Expression and targeting to the plasma membrane of xClC-K, a chloride channel specifically expressed in distinct tubule segments of *Xenopus laevis* kidney

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ClC-K channels are Cl^- channels specifically expressed in vertebrate kidneys. Although their heterologous functional expression is still controversial, indirect evidence points to them as major factors involved in Cl^- reabsorption in the nephron. We cloned xClC-K, an amphibian (*Xenopus*) homologue of mammalian ClC-K. The cDNA encodes a 77 kDa protein presenting 62% similarity with human ClC-Kb. The protein is monoglycosylated and is expressed primarily in the *Xenopus* kidney. It is localized in the basolateral membranes of proximal convoluted tubules of the nephron and in the apical region of the diluting segments. Heterologous expression of xClC-K in HEK-293 cells showed that the full-length protein is glycosylated and targeted to the cell membrane, but no associated Cl^- current could be observed with the patch-clamp recording technique. N-glycosylation of both the native kidney channel and the recombinant protein expressed in HEK-293 conferred on them anomalous behaviour in denaturing PAGE, which is indicative of strong interactions at the extracellular side of the plasma membrane. The expression of ClC-K channels in both mesonephric and metanephric kidneys will permit further comparative physiological studies of Cl^- permeabilities at the molecular level.

Key words: cDNA cloning, oocyte, Western blots.

INTRODUCTION

Members of the ClC family of Cl^- channels are found throughout the living world, including bacteria [1], yeast [2], plants [3] and animals [4]. Among several branches that can be distinguished in vertebrates, one consists of closely related kidney-specific channels, hence named ClC-K. At least two ClC-K forms are present in rat [5–7] and human [7], and one has been cloned from rabbit [8]. These channels are structurally so close that it has not been possible to make interspecies correlations on the basis of the comparison of their sequences. A large variety of Cl^- permeabilities is observed in kidney [9], reflecting the fine tuning required for the regulation of body fluids and salt balance, as well as for keeping its own cellular integrity under variable osmotic conditions. Unfortunately, the functional expression of cloned ClC-K in heterologous systems has rarely been obtained, so a correlation of their activation parameters with currents observed *in situ* still remains elusive [7,8]. Large Cl^- currents have occasionally been observed by expressing ClC-K1 or ClC-K2 from rat in *Xenopus* oocyte [5,6]. Both channels were open at all potentials studied and exhibited a slight outwardly rectifying current–voltage relationship. However, other groups have been unable to observe any current when expressing rat, human and rabbit ClC-K in *Xenopus* oocytes [7,8]. An anti-sense strategy and antibody blockade were used to probe the function of rabbit ClC-Ka. Both approaches promoted the specific inhibition of a specific Cl^- channel from kidney. The affected current is different from those observed when rat channels were expressed: it is potentiated by high cytosolic Cl^- concentrations and by cAMP [10]. The frequent failure of ClC-K to be functionally expressed has been attributed to a lack of expression of the protein or to incorrect post-translational processing.

Here we describe a ClC-K channel protein from *Xenopus laevis*, incidentally cloned from oocyte. Glycosylation of a single asparagine residue confers an unusual size heterogeneity on the channel protein. We show by heterologous expression of xClC-K in HEK-293 transfected cells that, although no Cl^- currents could be recorded, the full-length protein is glycosylated and targeted to the plasma membrane, suggesting that an association of xClC-K with other subunits could be a prerequisite for functional expression. The characteristic localization of xCLC-K observed in defined regions of *Xenopus* nephron will permit further physiological comparative studies of Cl^- conductances by using a molecular approach.

EXPERIMENTAL

Animals and cell cultures

Female *X. laevis* were purchased from the Centre de Recherche de Biochimie Macromoléculaire du CNRS (Montpellier, France). HEK-293 cells (ATCC CRL 1573) were obtained from the Laboratoire de Génétique Moléculaire des Eucaryotes (Strasbourg, France). Cells were grown at 37 °C under air/CO_2 (19:1) in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) decomplemented foetal calf serum. HEK-X6

Abbreviations used: PNGase F, peptide N-glycosidase F (EC 3.5.1.52); RACE, rapid amplification of cDNA ends.

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lines were grown in the presence of 250 μg/ml geneticin (Gibco/Life Technologies).

Oligonucleotides

Oligonucleotides (Eurogentec, Seraing, Belgium) were as follows, where the suffixes 's' and 'as' denote sense and anti-sense orientations respectively: CL1.s, 5′-CCGGATCCGGSTCYGGMMTCCCNGARMTGAARAC-3′ (740–765); CL3.as, 5′-CCGAATTCSACCTCNAWRCTRAAHAGSACNCC-3′ (1060–1037); P1C.as, 5′-CTCCGGAATGTGGAGTGATGCTCTG-3′ (740–716); P2C.as, 5′-GAGTGCTATTGGGTAGACAATCCAG-3′ (691–667). Map positions are given in parentheses (see Figure 1). CL1.s and CL3.as contained 5′ extensions of eight nucleotides with restriction sites *Bam*HI and *Eco*RI respectively.

