A short CIC-2 mRNA transcript is produced by exon skipping

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ABSTRACT

CIC-2 is a voltage- and volume-regulated chloride channel expressed in many tissues. We have shown that CIC-2 in rat lung airways is significantly down-regulated after birth [Murray, C.B. *et al.* (1995) *Am. J. Respir. Cell Mol. Biol.*, 12, 597–604]. During PCR amplification from rat lung cDNA, a second transcript was identified which is 60 bp shorter than the full length sequence. The peptide translated from this 60 bp sequence contains many positively charged amino acid residues. Rat genomic DNA sequencing showed that the 60 bp sequence is an intact exon. A 71% pyrimidine content and an AAG 3'-end splice site in the intron immediately upstream from the 60 bp sequence were identified which may account for the alternative splicing of the following exon. Human genomic sequence analyses demonstrated similar intron–exon arrangement. A high CT content and an AAG 3' acceptor site were conserved in the intron corresponding to the rat upstream intron. The presence of the full length short form transcript was confirmed in rat kidney by RT-PCR, and the ratio of the long and the short form transcripts varied significantly according to the tissues examined, with the lowest long/short form ratio found in the lung among the tissues studied. Our data demonstrated that the alternatively spliced short form (CIC-2S) is transcribed in many rat tissues, the ratio of the long/short form transcripts is lower in the lung compared with the brain, and the genomic organization in this area is conserved in rat and human.

INTRODUCTION

CIC-2 is a chloride channel belonging to a CIC family of chloride channels. More than 10 members of this family have been identified recently from a wide variety of species including yeast, fish and mammals. The first member of the CIC chloride channel family, CIC-0, was identified by expression cloning from *Torpedo marmorata* electric organ (2). Other members of the family were later cloned from yeast *Saccharomyces cerevisiae*, and from a number of rat and human tissues (3–9). These channels can be tissue-specific or ubiquitous, and from epithelial or non-epithelial

origins. Among all the CIC chloride channels, the CIC-2 gene encodes a voltage- and volume-regulated chloride channel whose mRNA can be detected in many tissues in rat and human (8,10). The predicted protein product has 12 putative membrane-spanning regions in the N-terminal two-thirds of the sequence followed by a C-terminal hydrophilic domain proposed to be cytosolic (8). Recently, two additional subforms of CIC-2, CIC-2G (CIC-2 α) and CIC-2 β , were identified from rabbit, the former in the stomach and heart, and the latter in the heart (11,12). Both are PKA activated. Interestingly, the rat CIC-2 produces a chloride current in *Xenopus* oocytes only when it is under control of an upstream sequence from CIC-0 (8), whereas the rabbit forms were expressed without the attachment of the CIC-0 sequence.

Although many chloride channels of the CIC family have been cloned and expressed in heterogenous systems, few studies have examined the regulation of their expression (3). Here we report the expression of a short form CIC-2 transcript (CIC-2S) by exon skipping, which is 60 bp shorter than the published sequence. The ratio of CIC-2/CIC-2S varies between the lung and other tissues. A high CT content and an AAG acceptor site in the upstream intron are conserved in rat and human. Our results suggest that alternative splicing may constitute one of the mechanisms that regulates CIC-2 expression and function in lung and other tissues.

MATERIALS AND METHODS

Animals and tissues

Adult rats and timed-pregnant dams were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN). Rats were euthanized by intraperitoneal injection of sodium pentobarbital. Tissues from fetuses and adults were harvested, pooled (fetal tissues, according to their gestation period), flash-frozen in dry ice, and stored at -70°C .

Cell cultures

The L2 cell line was derived from type II-like alveolar pneumocytes from normal adult female rat lung (13,14) and was obtained from American Type Culture Collection (ATCC, Rockville, MD). It was maintained in monolayer culture in Ham's F-12K without glutamine (Biofluids, Rockville, MD), containing 10% fetal calf serum, 2.5 $\mu\text{g/ml}$ fungizone, 100 U/ml penicillin G and 100 U/ml streptomycin. The cells were fed three times a week and split 1:10 when they reached 100% confluence. The

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human tracheal epithelial cell line 9/HTEo- (15) was obtained from Dr D. C. Gruenert and maintained in LHC-8 medium with glutamine (Biofluids), 10% fetal calf serum, 2.5 µg/ml fungizone, 100 U/ml penicillin G and 100 U/ml streptomycin. They were fed three times a week and split 1:5 when passed. Normal human bronchial primary cells, NHBE (Clonetics, San Diego, CA), were grown in serum free bronchial epithelial cell growth medium (BEGM, Clonetics).

