Identification of three novel members of the calcium-dependent chloride channel (CaCC) family predominantly expressed in the digestive tract and trachea[1]

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Abstract Three novel human sequences showing striking homology to the recently described bovine Ca^{2+}/calmodulin kinase II-dependent epithelial chloride channel bCaCC have been identified in an expressed sequence tags database. Full-length clones were isolated using a 5′ RACE approach. The encoded predicted proteins display 65% overall homology to bCaCC. Tissue expression patterns of the corresponding genes, designated as hCaCC-1, -2 and -3, appear to be highly restricted, with the first two genes primarily expressed in the digestive tract. Another original feature as compared to the CaCC family members is the fact that hCaCC-2 also shows expression in the brain. Taken together these findings demonstrate the existence of several CaCC-like genes in humans, some of which display distinct tissue specificity patterns within the CaCC subfamily of chloride channels.

Key words: Calcium-dependent chloride channel (CaCC); Intestine; Trachea; Chloride channel; Human

1. Introduction

Chloride channels form a large functional family with structurally diverse members, from neuronal ligand-gated channels such as $GABA_A$ and glycine receptors, to the CLC family of voltage-dependent channels found in a variety of tissues. Peripheral chloride channels are involved in the regulation of electrolytic fluxes, thereby modulating secretion, absorption, cell volume and membrane potential. These channels have received much attention since the discovery of an impaired chloride permeability in cystic fibrosis, the most common human fatal genetic disease. Genetic linkage studies and positional cloning eventually lead to the identification of the transmembrane conductance regulator (CFTR) gene, which was subsequently characterized as a cAMP-regulated chloride channel structurally related to the family of the ATP binding cassette transporters (for a review on CFTR, see [1]). Since then, other human diseases have also been found to result from mutations in chloride channels: Dent's disease, X-linked nephrolithiasis and X-linked recessive phosphataemic rickets with ClC-5 [2], and autosomal dominant myotonia congenita and autosomal recessive general myotonia with ClC-1 [3–6]. In a search for respiratory epithelium calcium-regulated chloride channels, a new channel was affinity-purified from bovine tracheal apical membrane vesicules and biochemically characterized as a 140 kDa complex [7]. According to electrophysiological studies on reconstituted planar lipid bilayers including the purified protein, this protein behaves as an anion selective channel [8]. This channel would be regulated by calcium, via a calmodulin kinase II-dependent mechanism [9]. Cloning and sequencing of the encoding gene, bCaCC, indicated that this protein is unrelated to previously described chloride channels, and that its expression would seem to be restricted to the trachea [10]. An outwardly rectifying DIDS-sensitive anion conductance was detected in bCaCC-sense cRNA injected Xenopus oocytes. Similarly, a Ca^{2+}-dependent anion conductance was observed in COS-7 cells transfected with a CaCC-encoding plasmid [10]. A related gene, Lu-ECAM, has been cloned from the bovine aortic endothelial cell line BAEC [11]. This gene is expressed in the lung and spleen but not in the trachea as assessed using RT-PCR. The family considerably enlarged by the end of 1998, with the publication of two additional bCaCC homologs, the mouse mCLCA1 [12] and the human hCLCA1 [13]. Both were shown to generate Ca^{2+}-dependent chloride currents upon transfection in HEK293 cells. In a search for human homologs of the bovine Ca^{2+}/calmodulin kinase II-dependent epithelial chloride channel, we have identified and cloned three human cDNAs bearing ~65% homology with bCaCC at the protein level. One of these eventually proved to be identical to hCLCA1. In this report, we present evidence that these genes form a family along with bovine CaCC, Lu-ECAM and mCLCA1. Previously reported bCaCC-related genes are mainly expressed in the respiratory epithelia. One of the human CaCC-like genes that we isolated is also primarily expressed in the trachea, but the other two display an original tissue distribution, with preferential transcription in the intestine.

2. Materials and methods

2.1. Sequencing

All DNA sequencing was performed by Genome Express (Grenoble, France).

2.2. Basic molecular biology techniques

Basic molecular biology techniques are described in Maniatis et al. [14].

2.3. Homology search and sequence analysis

Human Genome Sciences Expressed Sequence Tags database was screened with the published bovine CaCC protein sequence (SWISS-PROT P54281) using the Blast program [15]. Three clones, HSIAL89,

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[1] hCaCC-1, hCaCC-2 and hCaCC-3 sequences were deposited in GenBank database under accession numbers: AF127035; AF127036 and AF127980 respectively.