Purification and analyses of RNA

Total RNA was isolated from tissues by the method of Chirgwin et al. [11] and enriched in poly(A)$^+$ RNA by oligo(dT) chromatography [12]. *Xenopus* oocytes were defolliculated manually after digestion with collagenase before purification of RNA. First-strand cDNA was synthesized from poly(A)$^+$ RNA (2 μg) with random hexanucleotides as primers with the use of a reverse-transcription system (Promega) and recovered in 100 μl of water. PCR amplifications were performed on 5 μl of cDNA (30 cycles) or 10 ng of plasmid (10 cycles) in a total volume of 50 μl with Goldstar polymerase (Eurogentec) under conditions specified by the supplier of the enzyme (1.5 mM $MgCl_2$/200 μM dNTP/2 μM each oligonucleotide/1 unit of polymerase). Standard cycling conditions were: denaturation, 1 min at 94 °C; annealing, 1 min at 60 °C; extension, 1 min at 72 °C; final extension, 3 min at 72 °C. Northern blot analysis was performed essentially as described by Sambrook et al. [12]. Autoradiograms were exposed for up to 7 days.

cDNA library screening and sequencing

A size-selected directional library of *Xenopus* oocyte cDNA in λ Zap II was constructed by using the ZAP-cDNA synthesis kit (Stratagene). The recombinant library (3×10^5 plaque-forming units) was screened by three rounds of hybridization with the 330 bp PCR product, labelled with [^{32}P]dCTP by random priming. The phagemid was excised *in vivo* and sequenced [13] on both strands by using combinations of specifically designed primers and restriction enzyme deletions with the T7 sequencing kit (Pharmacia). Sequence compilations and analyses were performed with the GCG sequence analysis software package [14]. The 5′ part of the transcript was obtained by rapid amplification of cDNA ends (RACE) with the Marathon cDNA amplification kit (Clontec). The standard protocol for nested amplification was performed with P1C.as and P2C.as in succession as specific primers. PCR products were blunted and subcloned into the *Eco*RV site of pBluescript SK$^-$.

Fusion proteins, raising of antibodies and Western blotting

A 340 bp fragment (map position 2002–2343) was obtained by digestion with *Cla*I, Klenow repair and digestion with *Sac*I. It was subcloned in-frame into the *Bam*HI-blunted/*Sac*I sites of pQE30 (Quiagen). The fusion protein was obtained and purified under denaturing conditions by using the Quiaexpressionist kit (Quiagen). Antibody no. 1466 was raised in rabbit by Eurogentec (Belgium) by their standard immunization protocol. IgGs were purified from final bleed antiserum by Protein A–Sepharose chromatography [15]. Crude membranes were prepared as described in [16] for oocytes, as described in [17] for kidney and as described in [18] for other tissues and cultured cells. Protein content was assessed [19] and aliquots were stored at −80 °C. Digestions with peptide N-glycosidase F (PNGase F, EC 3.5.1.51; New England Biolabs) were performed as advised by the supplier with a preliminary denaturation step of 10 min at 37 °C. SDS/PAGE was performed by the method of Laemmli [20] with denaturation of samples for 10 min at 37 °C. Proteins were transferred to Hybond-ECL® membranes (Amersham) and probed with primary antibody no. 1466, a peroxidase-coupled goat anti-rabbit secondary antibody (Jackson Labs) and the chemiluminescence SuperSignal substrate (Pierce), by using the enhanced chemiluminescence protocol from Amersham.

Construction of eukaryotic expression plasmids and transfections in HEK-293 cells

The full-length xClC-K cDNA was reconstituted by successively subcloning the 5′ region (map coordinates 1–691) as a *Hin*dIII/*Not*I insert from the RACE/PCR product into pCDNA1, then the 1819 bp *Kpn*I fragment from X6 (661–2480) was ligated into this construct. Finally, the 5′ untranslated region (1–378) was excised by partial digestion with *Rca*I and complete digestion with *Hin*dIII. This region was replaced with a 90 bp *Hin*dIII/*Nco*I synthetic fragment shown to be efficient in eukaryotic expression constructs [21]. Poly(ethyleneimine)-mediated transfections of HEK-293 cells were performed as described [22], with 3 μg of plasmid per 35 mm plate containing 10^5–10^6 cells [molar ratio of poly(ethyleneimine) nitrogen to DNA phosphate = 6]. The transfection medium was replaced after 24 h with normal culture medium. Stable cell lines were established with a 5:1 mixture of pCDNA1-xCLC-K and pCMVNeo (pCDNA1 with the neomycin resistance gene under control of the cytomegalovirus promoter, a gift from Dr. Brigitte Kieffer) and resistant clonal colonies were selected with 250 μg/ml geneticin for several weeks.

