ClC-6 and ClC-7 are two novel broadly expressed members of the CLC chloride channel family

Silke Brandt, Thomas J. Jentsch*

Center for Molecular Neurobiology Hamburg (ZMNH), Hamburg University, Martinistrasse 52, D-20251 Hamburg, Germany

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Abstract We cloned two novel members of the CLC chloride channel family from rat and human brain. ClC-6 is a 97-kDa protein, and ClC-7 a 89-kDa protein roughly 45% identical with ClC-6. Together they define a new branch of this gene family. Both genes are very broadly expressed, e.g. in brain, testes, muscle and kidney. In mouse embryos, both genes are expressed as early as day 7. While the human gene for ClC-6 is located on human chromosome 1p36 and shares this region with hClC-Ka and hClC-Kb, ClC-7 is on 16p13. ClC-6 has a highly conserved glycosylation site between transmembrane domains D8 and D9, while ClC-7 is the only known eukaryotic ClC protein which lacks this site. Hydropathy analysis indicates that domain D4 cannot serve as a transmembrane domain. Both ClC-6 and ClC-7 cannot be expressed as chloride channels in *Xenopus* oocytes, either singly or in combination.

Key words: Chromosomal localization; Anion channel; Tissue distribution; Xenopus oocyte

1. Introduction

The expression cloning of the voltage-gated chloride channel CIC-0 from the electric organ of the marine ray Torpedo marmorata [1] led to the subsequent discovery of a large gene family of related proteins [2], which even includes members from Saccharomyces cerevisiae [3] and Escherichia coli [4]. In a single mammalian species, seven different ClC genes have been described so far [5-12]. These display differential tissue distribution and perform diverse functions. Thus, ClC-1, the main skeletal muscle chloride channel [5], is important for stabilizing the plasma membrane voltage of muscle. Its mutational inactivation both in animal models [13] and in humans [14] leads to myotonia, an inherited muscle disease caused by an electrical hyperexcitability of the muscle membrane. CIC-2 is an ubiquitously expressed chloride channel [6] which can be activated by hypotonic swelling and may be important for cell volume regulation [15] and regulation of neuronal excitability [16]. ClC-5, the most recently discovered member of this gene family, is highly expressed in kidney, but to some degree also in other organs, such as lung and brain [11,12]. Mutations in this chloride channel lead to X-linked kidney stone disease [11,17]. The function of other members of this gene family (ClC-Ka and ClC-Kb [7,8], ClC-3 [9,18] and ClC-4 [2,10]), however, is currently obscure. This problem is compounded by the fact that functional expression as chloride channels, as reported by one group [7,9], could not be reproduced by other groups [2,8,18]. Thus, they should presently rather be classified as putative chloride channels. Site-directed mutagenesis has begun to shed light on the structure-function relationship of the expressible channels [15,17,19,20].

In an attempt to learn more about this family of chloride channels, their functions and their possible role in disease, we have now cloned and analysed two novel mammalian ClC cDNAs. Starting from an established sequence tag (EST) from the genome project [21], we have cloned ClC-6. This putative channel was then used as the basis for a PCR-based strategy which led to the identification of ClC-7, which is only 45% identical with ClC-6. Both putative channels show a nearly ubiquitous tissue distribution, but cannot be expressed functionally as chloride channels in the *Xenopus* oocyte system.

2. Materials and methods

2.1. Cloning of ClC-6 and ClC-7 cDNAs

hClC-6 was cloned from a human frontal cerebral cortex cDNA library in λ ZAPII (Stratagene No. 936212) using three non-overlapping 42-bp oligonucleotides complementary to the EST with the accession No. D28475 [21]. They were end-labeled with ³²P and hybridized to the library at 42°C in 1 × SSC, 5 × Denhardt's and 0.1% SDS. Four independent cDNA clones hybridizing to all three probes were isolated and analysed. Two clones (HC11, 2525 bp; B2A, 4140 bp) covered the full coding region which was sequenced on both strands using an automated DNA sequencer (ABI 373) or manual sequencing, when appropriate. In the coding region overlapping with EST D28475, its sequence was fully confirmed. The sequence was deposited in the Genbank/ EMBL database, accession No. X83378.