Abbreviations: DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; ORF, open reading frame

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HAGDH75 and HWHPE04, were identified as new human homologs, retrieved and sequenced. Clone HSIAL89 contained a 2366 bp cDNA insert cloned in the *Eco*RI-*Xho*I sites of pBluescript SK$^-$ vector; HAGDH75 contained a 1473 bp cDNA insert cloned as the HSIAL89 insert, and HWHPE04 contained a 2912 bp cDNA inserted in *Sal*I-*Not*I sites of pCMVSport3.0 vector (LifeTechnologies). The genes encoding HSIAL89, HAGDH75 and HWHPE04 cDNAs were named hCaCC-1, hCaCC-2 and hCaCC-3, respectively. Basic nucleotide sequence analysis was performed using the Lasergene software (DNAstar). Signal peptide prediction was performed using G. von Heijne algorithm [16]. CaCC-related sequences were retrieved from GenBank database (accession numbers: bCaCC U36445; Lu-ECAM AF001261; mCLCA1 AF052746; *gob-5* AB017156; hCLCA1 AF039400). Protein sequences from members of the CFTR, CLC, glycine receptor and GABA-A receptor families were retrieved from the SWISS-PROT database. Protein sequence alignments were performed using CLUSTAL_W [17]. The phylogenetic tree was built following the neighbor-joining method with PHYLO_WIN [18].

2.4. hCaCC-1 and hCaCC-2 full-length cDNA cloning

HCaCC-1 and hCaCC-2 ORF 5′ ends were generated using the rapid amplification of cDNA ends (RACE) technique [19]. Two reactions were always run in parallel and subclones derived from them were sequenced in order to ensure the absence of mutations possibly introduced by polymerase chain reaction (PCR). Chameleon cDNA (0.5 ng) derived from human colon was amplified using the manufacturer's specifications (Clontech) with the following modifications: 2.8 U expand polymerase (Boehringer Mannheim) was used in reaction buffer containing 0.2 μM primers. Cycling conditions were: 94°C 30 s, (94°C 5 s; 72°C 4 min) 3 cycles, (94°C 5 s; 70°C 4 min) 3 cycles, (94°C 5 s; 68°C 4 min) 20 cycles, 68°C 10 min, using a Perkin Elmer 2400 thermocycler. One twentieth of the RACE product was amplified again under the same conditions using nested primers and the secondary PCR product was directly cloned into the TOPO cloning vector (InVitrogen). (1) *hCaCC-1*: Four clones containing the longest inserts (0.8 kb) were sequenced. These clones contained an ATG initiation codon near the 5′ end that was in-frame with HSIAL89 ORF, as assessed by alignment of the deduced protein sequences with HSIAL89 putative protein product. sscDNA was then synthesized from 50 ng human colon poly(A)+ RNA (Clontech) using superscript reverse transcriptase II according to the specifications of the manufacturer (Life Technologies). A PCR amplification was conducted on 1/10th of the sscDNA, using Pfu Turbo polymerase (2.5 U, Stratagene), and two hCaCC-1-specific primers designed to generate the same 800 bp fragment as obtained in the original RACE. Cycling was performed in a Perkin Elmer 2400 thermocycler as follows: 94°C 1 min, (94°C 10 s; 57°C 20 s; 72°C 4 min) 30 cycles, 72°C 15 min. A full-length hCaCC-1 cDNA was constructed by subcloning the PCR fragment in-frame with the insert of the HSIAL89 plasmid, taking advantage of the presence of a unique *Eco*RI site in the overlapping region between the PCR fragment and the plasmid insert. Full-length recombinant clones were selected based on their restriction digestion pattern. Four clones were partially sequenced and found to be identical. The insert of one of these clones was then entirely sequenced. (2) *hCaCC-2*: The cloning strategy used to generate a full-length hCaCC-2 cDNA was essentially the same as that described above, except for the following: RACE PCR product was 1.8 kb long; Pfu amplification cycling conditions were: 94°C 1 min, (94°C 10 s; 72°C 4 min) 30 cycles, 72°C 15 min; the Pfu PCR product was inserted in-frame of the insert of the HAGDH75 plasmid using a unique *Bsm*I restriction site in the overlapping region between the PCR fragment and the plasmid insert.