Techniques of microscopy

Adult *Xenopus* were anaesthetized by cooling (1 h 0 °C) and the kidneys were dissected from the abdominal cavity. Slices (approx. 3 mm) were immersed in 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.3, at 4 °C, cut manually in the transverse plane along the anteroposterior length and left for a further 2 h in fixative at 4 °C. The slices were then infiltrated with 20% sucrose in sodium phosphate buffer, pH 7.3 (overnight, 4 °C). Transverse cryostat sections 10 μm thick were cut and collected on gelatin-coated glass slides. Endogenous peroxidase was inactivated by treatment with 1% (v/v) H_2O_2 (30 min at room temperature) and non-specific sites were blocked for 1 h at room temperature in PBS containing 2% (v/v) goat serum and 0.3% BSA. The sections were incubated overnight with primary antibody no. 1466 and competitors at the indicated concentrations in PBS/0.1% (v/v) goat serum/0.3% BSA at 4 °C. After being rinsed three times in PBS, the slides were incubated for 1 h with biotinylated goat anti-rabbit IgG (dilution 1:200) in PBS at room temperature. The sections were subjected to the avidin/biotin/horseradish peroxidase protocol with the ABC kit of Vectastain™ and revealed by incubation for 50 s in 3,3′-diaminobenzidine tetrahydrochloride (Fast DAB; Sigma).

Preparation of cultured cells for immunofluorescence was performed as described [23] with 1 μg/ml primary antibody no. 1466. Images were taken with a confocal scanning laser microscope (Zeiss). Fluorescein-labelled cells were observed with an excitation wavelength of 488 nm and emission was filtered with a Zeiss 515-565 nm filter with a ×40 Planapo oil objective (numerical aperture 1.4). Negative controls were obtained both

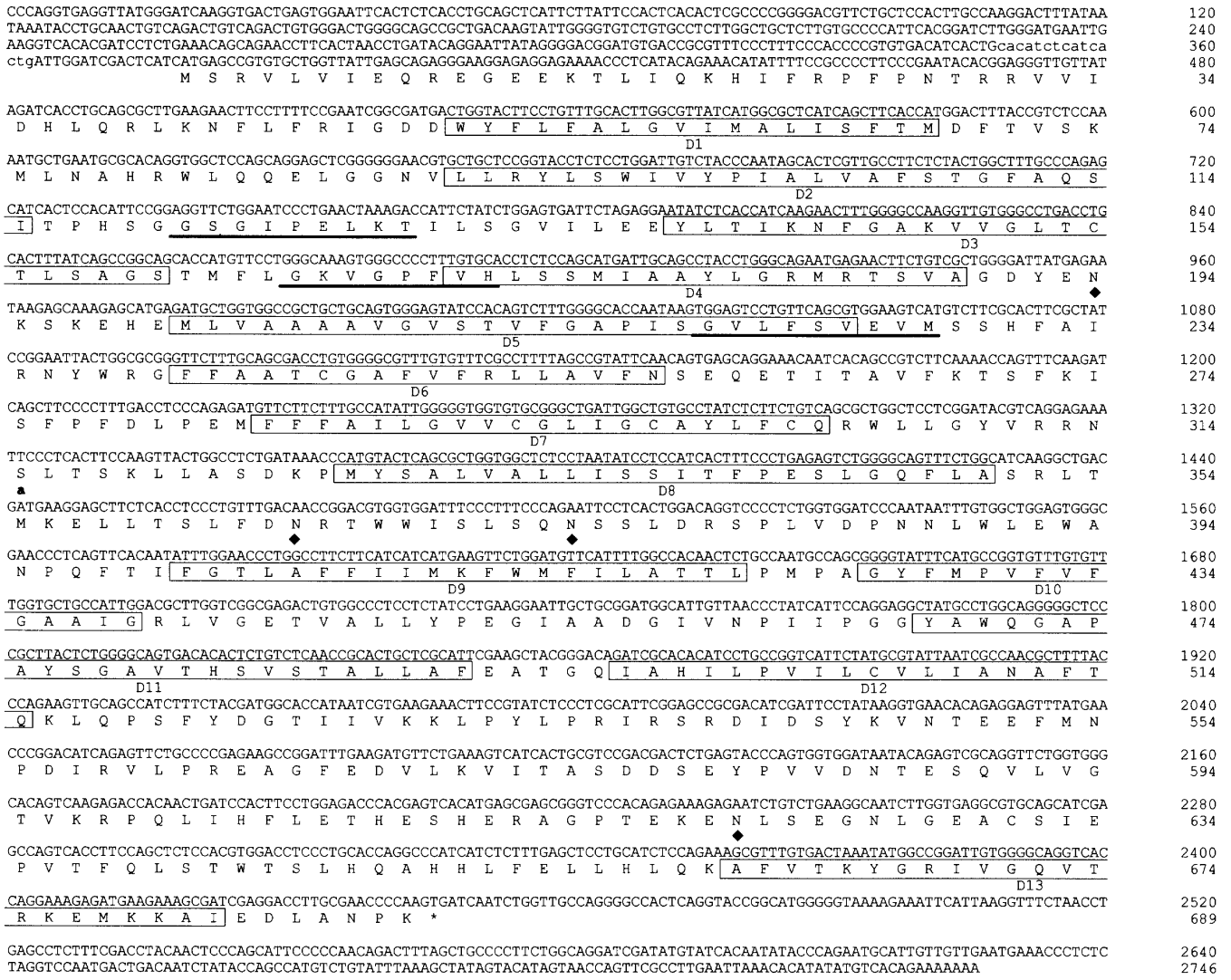

Figure 1 cDNA sequence and predicted amino acid sequence of xClCK

The nucleotide sequence 1–667 was obtained by 5′ RACE PCR and sequence 434–2746 derives from clone X6 obtained by library screening. Lower-case letters indicate a nucleotide stretch that was present in half of the sequenced clones. Boxed amino acid sequences labelled D1 to D13 are similar to the hydrophobic domains previously characterized in other members of the ClC family; underlined amino acid sequences are domains highly conserved throughout the ClC family. Symbols: ◆, potential N-glycosylation sites; **a**, potential protein kinase A phosphorylation site; *, termination codon.

by treatment with preimmune serum and without the addition of primary antibody.