To clone rClC-7, we designed several 'subfamily primer' pairs based on the principles outlined in section 3. The successful combination was directed against the CIC-6 amino-acid sequences EVKCYLNG and GKEGPMIH flanking domain D3. The degenerate primers represented all possible codons and incorporated 5' EcoRI sites for subcloning. Polymerase chain reaction (PCR) was performed on about 100 ng of rat brain cDNA using 60 cycles of 30 s at 94°C, 45 s at 53°C and 45 s at 72°C. Amplification products of the right size (161 bp) were cloned and sequenced. About half were rClC-6 fragments and the other class was different from, but homologous to, known ClC genes. This fragment was used to clone full-length ClC-7 from a rat brain cDNA library in λ ZAP (a gift from W. Meyerhof), a portion of which was then used to isolate a human CIC-7 cDNA (which lacks the 5' end) from the human cerebral cortex library. The sequences of these clones were deposited in the Genbank/EMBL database, accession No. Z67743 and Z67744.

2.2. Chromosomal localization

Genomic ClC-6 and ClC-7 clones were isolated from a human placenta library in λ FIXII (Stratagene No. 946206) and their inserts were subcloned into pBluescript plasmid. Their identity was confirmed by partial sequencing using internal primers. Chromosomal localization by fluorescent in situ hybridization (FISH) (BIOS Laboratories, New Haven, CT) was performed by labeling the genomic clones with biotin dUTP and hybridizing to normal metaphase chromosomes derived from PHA stimulated peripheral blood lymphocytes, subsequent incubation with fluoresceinated avidin and counterstaining with propidium iodide. This indicated that ClC-6 is located on chromosome 1p36 and ClC-7 on 16p13. The identity of the chromosomes was then confirmed by co-hybridizing the clones with respective chromosome-specific probes.

^{*}Corresponding author. Fax: (49) (40) 4717-4839.

2.3. Northern blots

Blots with 2 μ g polyA⁺ RNA from several human tissues (Clontech) or whole mouse embryos (embryonal days 7–17) were hybridized under high stringency conditions with ³²P-labeled cDNA inserts from hClC-6 or hClC-7 (or rClC-7 for the mouse blot) using standard conditions. Autoradiography was performed either with a phosphoimager (Fuji BAS1500) or films.

2.4. Expression in Xenopus oocytes

hClC-6 was assembled from two independent, overlapping clones by ligating the *EcoRI/NarI* fragment of the 5' clone HCl1 and the *NarI/ Hind*III fragment of clone B2a into the *EcoRI/Hind*III sites of the expression vector pGemHE (a gift from A. Baumann). The initiator ATG with its endogenous Kozak consensus site is the first ATG 30-bp downstream of the polylinker. rClC-7 has an *NcoI* site on its initiator ATG which was used to clone it into the *NcoI* site of PTLN (Lorenz and Jentsch, in prep.). Both vectors place the start site for translation downstream of the *Xenopus* β -globin 5' untranslated region [22]. Since our human ClC-7 clone lacks about 15 amino acids at the 5' end, we also constructed a chimeric rat/human ClC-7 in which the first 16 amino acids of rat ClC-7 were put before the first amino acid (aspartate) in front of human ClC-7 using recombinant PCR. Using Sp6 RNA polymerase, capped cRNA was prepared from these constructs after linearization. About 5–50 ng of cRNA was injected into *Xenopus* ooyctes prepared and handled as described [1]. After 2–3 days at 18°C, they were investigated by two-electrode voltage-clamping.