2.5. Northern and dot blot hybridizations

HSIAL89 *Ava*I 1.7 kb fragment, HAGDH75 *Eco*RI-*Xho*I 1.6 kb fragment, HWHPE04 *Hin*dIII 1.65 kb fragment and glyceraldehyde-3-phosphate-dehydrogenase cDNA fragment (Clontech) were radiolabeled by random priming [20] using α^{32}P-dCTP (NEN) and the Megaprime labeling kit (Amersham). Multiple tissue Northern blots, containing 2 μg poly(A)+ RNA per lane from a variety of human tissues, and Master dot blots, containing poly(A)+ from 50 human tissues in such quantities as to obtain similar hybridization signal intensities in the various tissues with a panel of 8 housekeeping genes (Clontech), were hybridized overnight in ExpressHyb (Clontech) hybridization solution at 68°C (MTN) or 65°C (dot blots) and washed to a final stringency of 0.1×SSC, 0.1% SDS, 50°C (MTN) and 0.1×SSC, 0.5% SDS, 50°C (Master). All steps were performed according to the specifications of the manufacturers. The hybridization signals were detected using a Storm 860 apparatus and quantified using ImageQuaNT software (Molecular Dynamics).

3. Results

3.1. Identification of CaCC-related genes and full-length cloning

Using a homology search approach, we identified three new human sequences in a proprietary EST database that displayed significant homology with the bovine Ca^{2+}/calmodulin kinase II-dependent epithelial chloride channel bCaCC. This finding was confirmed upon sequencing of the entire cDNA of the corresponding clones, which displayed ~65% overall homology with bCaCC at the protein level. The genes encoding these sequences were therefore named hCaCC-1, hCaCC-2 and hCaCC-3, respectively. Based on ORF prediction and alignment with bCaCC protein sequence, the 2.9 kb insert of hCaCC-3 appeared to encode a full-length protein, whereas hCaCC-1 and hCaCC-2 clones appeared to contain incomplete cDNAs at the 5′ end, missing at least 465 bp and 1770 bp, respectively. hCaCC-1 and hCaCC-2 5′ coding sequences were generated using a RACE approach, and the full-length cDNAs were obtained by in-frame insertion of the missing fragments in the original clone using restriction digestion. Reconstructed full-length hCaCC-1 cDNA was 2.8 kb

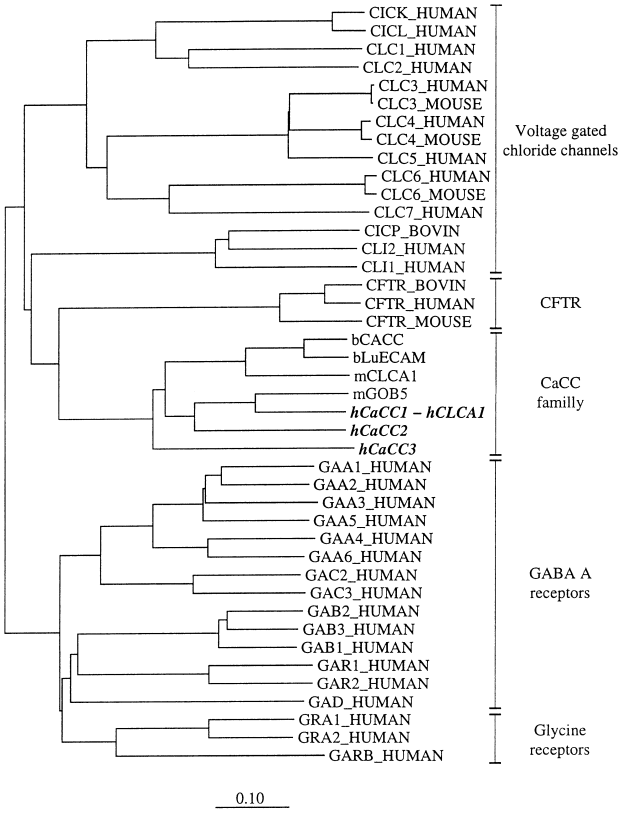

Fig. 1. Phylogenetic tree of chloride channels. A phylogenetic tree was built based on the deduced sequences of the putative protein products of bCaCC and related genes as well as on the protein sequences of various members of the CFTR, CLC, glycine receptor and $GABA_A$ receptor chloride channel subfamilies. Scale bar represents 10% diversity.