Electrophysiological analysis

Recordings were made with an Axopatch 200 A amplifier and pClamp6 software (Axon Instruments) in the whole-cell configuration of the patch-clamp technique. Sampling and low-pass filter frequencies were set to 10 and 1 kHz respectively. A minimal 75 % compensation of series resistance (less than 10 MΩ) and capacitative currents was achieved. Currents were evoked by 200 ms depolarizing and hyperpolarizing steps from 0 mV holding potential to voltages ranging from −100 to 100 mV (10 mV increments). Bath and pipette solutions were designed to record Cl^- currents in isolation. The bath solutions contained (in mM): 100 NaCl, 30 tetraethylammonium chloride, 10 Hepes, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose (adjusted to pH 7.4 with NaOH). The osmolarity of the external bath solution was adjusted to 300 m-osM with sucrose. In experiments without bivalent cations, $CaCl_2$ and $MgCl_2$ were omitted and 100 μM EGTA was added. The pipette solutions contained (in mM): 1 $MgCl_2$, 10 tetraethylammonium chloride, 10 Hepes, 3 $CaCl_2$ and either 100 CsCl, 5 EGTA (pCa 7) or 85 CsCl, 30 EGTA (pCa 8). The pH was adjusted to 7.2 with CsOH.

RESULTS

cDNA cloning and sequence analysis

Degenerate oligonucleotides CL1.s (512-fold degeneracy) and CL3.as (1536-fold degeneracy), designed against two highly conserved regions of ClC channels (Figure 1), were used to amplify cDNA from *Xenopus* oocyte. A PCR product of 330 bp, as expected for a member of the ClC family, was obtained and cloned. Two independent plasmids displayed identical sequences

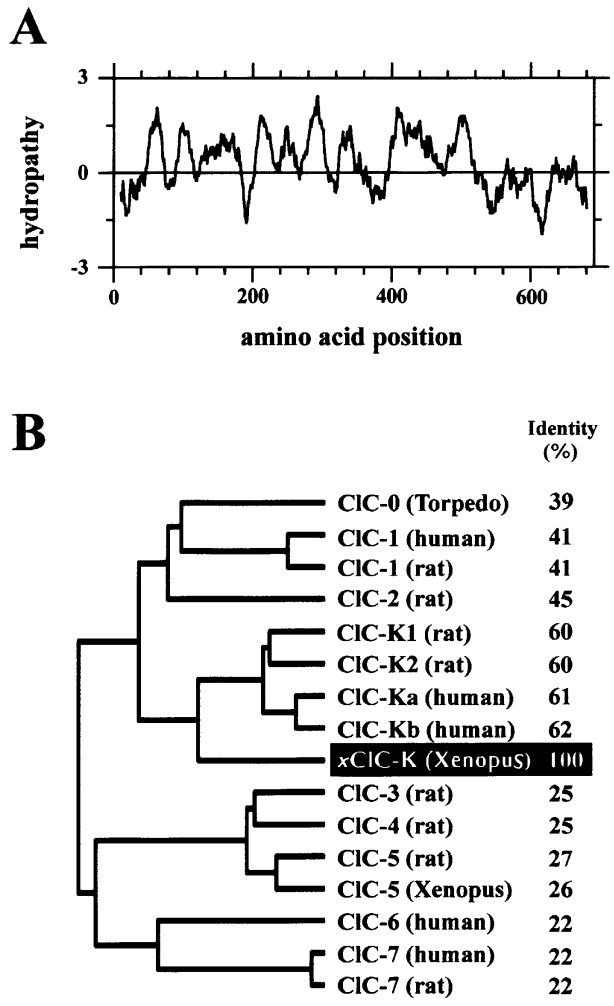

Figure 2 Sequence analysis and comparison of xClC-K

(**A**) Hydropathy plot of xClC-K by the Kyte–Doolittle method (window size, 20 residues). (**B**) Similarity of the xClC-K protein sequence to other members of the ClC family. The dendrogram was obtained by alignment of the sequences with the PILEUP program, followed by manual adjustments, running of the DISTANCE program to obtain an uncorrected distance matrix and construction of the tree with the GROWTREE program by using the UPGMA algorithm. GenBank/EMBL accession numbers are: ClC-0 (*Torpedo marmorata*), X56758; ClC-1 (human), Z25884; ClC-1 (rat), X62894; ClC-2 (rat), X64139; ClC-K1 (rat), D13927; ClC-K2 (rat), D26111; ClC-Ka (human), Z30643; ClC-Kb (human), Z30644; ClC-3 (rat), D17521; ClC-4 (rat), Z36944; ClC-5 (rat), Z56277; ClC-5 (*Xenopus*), Y09940; ClC-6 (human), X83378; ClC-7 (human), Z67744; ClC-7 (rat), Z67743.