	MANVSKKVSW	SGRDRD···G	· · · · · · · · · GQ ·	DEE·····	· · · · - · · - H	1 · · · · · · I · Q · N	N • • • • • • • • • • • • • • • • • • •	·EV····T··	78
hCLC-7 hCLC-6		DEEA	APLLERTARP	GGGTPLLNGA	GPGAARQSPR MAGCR	SALFRVGHMS	SVELDDELLD	POMDPPHPFF	ף זי 35
rCLC-2			м	AAATAAAATV	AGEGMEPRAL	QYEQTLMYGR	YTQELGAFAK	EEAARIRLGO	; 51
rCLC-7									158
hCLC-7	KEIPHNEKUL	SLKYESLDYD	NSENOLFLEE	ERRINHTAFR	TVEIKRWVIC	ALIGUITGLV	ACFLOLVMEN	LAGEKYRVIK	
rCLC-0	PEPWKGSPSA	RATHELLEYG	OSECARCETC	SVECHKELVS	RIMAVAMIVV RVG-FUMTEL		GUENDEFMEL	CLODODEMSE	2 114
	-0			011101111 210		D1		CDŐYŐŐutrot	130
rCLC-7	D		···s····	<u></u>	<u></u>	<u> </u>	·····	<u> </u>	238
hCLC-7	GNIDKFTEKG	GLEFSLILWA	GFNLTFVFLA	SVIMAFIEPV SLIM-LIEPV	AAGSGIPQIK	CFLNGVKUFH CYLNGVKWFG	VVRLKTLVIK IVRLRTLLCK	VISCVILLSVVG	193
rCLC-2	GLNT	NILLOYLAWV	TYPVVLITFS	AGFTQILAPO	AVGSGIPEMK	TILRGVVLKE	YLTUKIFVAK	VIGLTCALGS	204
		ببينندهم	D2					D3	,
rCLC-7 hCLC-7	GLAVGKEGPM	IHSGSVIAAG	ISQGRSSSIK	RDFKIFEYLR	RDTERRDFVS	AGAAAGVSAA	FGAPVGGVLF	SLEEGASFWN	318
hCLC-6	GLEVGKEGPM	IHSGSVVGAG	LPOFOSISIER	KIQFNEPYER	SORDKRDFVS	AGAAAGVAAA	FGAPIGGILF	SLEEGSSFWN	273
rCLC-2	GMPLGKEGPF	VHEASMCAAL	LSKFLS	LFGGIYE	NESRNTEMLA	AACAVGVGCC	FNAPICGVLF	SIEVISTEFA	277
rCLC-7						D	5		392
hCLC-7	OFLTWRIFFA	SMISTFTLNF	VLSIYHGNMW	D-LSSPGLIN	FGRFDSE	КМАУГТНЕ	IPVFIAMGVV	GGVLGAVENA	
hCLC-6	VENTWARCER	SMEATFTLNF	FREGIQEGS	GSFQLPGLLN	FGEFKCSDSD	KKCHLWITAMD	LGFFVVMGVI	GGLLGAIFIC	353
rulu-z	VIN IMPOLEN	D6	HAVWINKDEET	1 TALF KIRF -		REDFFFDEQE	CPAPAVIGIA	D7	346
rCLC-7	f iterfor			-M					449
hCLC-6	LINKRLAKYRM		PKPKLVRVLE	SULVEAVIAT	WEVASMVLG	ECROMSSSSQ	IGNESFOLOV	TEDVNSSIKT	427
rCLC-2	LNRKIVQVMR	KQKTINRFLM	KKRLLFPALV	TILESTINFP	PGEGQEMAGQ	LSQKETLVTL	FDNRTWVROG		416
		, –		D8			giye	l giyc	
rCLC-7	· <u>··</u> ···· <u>··</u> ·			<u>M</u>					529
hCLC-7	lfoadgeyns i	MAAAFFNTPE	KSVVSLFHDP	PGSYNPLITUG D	LFTLVYFELA	CWTYCLTWSA	GVEIPSLING	AAWGRLEGIS	020
hCLC-7 hCLC-6	LFCADGEYNS FFCPNDTYND	MAAAFFNTPE MATLFFNPOE	KSVVSLFHDP SAILQLFHQ- 1	PGSYNPLITIG IGTFSPVILA	LFTLVYFFLA LFFVLYFLLA	CWTYGLIVSA CWTYGISVPS	GVFIPSLUIG	AAWGRLFGIS AAFGRLVANV	506
hCLC-7 hCLC-6 rCLC-2	LFCADGEYNS FFCPNDTYND -LVEDLGAPS	MAAAFFNIPE MATLIFFNPQE ISQAWS	KSVVSLFHDP SAILQLFHQ- 1	PCSYNPLITIG DGTFSPVTIA PRANVFLITIV	LFTLVYFFLA LFTVLYFLLA LFILMKFWMS	CWTYGLIWSA CWTYGISVPS ALATTIEVPC	GVFIPSLING GLEVPSLLOG GAEMEVFVIG	AAMGRLFGIS AAFGRLVANV AAFGRLVGES	506 482
hCLC-7 hCLC-6 rCLC-2	LFCADGEYNS FFCPNDTYND -LVEDLGAPS	MAAAFFNITPE MATLFFNPOE ISQAWS	KSVVSLFHDP SAILQLFHQ- 1	PGSYNPLITIG DGTFSBVTLA PRANVFLITLV	LFTLVYFFLA LFFVLYFILA FILMKFWMS	CWTYGLIVSA CWTYGISVPS ALATTIPVPC D9-D	GVFIPSLUIG GUFVPSLUCG GAFMEVFVIG 10	AAWGRLFGIS AAFGRLVANV AAFGRLVGES	506 482 602
hCLC-7 hCLC-6 rCLC-2 rCLC-7 hCLC-7	LFCADGEYNS FFCPNDTYND -LVEDLGAPS	MAAAFFNTPE MATLFFNPOE ISQAWS	KSVVSLFHDP SAILQLFHD- P	CLCCIVRMTL	EFTLVYFELA EFFVLYFILA FILMKFWMS	CWTYGLIWSA CWTYGISVPS ALATTIPVPC D9 - D SNVTYGPPIM	GVFIIPSLING GLEVPSLIGG GAEMPVFVIG 10 LVLMIAKIVG	AAWGRLFGIS AAFGRLVANV AAFGRLVGES	506 482 602
hCLC-7 hCLC-6 rCLC-2 rCLC-7 hCLC-7 hCLC-6 rCLC-6	LFGADGEYNS FFGPNDTYND -LVEDLGAPS I-SYLTGA LKSYIGLG	MAAAFFNTPE MATLFFNPOE TSQAWS AIWADP HIYS TDSSTYRIVP	KSVVSLFHDP SAILQLFHQ- P GKYAIMGAAA GTFALIGAAA	PGSYNFILTIG I DGTFSEVITIA PRANVFILTIV OLGGIVRMTL FLGGVVRMTL TLGGVVRMTL	FTLVYFFLA FFVLYFILA GILMRFWMS SLTVIMMEAT SLTVILIEST	CWTYGLIUSA CWTYGISVPS ALATTIFVPC D9 - D SNVTYGFPIM NEITYGLPIM COLAHUURW	GVFIIPSLING GLEVIPSLICG GAEMEVFVIG 10 LVEMTARIVG VTEMVARWIG IAVILIAVAVA	AAMGRIFGIS AAFGRIVANV AAFGRIVGES	506 482 602 578 561
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Fig. 1A. Sequence comparison of ClC-6, ClC-7 and ClC-2. Shown are the rat (prefix r) and human sequences (prefix h). For rat rClC-7, only those amino acids differing from hClC-7 are shown. Identical amino acids are indicated by dots. For hClC-7, we still miss the 5' end. ClC-6 and ClC-7 are approximately 45% identical and share about 21% identity with the ubiquitous volume-activated channel ClC-2 [6]. Residues identical between ClC-6 and ClC-7, or between all three proteins, are boxed. Putative transmembrane domains D1–D12 are highlighted by solid lines, and the highly conserved cytoplasmic domain D13 is indicated by a shaded bar. N-linked glycosylation consensus sites predicted to be extracellular are indicated. Note that ClC-7 lacks N-linked glycosylation sites between D8 and D9. All other known eukaryotic ClC proteins have such sites at this position, which are known to be used in the ClC-K proteins [8] and ClC-0 [24].