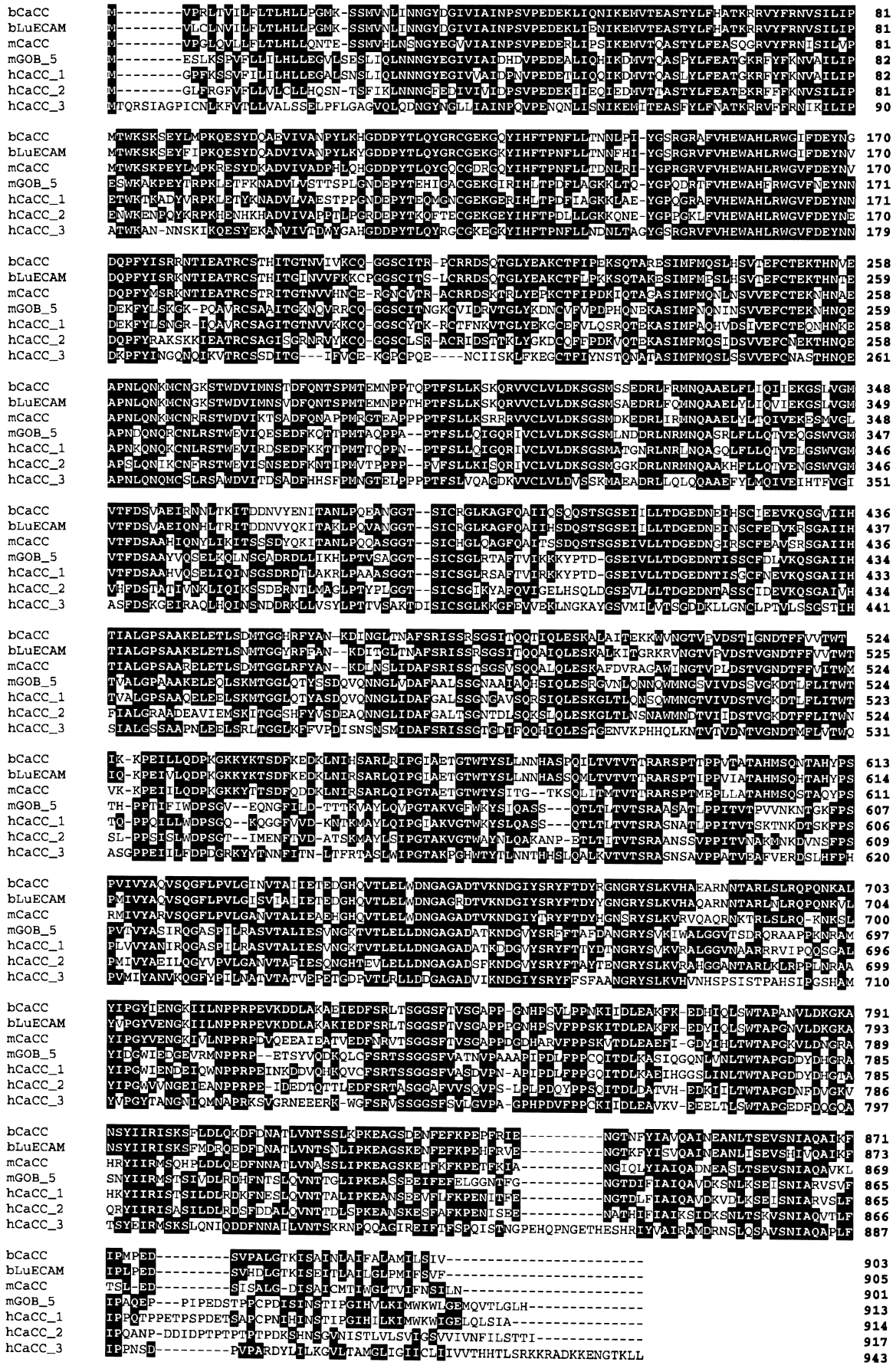

Fig. 2. Protein sequence alignment of hCaCC-1, -2 and -3 with bCaCC-related sequences. Conserved residues are highlighted by black boxes. Residues are numbered according to their position in the sequence.

long, whereas hCaCC-2 cDNA was 3.3 kb long. The sizes of the predicted hCaCC-1, hCaCC-2 and hCaCC-3 protein products, 914 amino acids (aa), 917 aa and 943 aa, respectively, were similar to that of the bovine CaCC, a 903 aa polypeptide. All three putative proteins were predicted to have a signal peptide, similarly to the bCaCC (data not shown).