and eight additional clones were characterized by PCR amplification with CL1.s/CL3.as and subsequent digestion with *Xba*I and *Pst*I. All clones except one yielded the expected pattern of amplified products and restriction digests, indicating that PCR generated a single major product (results not shown). The 330 bp fragment was used to screen a size-selected cDNA library from *Xenopus* ovary in λ Zap II. A single clone, X6, of 2.3 kb was obtained, which proved to be truncated at the 5′ end. A 700 bp fragment overlapping with X6 was obtained by nested 5′-anchored PCR from oocyte cDNA. Four independent subclones of the 5′ region gave nearly identical sequences. Four point variabilities, all in one clone out of four, were found at positions 191, 264, 269 and 403, caused by polymerase infidelity. An insert of 15 bp was observed in two clones at positions 349–363 that could have been due to misreading of the polymerase in a

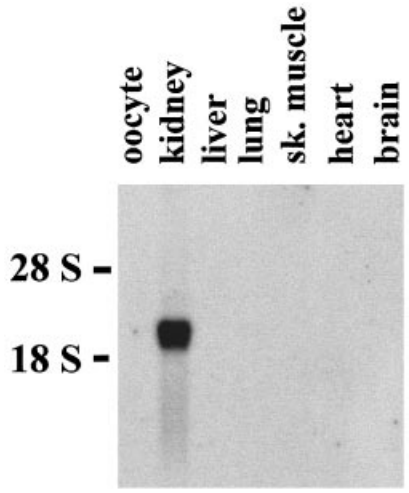

Figure 3 Tissue distribution of xClC-K transcripts

Northern blots [2 μg of poly(A)$^+$ RNA per lane] were probed with the [^{32}P]dCTP-labelled *Cla*I/*Sac*I 340 bp fragment (map coordinate 2002–2343). Lanes loaded with 10 μg of rat brain total RNA were run on each side of the gel; the migrations of 18 S and 28 S rRNA were used as molecular mass standards. The autoradiogram was scanned digitally with an Agfa Arcus II scanner and processed with Adobe Photoshop 4.0 in a PC environment.

repetitive region, to alternative splicings or to allelic variation. The total sequence of the transcript was 2739 bp long, down to the first nucleotide of the poly(A) sequence (Figure 1). A 2047 nt open reading frame starting at an ATG codon (380) and ending at a TGA codon encoded a 689-residue polypeptide. The polypeptide displayed the primary structure of a member of the ClC family. It contained the characteristic 13 hydrophobic domains (Figure 2A) and the three consensus sequences, between domains D2 and D3, at the N-side of D4 and at the C-side of D5. A sequence comparison with other members of the family showed that the protein is closest to the ClC-K subset specifically expressed in kidneys (Figure 2B). Potential N-glycosylation sites were found at positions Asn^{194}, Asn^{365}, Asn^{375} and Asn^{634}. A potential protein kinase A phosphorylation site, not conserved in other ClC family members, was also found at residue Ser^{315}.

Tissue distribution

We performed Northern analysis on different *Xenopus* tissues, using a restriction fragment that was most specific for the transcript. As shown in Figure 3, expression was restricted to *Xenopus* kidney; there was no detectable signal in the other tissues tested, even after longer autoradiography. This result confirmed that we had cloned a batrachian homologue of ClC-K channels shown to be specific for kidney in mammals [5–8]. We named it xClC-K in accordance with the current nomenclature of ClC channels. More surprising was the absence of signal from oocyte mRNA in which xClC-K had been cloned. We hypothesized that the channel could be synthesized at earlier stages of maturation and that the transcript was no longer available. Alternatively it could be expressed in associated cells of the ovary, which could not be entirely dissected away in some of our preparations. Northern blotting on mRNA from total ovary cells did not improve the signal (results not shown). To study the protein distribution, we raised antibodies against a fusion protein containing a C-terminal fragment of xClC-K, in which similarity to other ClC members was the lowest. SDS/PAGE analysis and