3. Results

Search of the Genbank/EMBL database using the TBlastN algorithm revealed a partial human cDNA sequence of 4815 bp (accession No. D28475) with significant homology to the ClC family of chloride channels. It represents a randomly picked cDNA clone from the human immature myeloid cell line KG-1 [21]. The protein sequence predicted by this partial clone begins at putative transmembrane span D5 and extends beyond the stop codon. Thus, we predicted that it lacks about 500 bp of 5' sequence, including the coding region for the first 4 transmembrane domains. To conform with the nomenclature for the ClC family [2], we named this protein hClC-6 (prefix h for human).

Using specific oligonucleotides we isolated 5 independent ClC-6 clones from a human brain cDNA library. Two overlapping clones covered the entire coding region. The initiator methionine was assigned to the first ATG in frame. Its surroundings conform to the consensus sequence of eukaryotic initiation of translation [23] and is preceded by a stop codon in frame. Its coding region predicts a 869 amino-acid protein with a molecular weight of 97 kDa (Fig. 1). Significant homology to other ClC proteins is spread over most of the protein, but is higher in putative transmembrane domains and neighbouring regions. In the central portion encompassing the transmembrane regions, ClC-6 is only roughly 29% identical with the



Fig. 1B. Dendrogram graphically showing the similarities between mammalian ClC proteins. Note that ClC-6 and ClC-7 form a distinct subbranch of the family, which is most closely related to the ClC-3, ClC-4 and ClC-5 group. This plot has been generated using the Pileup program of the GCG software package.



Fig. 2. Hydropathy analysis of ClC-6, ClC-7 and, for comparison, ClC-2. The Kyte-Doolittle scale was used with a window size of 19 residues. Note that the hydrophobic peak leading to the prediction of a putative transmembrane domain D4 is present in ClC-2, but is absent in ClC-6 and ClC-7. The weakly hydrophobic, but conserved domain D13 is now known to be cytoplasmic [15]. ClC-7 is shorter than ClC-6 primarily because of a shorter stretch between D12 and D13.