3.2. Homology between the CaCC family members

All three human predicted protein sequences display a similar degree of overall homology with bCaCC putative protein product: 51% to 54% identity and 65% to 68% similarity. Amongst the human proteins, hCaCC-1 and hCaCC-2 appeared to be closer, with a global 62% identity and 74% similarity. hCaCC-3 showed only 45% identity and 63% similarity to hCaCC-1 and -2. Using multiple protein sequence alignment algorithms, the three human predicted protein sequences were compared with bCaCC, Lu-ECAM, mCLCA1, hCLCA1, as well as to a variety of chloride channels such as CFTR, ClCs and $GABA_A$ receptors, included as reference chloride channel family members. hCLCA1, a very recently published human bCaCC-like sequence [13], was included in this analysis, as well as mouse *gob-5* sequence [21], which we found to be a new CaCC-related sequence in a routine homology search of public databases. The resulting phylogenetic tree clearly shows that hCaCC-1 and hCLCA1 are identical, and that hCaCC-1/hCLCA1, hCaCC-2 and hCaCC-3 form a subfamily together with bCaCC, Lu-ECAM, mCLCA1 and *gob-5* (Fig. 1). This tree also illustrates how structurally distant chloride channel subfamilies are from each other. Within the CaCC family, mCLCA1 appears to be closer to bCaCC and Lu-ECAM, whereas *gob-5* is mostly related to hCaCC-1, suggesting that it may encode the murine homolog of this human gene. Examination of the sequence alignment of the bCaCC-related proteins (Fig. 2) reveals significant conservation level throughout the entire sequences, with the exception of the N- and C-termini. Some regions are particularly well conserved, forming sequence motifs that may have significance at the structural or the functional level. The most conserved region, from residue 153 to residue 169 of bCaCC, contains an HEXXH motif. Such a motif is found in a number of metal binding proteins, where it forms part of the metal atom binding site. In a number of families, X-ray analysis indicated that the two H residues of the motif are involved in Zn^{2+} binding, together with another E or H, located in the next 50 residues [22]. Interestingly, a conserved E is found in the next 10 residues of the HEXXH motif of all CaCC-related sequences.

3.3. Tissue distribution of the hCaCCs

3.3.1. General. The tissue distribution of these new genes was determined using Northern analysis (Fig. 3) as well as RNA dot blot hybridization (Fig. 4). Using an hCaCC-1-derived probe, a unique 2.9 kb band was detected, whereas hCaCC-2 and hCaCC-3 probes revealed 3.5 kb and 4.2 kb bands, respectively. These transcript sizes are similar to that of bCaCC, which is 3.1 kb. An additional minor 8.0 kb band was also detectable in the brain with the hCaCC-2 probe. Northern blot experiments suggested that the expression of these three genes would be spatially restricted. Indeed, no signal was found in organs such as heart, lung, liver, kidney or pancreas (Fig. 3). This finding was confirmed on the RNA dot blots, which are optimized for the comparison of gene expression between 50 different human tissues (Fig. 4).

3.3.2. hCaCC-1. Quantification of the hybridization signals obtained with an hCaCC-1 probe on the RNA dot blot indicated that hCaCC-1 mRNA was essentially present in the digestive tract. Highest expression was found in the colon, small intestine and appendix. Hybridization signal intensities were quite high in these tissues, suggesting that the transcript would be rather abundant in the intestine. These findings were in good agreement with the fact that most hCaCC-1 ESTs found in databases originated from sequencing of small intestine- and colon-derived cDNA libraries. There was also a good correlation with the Northern blot data, where an intense band was seen in small intestine and colon. Expression of hCaCC-1 was also assessed in a fourth region of the digestive tract, the stomach. A very low signal was detectable on both types of blots. Quantification on the RNA dot blot indicated that hCaCC-1 expression in this tissue would be about 100 times lower than that detected in colon.

Three other adult tissues were labeled on the RNA dot blot, indicating that expression of hCaCC-1 gene is not strictly restricted to the digestive tract. These tissues, namely uterus, testis and kidney, belong to the urogenital tract. Signal intensities were very low, about 2 to 3 orders of magnitude below