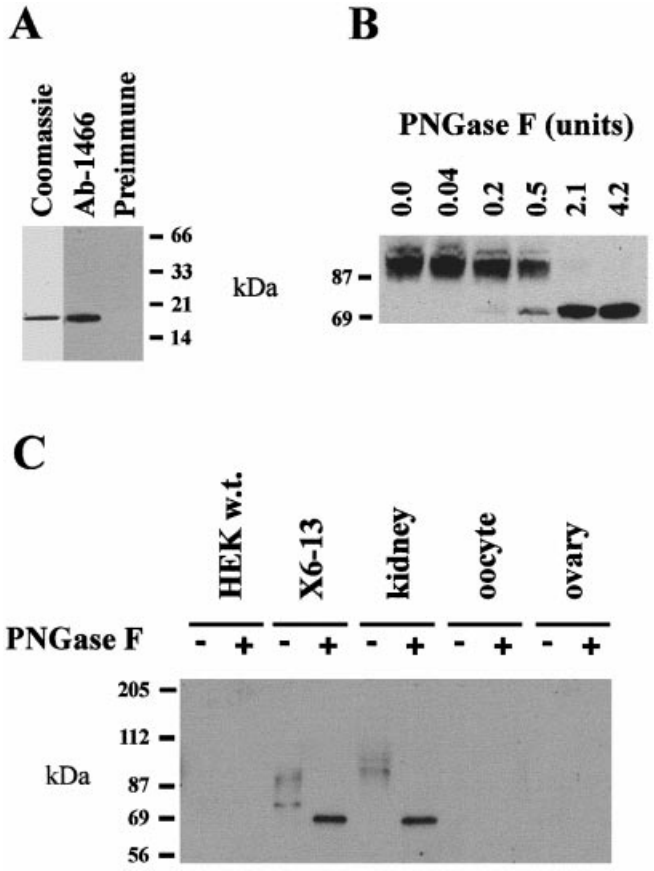

Figure 4 Western blot analysis with antibody no. 1466

(**A**) Efficiency of the antibody checked on the His_6-tagged polypeptide [15% (w/v) polyacrylamide gel; 0.5 μg of purified protein per lane]. (**B**) Digestion of crude membranes from *Xenopus* kidney with increasing amounts of PNGase F [30 min digestion; 7.5% (w/v) polyacrylamide gel; 40 μg of protein per lane]. (**C**) Western blot analysis of glycosylated and deglycosylated xClC-K in different tissues and cell lines; the amounts of protein were as follows: HEK wild-type (w.t.), 12 μg; X6-13 (stably transfected HEK cell lines expressing xCLC-K), 2 μg; *Xenopus* kidney, 50 μg; *Xenopus* oocyte, 30 μg; *Xenopus* ovary, 50 μg [7.5% (w/v) polyacrylamide gel]. The picture on film was processed digitally as in Figure 3.

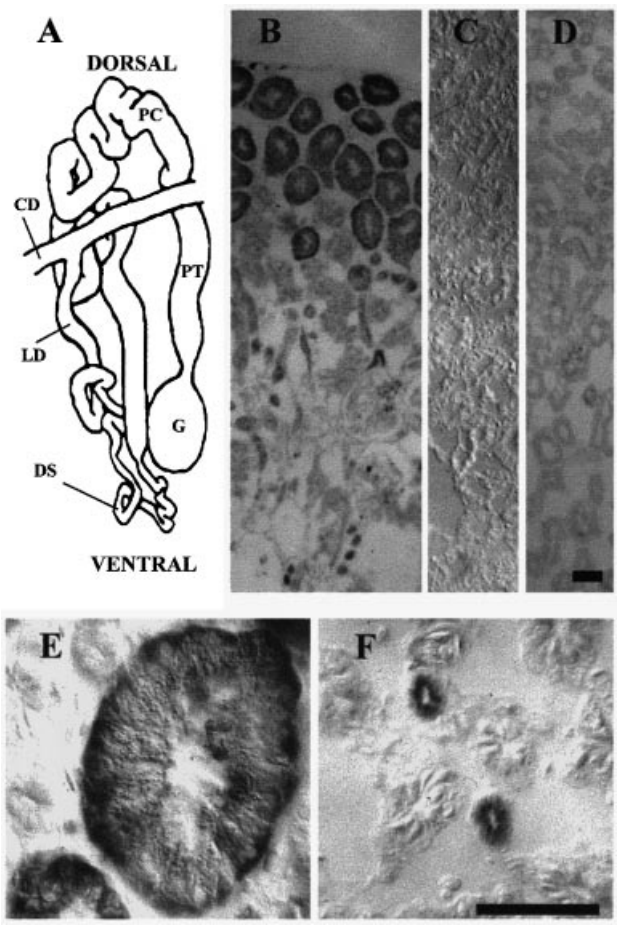

Figure 5 Immunohistochemical localization of xClC-K in *Xenopus* kidney

Transverse sections (10 μm thickness) were incubated with the indicated amounts of antibody no. 1466 and competitor and revealed with peroxidase-conjugated secondary antibody. (**A**–**D**) Diagram (**A**) and photomicrographs (**B**–**D**) (all at the same scale) oriented along the same dorsoventral axis. (**A**) Schematic drawing adapted from [26] showing the orientation of a batrachian nephron. G, glomerule; PT, proximal tubule; PC, proximal convoluted tubule; DS, diluting segment; LD, late distal tubule; CD, collecting duct. (**B**) After incubation with antibody no. 1466 (dilution 1:50); (**C**) after incubation without primary antibody; (**D**) after incubation with antibody no. 1466 (dilution 1:50) and competing His_6-tagged polypeptide (1 μg/ml). (**E**, **F**) Enlargement (to the same scale) of tubules labelled under the same conditions as in (**A**): (**E**) large dorsal tubules (proximal convoluted); (**F**) small medio-ventral tubules (diluting segments). Scale bars, 50 μm. Photomicrographs were processed digitally and assembled as in Figure 3.

