages that may be useful for understanding the fundamental biological function of ClC-5 proteins and the pathological conditions that result from their malfunction.

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REFERENCES