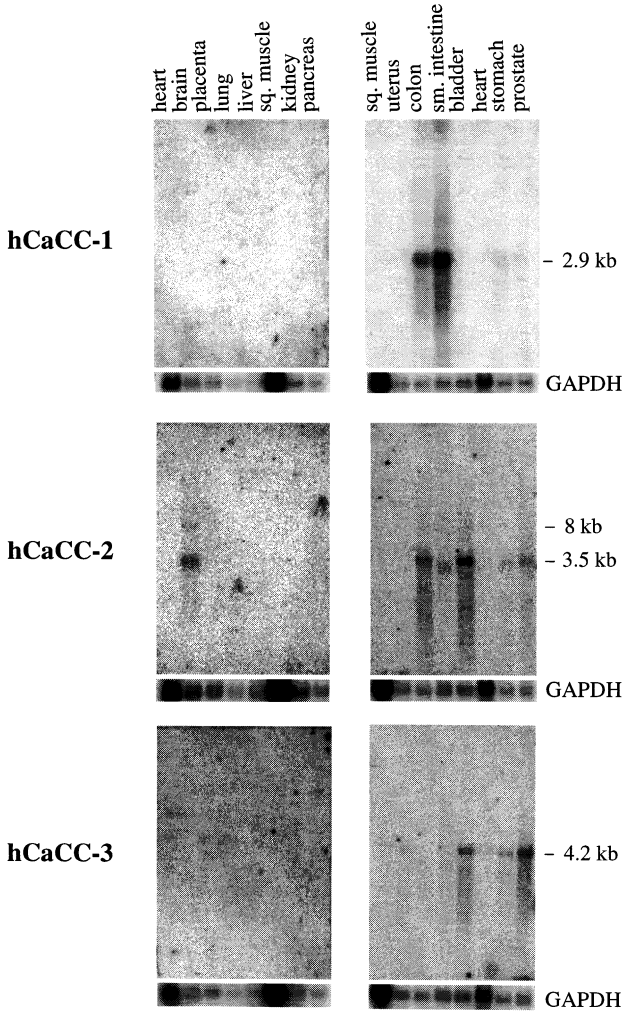

Fig. 3. Northern analysis of hCaCC-1, -2 and -3 tissue distribution.

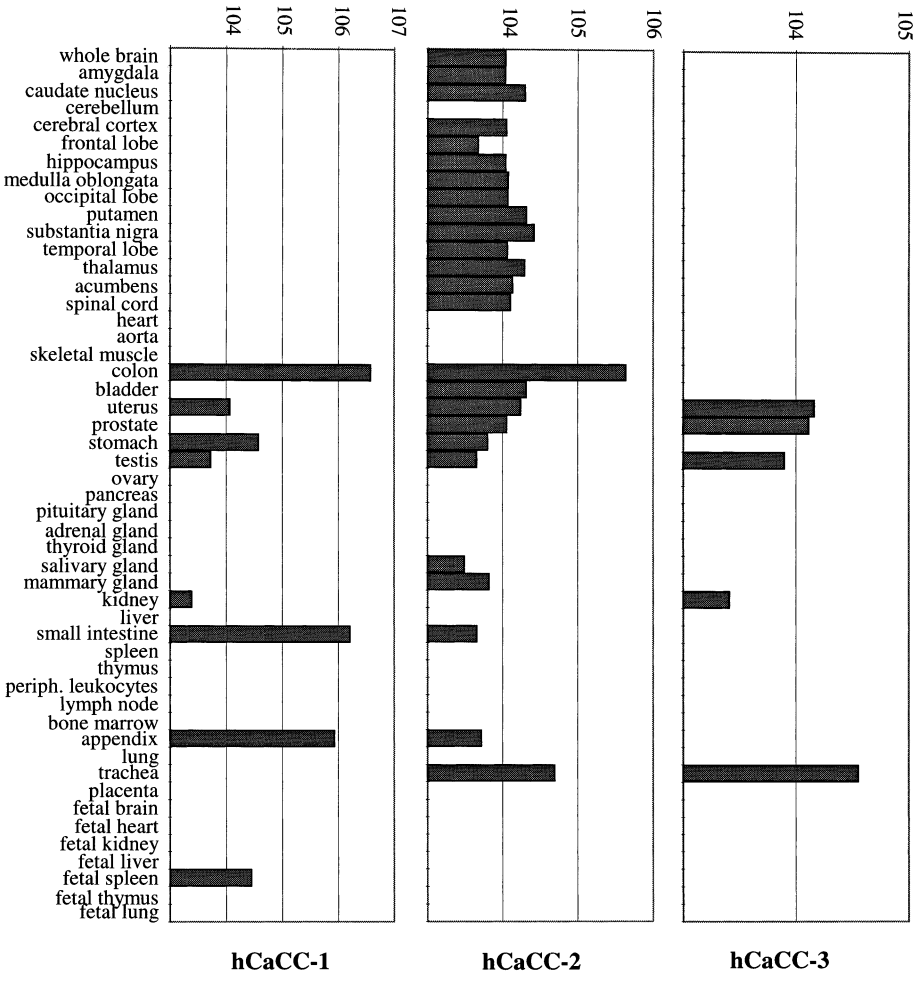

Fig. 4. RNA dot blot analysis of hCaCC-1, -2 and -3 tissue distribution. Hybridization signal intensities are expressed as arbitrary units and displayed using a log scale.