Western blotting showed that antibody no. 1466 specifically recognized the fusion protein (Figure 4A). Crude membranes from *Xenopus* kidney were analysed by SDS/PAGE and Western blotting and displayed a heterogeneous pattern of bands in the size range 90–110 kDa (Figures 4B and 4C). Prolonged denaturation at high temperature (15 min at 95 °C) tended to decrease the amount of immunoreactive proteins markedly without improving the resolution. Controls with preimmune serum did not reveal any signal (results not shown). Deglycosylation with PNGase F decreased the pattern to a single band of 70 kDa, close to the computed size of 77 kDa (Figures 4B and 4C). Partial deglycosylation displayed a simple pattern with no intermediate band between the fully glycosylated and the deglycosylated species, indicating that a single asparagine residue was effectively glycoconjugated (Figure 4B). Studies on other ClC channels have shown that asparagine residues corresponding to Asn^{194} are not glycosylated [7]; neither are the sites present in the same cytoplasmic domain as Asn^{634} [24]. Either or both of the counterparts of the Asn^{365} and Asn^{375} sites are conserved throughout the ClC family and have been demonstrated to be effectively glycosylated in ClC-0 [25], ClC-1 [24], ClC-K1 and ClC-K2 [7]. Consequently, either Asn^{365} or Asn^{375} is most probably glycosylated in xClC-K. No signal was observed after Western blotting of oocyte and ovary membrane proteins, indicating that the protein is not significantly expressed in this organ (Figure 4C).

Localization in kidney tubules

We took advantage of the regular dorsoventral orientation of frog nephrons [26,27] to assess the presence of xClC-K in the different tubule segments. As shown in Figure 5, we observed a significant labelling of large tubules confined to the dorsal side of the kidney, where xClC-K was essentially expressed on the basolateral side of the epithelium. Both the position and the large diameter of the tubules allowed their straightforward identification as proximal convoluted tubules. In addition, xClC-K immunoreactivity was also observed on the apical side of the

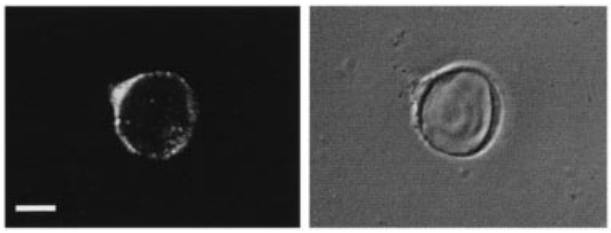

Figure 6 Subcellular localization of xClC-K in the X6-13 cell line

Cells labelled with antibody no. 1466 were examined by confocal microscopy. Left panel: immunofluorescent confocal image (0.2 μm horizontal optical section). Right panel: the corresponding phase image of the cell as obtained without modification of the vertical focus. Note the annular distribution of antigen at the plasma membrane. Scale bar, 10 μm. Digital images were processed as in Figure 3.

epithelial cells lining the lumen of the thinnest tubules present in *Xenopus* kidney. Their size and distribution in the medial and ventral parts are indicative of the so-called diluting segment of amphibian kidney.

Expression of xCLC-K in HEK-293 cells

A eukaryotic expression plasmid that contained the full-length open reading frame of xClC-K was constructed in pCDNA1 and stably transfected into HEK-293 cells. Two clonal cell lines, X6-10 and X6-13, were positive for antigen no. 1466. Clone X6-13 was generally used for further studies. When observed under confocal fluorescence microscopy, most of the fluorescent signal was associated with the plasma membrane of transfected cells (Figure 6), whereas no fluorescence was observed on wild-type HEK-293 cells (results not shown), indicating that xClC-K was efficiently targeted to the plasma membrane. Western blot analysis on crude membrane proteins from X6-13 revealed the expression of a polypeptide displaying the same heterogeneous appearance as did the native *Xenopus* kidney protein (Figure 4C). An additional immunoreactive band of 72 kDa, representing core glycosylated protein in transit through the intracellular compartments, was indicative of its high rate of synthesis. After deglycosylation with PNGase F a single band of 70 kDa was revealed as for the native protein, indicating that the full-length polypeptide was expressed by the transfected cells (Figure 4C).

Functional studies

Patch-clamp studies in the whole-cell configuration were performed on X6-13 cells in conditions under which Cl^- currents could be recorded in isolation (see the Experimental section). No significant current was detected in the range of -100 to $+100$ mV in the presence of either 10 or 100 nM intracellular free calcium. Cl^- currents, presumably not mediated by cystic fibrosis transmembrane conductance regulator, have been shown to be induced by cAMP-dependent protein kinase in several tissues and cell lines including kidney [9]. Furthermore, a perfect consensus site of phosphorylation by protein kinase A (Arg-Arg-Xaa-Ser), which is only partly conserved in other ClCs, was found at Ser^{315}. This led us to test the sensitivity of xClC-K to conditions promoting cAMP-dependent phosphorylation. After treatment with 10 μM forskolin or 1 mM 8-bromo-cAMP for 10 min, no current was observed. We also tested the effect of transient elevation of intracellular Ca^{2+} in HEK-293 transfected cells co-expressing xClC-K and voltage-activated Ca^{2+} channels without observing any current in addition to the Ca^{2+} flux (results not shown).