CIC-3, CIC-4 and CIC-5 branch and even less so to CIC-1, CIC-2 and CLC-K (~23%). Thus, it may represent the first member of a new subfamily of CIC proteins.

We pursued this hypothesis by designing 'subfamily' primers for RT-PCR cloning experiments. We first identified regions which are highly conserved within a given branch of the ClC family, but show significant divergence between branches. We designed degenerate oligonucleotide primers which allowed for all possible triplets encoding the amino acids found in CIC-6 in these specific regions. These primers will not recognize members of the ClC-1 and ClC-3 branches, but should amplify ClC-6 and hopefully other members of a new branch defined by ClC-6. Using several primer combinations based on this novel principle, we performed RT-PCR amplifications on rat brain cDNA. Indeed, we cloned a fragment encoding a novel ClC protein. It was used to isolate a full-length clone from a rat brain cDNA library. In addition, we isolated a human ClC-7 cDNA which, however, still lacks coding sequence for about 15 amino acids at the 5' end. Both clones were fully sequenced (Fig. 1A) and are 96% identical at the protein level. rClC-7 is predicted to be a 89-kDa protein having 803 amino acids.

Comparison with other CIC proteins indicates that CIC-6 and CIC-7 (which share about 45% identity) belong to a common, new branch of the CIC family (Fig. 1B). Compared with CIC-6, CIC-7 is smaller by more than 60 amino acids because of a shorter cytoplasmic stretch between D12 and D13. This stretch is poorly conserved between different CIC genes and differs in length between other CIC proteins as well.

ClC-6 has two consensus sites for N-linked glycosylation between putative transmembrane domains D8 and D9. In this extracellular loop, glycosylation sites are present in all published eukaryotic ClC proteins, including the yeast homologue



Fig. 3. Tissue distribution of ClC-6 and ClC-7 and their expression during embryonic development. Northern blots with $2 \mu g$ polyA⁺ RNA from several human tissues were probed with a human ClC-6 (A) or a human ClC-7 cDNA (B). Expression during mouse development was studied by analysing hybridization to $2 \mu g$ each of polyA⁺ RNAs from whole mouse embryos at days 7, 11, 15 and 17. ClC-6 message (C) increases slightly during the first 2 weeks, while ClC-7 (D) rather decreases. The blots were probed with human ClC-6 and rat ClC-7, respectively. Blots in A and B and in C and D are identical, respectively, and were reprobed after stripping.

scClC-a [3]. With ClC-K1 and ClC-K2, site-directed mutagenesis indicated that these sites are used [8] and biochemical analysis of the native ClC-0 *Torpedo* channel came to the same conclusion [24]. ClC-7 is the first ClC protein lacking such a glycosylation site. There is no consensus sequence in other predicted extracellular loops which could serve as a substitute.

There are still other features of ClC-7 which are unique. In contrast to all other ClC proteins, including those from yeast and bacteria, it does not have a glutamic acid at position 208, but glutamine, and also lacks a proline at position 508 (replaced by serine). It is this highly conserved proline, which, when mutated to leucine in the muscle channel ClC-1, causes Thomsen's disease (autosomal dominant myotonia) in Dr. Thomsen's own family [19].

Hydropathy analysis of CIC-6 and CIC-7 reveals roughly the same pattern as for other members of this gene family, e.g. CIC-2 (Fig. 2). In contrast to CIC-2, however, they lack the hydrophobic peak which suggested that D4 spans the membrane [1]. The present data, thus, support our more recent model [2,20]. This changed topology model is also consistent with an extracellular localization of the loop between domains D8 and D9, which is necessary to explain its glycosylation [8,24].

Northern analysis suggests that both genes are broadly expressed. The ClC-6 probe recognizes mainly a ~6-kb message in human tissues (Fig. 3A), which seems slightly smaller in mouse (Fig. 3C). In both species, there is also a larger minority transcript which may be due to alternative polyadenylation. Expression is most prominent in testis, ovary, small intestine, brain and skeletal muscle, but faint bands are detectable in every tissue examined. ClC-7 recognizes a ~4.2-kb message which is also broadly expressed, including again testis, brain and skeletal muscle (Fig. 3B). As compared with ClC-6, expression is stronger in pancreas, kidney, liver and lung. During development in mice, ClC-6 and ClC-7 transcripts are present early on (Fig. 3C,D). They could be detected in whole embryos at days 7, 11, 15 and 17. While ClC-6 expression increases during the first 2 weeks, ClC-7 mRNA levels decrease.