that of colon. In addition, a very faint spot was detected in the prostate poly(A)+ dot, that could not be quantified and therefore does not appear on the histogram Fig. 4. Expression in this gland was confirmed on the Northern blot (Fig. 3). The correlation between the Northern and the RNA dot blot data was not as good for the very low level signals as for the intense ones, as no band could be detected either in uterus or kidney on the Northern blot. More generally, low level signals, below 10 000 arbitrary units, are to be interpreted with caution and would need to be confirmed using a different approach. In fetal tissues, hCaCC-1 probe labeled a unique dot, corresponding to spleen.

3.3.3. hCaCC-2. Similarly to the hCaCC-1 probe, the hCaCC-2-derived probe labeled colon poly(A)+ RNA to the highest extent in RNA dot blot hybridizations. However, its labeling pattern within the digestive tract tissues was considerably different. Small intestine, appendix and stomach signals were among the weakest, about 50 to 100 times lower than that of colon. The second major signal was observed in trachea. It was estimated to be about 10 times less intense than the signal detected in colon. A number of other minor but significant spots were detected in a variety of tissues such as brain and the urogenital organs, bladder, prostate and uterus. Weak spots were also observed in the testis, as well as salivary and mammary glands. These findings were in good agreement with the Northern data, when assessable, except for the fact that no signal was observed in the uterus sample. On the Northern, the size of the band that was observed in the poly(A)+ from the small intestine appeared to be lower than in the other tissues. This band would possibly correspond to an alternative transcript or to limited cross-hybridization with hCaCC-1 mRNA, although this second hypothesis would seem unlikely given the low level of conservation between the two sequences. According to the RNA dot blot analysis, expression in the brain would seem to be rather uniform, apart from cerebellum where no signal was detected. These data were confirmed on Northern blots containing poly(A)+ RNA from the same 15 brain regions that are present on the RNA dot blot. The same two 3.5 kb and 8 kb bands seen in the whole brain sample of the Northern blot shown in Fig. 3 were detected with similar intensities in all brain regions, except for the cerebellum where it was notably fainter (data not shown).

3.3.4. hCaCC-3. The tissue distribution of hCaCC-3 was analyzed by hybridizing an RNA dot blot with a hCaCC-3-derived cDNA probe. The main signal was observed in the trachea (Fig. 4) whereas very faint spots were detectable in uterus, prostate, testis and kidney. On Northern blots, the

hCaCC-3 probe revealed a 4.2 kb band in bladder, stomach and prostate, whereas no signal could be seen in uterus or kidney.

4. Discussion

We have identified and isolated three new human genes, which are related to the bovine Ca^{2+}/calmodulin kinase II-activated epithelial chloride channel bCaCC [10]. One of these, namely hCaCC-1, proved to be identical to hCLCA1, a very recently published bCaCC-related chloride channel [13]. The degree of sequence conservation between these human genes and the bovine CaCC, about 65% at the protein level, would suggest that they form a structural subfamily. This new protein subfamily seems to be conserved not only between man and bovine, but also in rodents as a bCaCC homolog, mCLCA1, showing 80% homology at the protein level was recently described in the mouse [12]. In addition, we have found that the mouse *gob-5* gene, whose sequence was recently deposited in public databases [21], is a close homolog of hCaCC-1. However, hCaCC-1, -2 or -3 would not seem to encode a human counterpart of bCaCC, as if so, the degree of homology would be expected to be at least 80%. Therefore, a fourth human CaCC sequence most probably remains to be identified. The existence of CaCC-related genes in several mammalian species would suggest that the biological function of this family of proteins was evolutionarily conserved. However, no CaCC type sequence could be found in yeast genome using a homology search approach, suggesting that this family emerged after or with the metazoans.

One of the most impressive features of these new human genes is their expression pattern. One of these genes, hCaCC-3, is expressed in the trachea. Noteworthy, Ca^{2+}-stimulated chloride channels, presently unidentified, have been reported in the apical membrane of human airway epithelia. These channels, that are not affected in cystic fibrosis patients [23], may represent potential therapeutic targets to treat this disease. Such a hypothesis is based on the finding that an up-regulation of Ca^{2+}-activated Cl^- fluxes takes place in the nasal epithelium of knock-out $cftr^{-/-}$ mice, and accounts for the absence of any significant pathology in these model animals [24,25]. Given their relatedness to the Ca^{2+}-activated chloride channel bCaCC and their transcription in human trachea, it is tempting to speculate that hCaCC-2 and hCaCC-3 might be encoding such channels.