DISCUSSION

A single cDNA clone encoding a ClC channel was isolated from *Xenopus* oocyte. The transcript proved to be poorly expressed and its protein to be absent from this cell type, but sequence comparison and organ localization demonstrated that we cloned a *Xenopus* homologue of mammalian ClC-K channels. We expressed xClC-K in HEK-293 cells and isolated stable transfected cell lines, because the homogeneity of the cells and the unlimited supply of material are convenient for patch-clamp studies and biochemical and immunochemical analyses. We showed that HEK-X6 cell lines synthesized a full-length glycosylated xClC-K, targeted to the plasma membrane, but, under all conditions investigated, no specific Cl^- current could be recorded. The most likely explanation for non-functional expression might relate to the multimeric assembly of ClC channels. Biochemical and functional analyses have shown that many ClC channels display a dimeric quaternary structure [25,28–30], suggesting that dimerization is probably a general feature of the ClC channel's architecture. Functional expression of ClC-K might therefore depend on its association with another ClC subunit of as yet unknown nature. However, combinations of different mammalian ClC-K in *Xenopus* oocytes did not generate any measurable current [7]. The possibility remains, which has not been yet investigated to our knowledge, that ClC-K should be associated with regulatory subunits other than a ClC to generate Cl^- currents. Another possibility would be that ClC-K are functional channels in which the opening is mediated by an as yet uninvestigated second messenger pathway. However, this seems unlikely in view of the multiple trials of functional expression performed with ClC-K of diverse origins by our group and others [7,8] under conditions similar to those that activate Cl^- channels in renal epithelia. Moreover, a small background activity of the channel would be expected, even without appropriate stimulation, because heterologous systems generally overexpress the proteins of interest. A combination of biochemical analyses of the native complexes, reconstitutions and multivariate electrophysiological recordings is therefore still needed for a description of the functioning of ClC-K channels.

The behaviour of xClC-K on SDS/PAGE is unusual in that a single glycoconjugate generates a heterogeneous pattern of bands with a large contribution of up to 40 kDa to the apparent size. Moreover, the glycosylated xClC-K protein systematically displayed a fainter signal than PNGase F-treated samples on Western blot analysis. As the antibody recognizes epitopes remote from potential glycosylation sites, direct interference with glycosylated residues can be excluded. The glycoconjugate is therefore probably strongly associated with other elements at the extracellular side of the membrane bilayer. As this anomalous behaviour in denaturing conditions was similar for native xClC-K isolated from *Xenopus* kidney and the recombinant protein expressed in the cell line, we suggest that essential covalent post-translational modifications of xClC-K are conserved in the heterologous system.

The amphibian kidney has long been recognized as a pivotal model in kidney physiology. It displays evolutionary, morphological and functional properties that led to fruitful comparative studies and in several cases enabled pioneering experiments in which mammalian preparations were not practically suitable [31,32]. Ontogenically, the vertebrate kidney is characterized by the developmental succession of up to three renal structures: the pronephros, the mesonephros and the meta-

nephros. Whereas the mammalian kidney develops into a typical metanephros, the batrachian kidney stops its development at the mesonephros stage [33]. XClC-K is the second Cl^- channel predominantly expressed in kidney that has been cloned from *Xenopus* after xClC-5 [34], the homologue of mammalian ClC-5 channels [35]. Because both display properties very similar to their respective mammalian counterparts, this suggests that Cl^- transport relies on a common set of ClC channels acting in a similar fashion in both primitive and more evolved kidneys. Thus future insights obtained in the amphibian model at the molecular level might easily be extended to higher vertebrates.

Localization performed on rat kidney by reverse-transcriptase-mediated PCR and immunohistochemistry has pointed to a differential expression of its two known ClC-K forms. ClC-K2 displayed a widespread distribution in all tubules, whereas ClC-K1 was restricted to the proximal straight tubule and the thick ascending limb of the loop of Henle [7,36]. Both channels were essentially associated with the basolateral side of the epithelium. At the morphological level, the batrachian kidney is less complex than the mammalian. It does not provide a juxtaglomerular apparatus and the nephrons, which do not have a true loop of Henle, are regularly oriented along a dorsoventral axis (see Figure 5). We have shown here that xClC-K is expressed in two distinct regions of the *Xenopus* nephron: the proximal convoluted tubule, whose role in salt reabsorption is similar to the mammalian cortical proximal tubule, and the diluting segment, which displays transport properties close to those of the thick ascending limb of the loop of Henle in mammals [37]. In the proximal tubule, the channel resides at the level of the basolateral membrane, but the polarity reverses in the diluting segment where most of the labelling is found with an apical distribution. Such a differential intracellular sorting, which probably reflects the different functional specializations of each segment related to Cl^- permeability, was unexpected for a single protein. We cannot exclude the possibility that our antibody recognizes two or more related ClC-K forms bearing different sorting signals. In this case, however, both these proteins and their transcripts should be of very similar sizes.

The *Xenopus* kidney regulates body fluids and salt balance in an aquatic environment and excretes diluted urine, whereas the kidney of mammals adapted to terrestrial life actively reabsorbs water to avoid dehydration. Consequently, the availability of homologous mediators of ionic fluxes such as ClC-K channels will be helpful in improving our understanding of their function at the molecular level as well as of differences required for environmental adaptation.

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