Since mutations in ClC-1 and ClC-5 cause inherited disease in humans [14,17,19], we localized ClC-6 and ClC-7 in the human genome to see whether they could be candidate genes for human disease. FISH to human metaphase chromosomes localized ClC-6 on chromosome 1p36, while ClC-7 is encoded on 16p13.

CIC-6 and CIC-7 belong to a gene family which includes several well-characterized chloride channels [1,5,6]. We, therefore, tried to express them functionally in Xenopus oocytes. We cloned the cDNAs into the highly efficient expression vectors PTLN and pGEMHE, transcribed cRNA in vitro and injected it into Xenopus oocytes. After 2-3 days, they were examined by two-electrode voltage clamp. However, similar to our previous experience with the ClC-K channels [8] and with ClC-3 and ClC-4 [2], we were unable to detect novel chloride currents with hClC-6, rClC-7 and also with hClC-7 to which we had added the first 16 amino acids from the rat clone. Injecting large amounts of cDNA, in contrast, yielded anion currents with an iodide > chloride conductivity sequence which activated slowly upon strong hyperpolarization (in excess of -100 mV) (data not shown). However, similar currents are endogenous to Xenopus oocytes [25] and can also be provoked by overexpression of phospholemman [26], Mat-8 [27], minK (also named I_{SK} [28]) [29] and mutants of ClC-1 which are closed in that voltagerange [19]. Thus, it seems that these currents are just an artifact of overexpressing membrane proteins in Xenopus oocytes [30].

Since ClC-1 is functional as a (homo)multimer [19], we also

explored the possibility whether ClC-6 or ClC-7 together can form functional heterooligomers, but again with negative results. Also co-expression with several other members of this gene family (ClC-K, ClC-3 and ClC-4) as well as attempts to activate channels by second messengers proved to be unsuccessful in preliminary experiments.

4. Discussion

With ClC-6 and ClC-7, we have identified and characterized two new members of the expanding ClC superfamily of chloride channels. These two genes define a new subbranch which is most closely related to ClC-3, ClC-4 and ClC-5, with which they share about 30% identity. ClC-6 and ClC-7 are only 45% identical with each other and, thus, are less related than e.g. ClC-1 is to ClC-2.

While hydropathy analysis of the ClC-0, ClC-1 and ClC-2 chloride channels previously suggested a putative transmembrane domain D4 [1,5], the corresponding regions in ClC-6 and ClC-7 are clearly not hydrophobic enough to support that conclusion. Elimination of D4 as a transmembrane domain supports a revision of the original topology model [1] for ClC chloride channels, which puts the loop between D8 and D9 outside the cell [20]. This loop is known to be glycosylated both in the ClC-K proteins [8] and in ClC-0 [24]. Glycosylation consensus sites are present in this same loop in all previously known eukaryotic ClC proteins, including yeast, but surprisingly not in ClC-7. When compared with ClC-6, this is due to two small deletions, which can be found both in rat and human ClC-7.

hClC-6 is located on human chromosome 1p36, while the hClC-7 locus is on 16p13. No diseases have been mapped to these loci for which ClC-6 or ClC-7 would be obvious candidate genes. Interestingly, the loci for the human kidney-specific channels hClC-Ka and hClC-Kb are also on 1p36 (Grimm, Kieferle and Jentsch, unpubl. data). Thus, there is a cluster of ClC genes in this chromosomal region. For ClC-Ka and ClC-Kb, this is not surprising, as they are 90% identical and may have arisen by a comparatively recent gene duplication. However, ClC-6 belongs to a distant branch of this family and its closest known cousin is on chromosome 16. The other ClC genes are located on different human chromosomes: ClC-1 on 7p35 [14], ClC-2 on 3q26-3qter [31], ClC-3 on chromsome 4 [18], ClC-4 on Xp22.3 [10] and ClC-5 on Xp11.22 [11].

As with other members of this gene family (ClC-K, ClC-3 and ClC-4) [8,2,18], we were unable to express ClC-6 and ClC-7 functionally as chloride channels in *Xenopus* oocytes. Since ClC-1 functions as a homomultimer [19], we speculated that different ClC proteins may also combine to form heteromultimeric channels. We, therefore, examined ClC-6/ClC-7 co-injections and also tried co-expression with other ClC proteins. However, none of these procedures elicited any novel currents.