RNA and cDNA preparation

Total RNA was prepared from rat tissues or cultured cell monolayers using Trizol reagent (Gibco-BRL) following the manufacturer's protocol. The quality and quantity of the total RNA were examined by agarose gel electrophoresis and UV spectrophotometry. cDNA was prepared from 0.1 µg of total RNA with the cDNA Cycle Kit (Invitrogen, San Diego, CA), using the oligo dT primer. For subsequent PCR amplification, 20% of the prepared cDNA was used in each 50 µl reaction as the template.

PCR amplification

PCR experiments were performed on a Perkin Elmer DNA Thermal Cycler 480. Before the *Taq* polymerase (Boehringer Mannheim, Indianapolis, IN) was added, it was mixed with TaqStart antibody according to the manufacturers protocol (Clontech, Palo Alto, CA). Primers used in RT-PCR are listed in Table 1. Among all the primers, HRC5' and HRC3' were also used to evaluate the ratio of the two forms of the CIC-2 transcript. Their sequences are identical to human CIC-2 at nt 2177–2195 and 2347–2369 respectively in the coding sequence (10). For evaluation of the ratio of the two forms, HRC5' was labeled with blue fluorescent dye FAM (Applied Biosystems, Inc., Foster City, CA) (16), and 25–31 cycles or 29–37 cycles of amplification were performed depending on the yield of the PCR products. In each cycle, the reactions were denatured at 94°C for 1 min, annealed at 57°C for 40 s and extended at 72°C for 40 s. PCR products were separated on a Model 373 ABI DNA Sequencer and quantified by GeneScan software (Applied Biosystems). In each PCR reaction, the quantity of the fluorescence detected by the scanner was plotted on a log scale against the number of cycles. Only those points which were on a linear curve (before reaching the plateau area) were used for further analysis. The long/short form ratio of the CIC-2 products in each organ is the mean of three individual reactions each representing a different animal or cell culture. The one-way ANOVA followed by the Bonferroni were used to analyze these results.

Cloning and sequencing of PCR fragments

PCR products were separated by agarose gel electrophoresis and the gel slices containing the bands of interest were excised. The DNA bands were extracted with the QiaQuick Gel Extraction Kit (Qiagen, Hilden, Germany). The DNA fragments were then cloned into the pCR3 plasmid (Invitrogen). DNA sequencing was performed on the ABI DNA Sequencer Model 373. For direct sequencing of PCR fragments, the DNA in 50–100 µl of PCR reactions was concentrated by ethanol precipitation and resuspended in a 10 µl volume. The resuspended DNA was electrophoresed on a 3% NuSieve GTG agarose gel (FMC, Rockland, ME), electroeluted from the excised gel slice, and used as sequencing template.

Figure 1. Agarose gel electrophoresis of PCR products of adult rat CIC-2 cDNA using HRC5' and HRC3' with 35 cycles. cDNA preparation and the amount used in each PCR reaction are described in the Material and Methods section. Outer lanes on both sides are molecular weight markers. Arrows indicate number of base pairs of the bands in the marker lanes. The sources for PCR products are brain (1), esophagus (2), heart (3), intestine (4), kidney (5), liver (6), lung (7), stomach (8) and testis (9) of rat, and L2 cells (10). The two major products are of 202 and 142 bp.

Table 1. Primers used in PCR experiments

Name	Sequence	Location ^a
nt5'out	AGCTGAGTCAAGGCCAGA	–104 to –87
ct3'out	CACAGAACCCCCACCTCCT	2979 to 2962
A5'in	AGAAGCAAGAGGAGGCAA	–35 to –18
ct3'in	GTGTTGAAAATGGGGGAG	2930 to 2913
C2in5'	TACTGGGAGCACAGCTGAGT	1922 to 1941
C2in3'	GAAGACAGGCCGTCCAGTAT	2749 to 2730
HRC5'	GCCTCTTCTGTGGCAGTCC	2195 to 2213
HRC3'	ACCCGCTTCAGCTTGC	2396 to 2347

^aThe first base in the published rat CIC-2 coding sequence is designated as +1 (8). The base 5' to +1 is –1. The primers on the opposite strand are assigned the numbers on the coding strand.

RESULTS

A short form of CIC-2 transcript is produced in rat tissues

During PCR amplifications of the 3'-portion of rat lung cDNA, in addition to the expected 202 nt fragment, a second smaller band was consistently amplified. The possibility of non-specific amplification was less likely because the double-band pattern was observed using three different primer pairs (C2in5'/C2in3', HRC5'/C2in3' and HRC5'/HRC3'). PCR sequencing results localized the origin of the long and short PCR fragments to a segment of 160 bp between oligos HRC5' and HRC3' or A₂₂₁₄ to A₂₃₇₃ in the CIC-2 coding sequence (8) (GenBank accession no. X64139). Although the two-band pattern appeared in many different rat tissues, the ratio of these two bands varied reproducibly depending on the source of rat cDNA, suggesting a tissue-dependent expression pattern (Fig. 1).