Besides, although tissue distribution of hCaCC-3 is somewhat reminiscent of that of bCaCC as it is mainly expressed in the trachea, hCaCC-1 and hCaCC-2 present an expression pattern that is unique compared to that of Lu-ECAM, bCaCC and mCLCA1. hCaCC-1 transcription is strikingly associated with the digestive tract. Among 50 different human tissues, very few structures other than those belonging to the digestive tract were found to express this gene, and always at a very low level compared to the intestine. Within the digestive tract, hCaCC-1 mRNA distribution was not uniform, with strikingly higher expression in small intestine, colon and appendix compared to stomach. These findings are in good agreement with the previously reported tissue distribution of hCLCA1 which was described by Gruber et al. (1998) as specifically expressed in the colon and small intestine based on the Northern blot technique and on in situ hybridization [13]. hCaCC-2, the second new human gene that we identified, is more widely distributed. However, its expression is prominent in the colon, with only minor expression in the other tissues tested. Contrary to hCaCC-1, hCaCC-2 is only weakly expressed in the small intestine and appendix, indicating that the expression of these two homologous human genes would be regulated independently in the gut.

Expression of hCaCC-1 and hCaCC-2 in the intestine is very interesting with respect to their potential Ca^{2+}-dependent chloride channel function. Indeed, chloride channels play an important role in the regulation of water and electrolytic fluxes in the intestinal epithelium. Both cAMP- and Ca^{2+}-dependent Cl^- currents have been described in animal intestinal epithelial cells, as well as in human and rodent colon-derived cell lines [26–29]. A Ca^{2+}/calmodulin kinase II-dependent Cl^- current, reminiscent of the bCaCC type, has been reported in the T84 colonic tumoral cell line [30,31]. However, Anderson and Welsh [32,33] reported that there are no Ca^{2+}-dependent but only cAMP-dependent Cl^- channels at the apical surface of intestinal mucosa, a crucial issue with respect to the potential biological role of these channels in the intestine. Indeed, apical Cl^- channels would regulate fluid and electrolyte transport in secretory epithelia according to the current hypothesis. Localizing hCaCC-1 and -2 proteins within the digestive wall, together with assessment of their putative chloride channel function, would be of primary interest in this controversy.

Another most interesting characteristic of hCaCC-2 expression is its transcription in the brain, a unique feature among the bCaCC family. Ca^{2+}-dependent chloride channels have been characterized in olfactory sensory neurons, where they would play a role in odor detection [34,35], and they have also been described in microglia [36], but to our knowledge there is no evidence to date of a Ca^{2+}/calmodulin kinase II-dependent channel in the brain. Therefore, in order to better understand the potential role of hCaCC-2 in this organ, it would be interesting to determine its cell type specificity in the central nervous system.

An intriguing question about the hCaCCs, and more generally about all new bCaCC-related genes is their biological function. Indeed, although bCaCC and more recently mCLCA1 and *hCLCA1* were characterized as Ca^{2+}-dependent chloride channels, the close homolog Lu-ECAM was identified as an adhesion molecule, mediating adhesion of tumor cells to lung epithelium [37]. Evidence that bCaCC, mCLCA1 and hCLCA1 encode chloride channels is based on electrophysiological studies on bCaCC cRNA injected Xenopus oocytes, as well as on bCaCC, mCLCA1 and hCLCA1 transiently transfected mammalian cells [10,12,13]. The possibility remains that the observed currents were not directly associated with the expression of the various bCaCC family members, but were rather due to activation of an endogenous channel by an indirect mechanism, as suggested for pI(Cln) [38]. Such a hypothesis would suggest that expressed bCaCC might in fact play the role of a regulatory protein required for the activation of endogenous chloride channels. Conversely, multifunctional proteins are well known, and it is also conceivable that a channel that is present at the cell surface might also serve as an adhesion molecule in some conditions. Such a protein with a double function associating a channel activity to an adhesion function has previously been described in *C. elegans*, where the *unc-105* gene encodes an epithelial Na^+ channel of the degenerin family that interacts with type IV

collagen [39]. Clearly, one of the major directions for further investigations concerning the hCaCC proteins is the characterization of their in vivo biological functions.

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