There are several possible ways to explain why ClC-6 and ClC-7 (and other members of the gene family) cannot be functionally expressed as chloride channels. We could miss another subunit necessary for its functional expression, either belonging to the same gene family, or representing a different ' β -subunit' as for other channels [32]. Second, the expression system (*Xenopus* oocytes) may not be appropriate. Third, ClC-6 and ClC-7 may be channels which are regulated in a complicated way which we have not yet understood. In fact, many patch-clamp studies have revealed chloride channels which can only be observed after patch-excision [33]. Fourth, these channels may reside in intracellular organelles, which are known to possess chloride channels as well [34-36]. The iron-repressible petite phenotype of yeast mutants lacking a ClC homologue (which is structurally close to the ClC-3 to ClC-7 branch) may point into that direction [3]. This question should be addressed by immunofluorescence once good antibodies are available. For CIC-K proteins, however, which cannot be expressed as chloride channels in our hands [2,8] (but see [7,37]), immunofluorescence has shown its presence in the plasma membrane [37]. It is also interesting to note that ClC-5 reproducibly yields large chloride currents in oocytes [12,17], but that the highly related (>85% identity) ClC-3 and ClC-4 proteins do not elicit chloride currents [2,18] (but see [9]). It would seem unlikely, but not impossible, that these highly related (>85% identity) proteins would be sorted to different compartments. Finally, ClC-6 and ClC-7 may not be chloride channels, but perform other functions. Although seemingly far-fetched, there is at least one precedent: CFTR, the cystic fibrosis transmembrane conductance regulator, functions as a chloride channel even though it belongs to a superfamily of transport ATPases [38].

Similar to CIC-2 [6], both CIC-6 and CIC-7 are quite ubiquitously expressed, although they differ in detail. Both genes are also expressed in rather early stages of embryonic development (day 7 for mouse embryos). These observations may suggest house-keeping functions for both proteins. When expressed in Xenopus oocytes, ClC-2 can be activated by hypotonic swelling [15], but this protocol did not elicit currents in oocytes expressing ClC-6 or ClC-7. The very broad expression of ClC-6 and ClC-7 implies that there are many tissues which express several different CIC genes at the same time. For instance, skeletal muscle expresses at least ClC-1, ClC-2, ClC-3, ClC-4, ClC-6 and ClC-7. The function of ClC-1 in stabilizing the membrane voltage is well-understood, especially because mutations in this gene lead to myotonia [13,14]. The function of ClC-2 appears unclear in that particular tissue, because the swelling activation of a new chloride conductance on top of the high conductance mediated by CIC-1 will not contribute significantly to volume regulation. The functions of the remaining ClC proteins, including ClC-6 and ClC-7, is still obscure in this and other tissues. Their elucidation is a challenging problem for the future.

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References

- Jentsch, T.J., Steinmeyer, K. and Schwarz, G. (1990) Nature (London) 348, 510–514
- [2] Jentsch, T.J., Günther, W., Pusch, M. and Schwappach, B. (1995) J. Physiol. 482, 19S–25S.
- [3] Greene, J.R., Brown, N.H., DiDomenico, B.J., Kaplan, J., and Eide, D.J. (1993) Mol. Gen. Gen. 241, 542–553.
- [4] Fujita, N., Mori, H., Yura, T. and Ishihama, A. (1994) Nucl. Acids Res. 9, 1637–1639.
- [5] Steinmeyer, K., Ortland, C. and Jentsch, T.J. (1991) Nature (London) 354, 301–304.
- [6] Thiemann, A., Gründer, S., Pusch, M. and Jentsch, T.J. (1992) Nature (London) 356, 57–60.

- [7] Uchida, S., Sasaki, S., Furukawa, T., Hiraoka, M., Imai, T., Hirata, Y. and Marumo, F. (1993) J. Biol. Chem. 268, 3821–3824.
- [8] Kieferle, S., Fong, P., Bens, M., Vandewalle, A. and Jentsch, T.J. (1994) Proc. Natl. Acad. Sci. USA 91, 6943–6947.
- [9] Kawasaki, M., Uchida, S., Monkawa, T., Miyawaki, A., Mikoshiba, K., Marumo, F. and Sasaki, S. (1994) Neuron 12, 597-604.
- [10] van Slegtenhorst, M.A., Bassi, M.T., Borsani, G., Wapenaar, M.C., Ferrero, G.B., de Conciliis, L., Rugarli, E.I., Grillo, A., Franco, B., Zoghbi, H.Y. and Ballabio, A. (1994) Hum. Mol. Genet. 3, 547–552.
- [11] Fisher, S.E., Black, G.C.M., Lloyd, S.E., Hatchwell, E., Wrong, O., Thakker, R.V. and Craig, I.W. (1994) Hum. Mol. Genet. 3, 2053–2059.
- [12] Steinmeyer, K., Schwappach, B., Bens, M., Vandewalle, A. and Jentsch, T.J. (in press) J. Biol. Chem.
- [13] Steinmeyer, K., Klocke, R., Ortland, C., Gronemeier, M., Jockusch, H., Gründer, S. and Jentsch, T.J. (1991) Nature (London) 354, 304–308.
- [14] Koch, M.C., Steinmeyer, K., Lorenz, C., Ricker, K., Wolf, F., Otto, M., Zoll, B., Lehmann-Horn, F., Grzeschik, K.H. and Jentsch, T.J. (1992) Science 257, 797–800.
- [15] Gründer, S., Thiemann, A., Pusch, M. and Jentsch, T.J. (1992) Nature (London) 360, 759–762.
- [16] Smith, R.L., Clayton, G.H., Wilcox, C.L. and Staley, K.J. (1995) J. Neurosci. 15, 4057–4067.
- [17] Lloyd, S.E., Pearce, S.H.S., Fisher, S.E., Steinmeyer, K., Schwappach, B., Scheinman, S.J., Harding, B., Bolino, A., Devoto, M., Goodyer, P., Rigden, S.P.A., Wrong, O., Jentsch, T.J., Craig, I.W. and Thakker, R.V. (submitted).
- [18] Borsani, G., Rugarli, E.I., Taglialatela, M., Wong, C., and Ballabio, A. (1995) Genomics 27, 131–141.
- [19] Steinmeyer, K., Lorenz, C., Pusch, M., Koch, M.C. and Jentsch, T.J. (1994) EMBO J. 13, 737–743.
- [20] Pusch, M., Ludewig, U., Rehfeldt, A. and Jentsch, T.J. (1995) Nature (London) 373, 527–531.

- [21] Nomura, N., Nagase, T., Miyajima, N., Sazuka, T., Tanaka, A., et al. (1994) DNA Res. 1, 223–229.
- [22] Krieg, P.A. and Melton, D.A. (1984) Nucl. Acids Res. 12, 7057– 7070.
- [23] Kozak, M. (1991) J. Cell Biol. 115, 887-903.
- [24] Middleton, R.E., Pheasant, D.J. and Miller, C. (1994) Biochemistry 33, 13189–13198.
- [25] Kowdley, G.C., Ackerman, S.J., John, E., Jones, L.R and Moorman, J.R. (1994) J. Gen. Physiol. 103, 217–230.
- [26] Moorman, J.R., Palmer, C.J., John II, J.E., Durieux, M.E. and Jones, L.R. (1992) J. Biol. Chem. 267, 14551–14554.
- [27] Morrison, B.W., Moorman, J.R., Kowdley, G.C., Kobayashi, Y.M., Jones, L.R. and Leder, P. (1995) J. Biol. Chem. 270, 2176– 2182.
- [28] Takumi, T., Ohkubo, H. and Nakanishi, S. (1988) Science 242, 1042–1045.
- [29] Attali, B., Guillemare, E., Lesage, F., Honoré, E., Romey, G., Lazdunski, M. and Barhanin, J. (1993) Nature (London) 365, 850–852.
- [30] Tzounopoulos, T., Maylie, J. and Adelman, J.P. (1995) Biophys. J. 69, 904–908.
- [31] Cid, L.P., Montrose-Rafizadeh, C., Smith, D.I., Guggino, W.B. and Cutting, G.R. (1995) Hum. Mol. Genet. 4, 407–413.
- [32] Isom, L.L., De Jongh, K.S. and Catterall, W.A. (1994) Neuron 12, 1183–1194.
- [33] Kunzelmann, K., Tilmann, M., Hansen, C.P. and Greger, R. (1991) Pflügers Arch. 418, 479–490.
- [34] Bae, H.-R. and Verkman, A.S. (1990) Nature (London) 348, 637-639.
- [35] Mulberg, A.E., Tulk, B.M. and Forgac, M. (1991) J. Biol. Chem. 266, 20590–20593.
- [36] Reeves, W.B. and Gurich, R.W. (1994) Am. J. Physiol. 266, C741– C750.
- [37] Uchida, S., Sasaki, S., Nitta, K., Uchida, K., Horita, S., Nihei, H. and Marumo, F. (1995) J. Clin. Invest. 95, 104–113.
- [38] Riordan, J.R. (1993) Annu. Rev. Physiol. 55, 609-630.