Two methods, DNA sequencing and size determination of fluorescent-labeled PCR products, were used to investigate whether both PCR products were derived from CIC-2. In the first approach, the amplified fragments from the adult rat lung were sequenced both after subcloning and directly from the PCR products. DNA sequencing data showed that the short fragment is identical to the long fragment except for the absence of a 60 bp sequence (Fig. 2). In the second approach, the long and short fragments were amplified from adult rat testes, trachea, intestine, kidney, liver, lung, brain, 19- and 21-day gestation fetal rat lungs and L2 cells with fluorescent labeled HRC5' and unlabeled

Figure 2. (A) Sequence alignment of the rat and human CIC-2 and the short form rat CIC-2 amplified by RT-PCR using the primers HRC5' and HRC3'. Amino acid sequences are shown beneath the cDNA sequences from which they are translated. Locations where introns are found are indicated by '*'. HRC5' and HRC3' sequences are double underlined. (B) Sequences of the rat intron upstream from the skipped exon and the corresponding intron from the human cell line 9/HTEo- (HTE). Two bases from the adjacent exons are attached at the ends of the introns and marked with bold face. Possible branch point sequences are underlined. (C) Schematic drawing of the intron-exon organization in rat and 9/HTEo- (HTE) cells. Boxes indicate exons and straight lines indicate introns. Lines above the exons and introns indicate splicing events.

HRC3'. The exact size of each product in number of the bases was measured with fluorescent labeled molecular weight standards and GeneScan software after separation on a sequencing gel. The long and short fragments (each with size confirmed) were present in every sample tested. As predicted from the DNA sequence, deletion of the 60 bp fragment would not cause any frame shift or termination but would produce a shorter form of CIC-2 protein by ~2 kDa (the predicted molecular weight of the published rat CIC-2 protein is 99 kDa). The 60 bp fragment codes for a 20 amino acid residue peptide in the CIC-2 protein from L₇₄₇ to A₇₆₆ which is the most positively charged region in the protein. This region is located in the proposed C-terminal cytosolic domain, a region with little sequence homology to other members of the CIC chloride channel family.

To confirm the presence of a full length CIC-2 transcript differing from the published sequence by the 60 bp deletion, nested RT-PCR was performed on cDNA from rat lung and kidney. The first amplification with an outer primer pair (nt5'out and ct3'out) was followed by nested amplification with an inner primer pair (A5'in and ct3'in). A predominant band of ~3 kb was obtained from both rat lung and kidney. The PCR products were then cloned into vector plasmid pCR3 (Invitrogen) and two clones, pRK and pRL from rat kidney and lung respectively, were used for further analyses. DNA sequencing of the entire inserts in these two clones demonstrated that the majority of the sequence is identical to the published sequence except for two silent mutations (C₅₆₄ to A₅₆₄, A₁₄₂₂ to G₁₄₂₂) and three missense mutations (C₈₃₀ to T₈₃₀, T₉₃₂ to C₉₃₂, G₁₂₈₅ to A₁₂₈₅) in pRL, and two missense mutations (G₁₁₆₉ to T₁₁₆₉, A₂₀₅₃ to G₂₀₅₃), one

single base pair deletion (C₄₇₅) and one 60 bp deletion (T₂₂₃₉ to G₂₂₉₈) in pRK. While the single base changes are likely the results of PCR error, the 60 bp deletion in pRK is identical to that found in the short form PCR product amplified by HRC5' and HRC3', suggesting that the short form CIC-2 transcript (CIC-2S) is present in rat tissues with only the 60 bp sequence deletion, but no other alternative splicing.

The short form CIC-2 transcript is produced by exon skipping

The genomic DNA structure in the relevant region was then investigated by cloning and sequencing. A rat genomic clone containing part of the CIC-2 gene was selected from a rat genomic DNA library constructed in P1 phage (17). The subclones were prepared in plasmid pTZ19R (Pharmacia) and sequenced. The results revealed additional sequences at both ends of the 60 bp sequence characteristic of introns, which begin with GT at the 5' end and end with AG at the 3' end, suggesting that the 60 bp sequence is an intact exon (Fig. 2). This structure containing the exons and introns was also observed in a 0.6 kb PCR fragment amplified from adult rat lung cDNA using primers HRC5' and HRC3', indicating that this 0.6 kb fragment might have been amplified from incompletely processed primary CIC-2 RNA or contaminating genomic DNA. Although no obvious CT tracts were found, a high CT content was observed in the upstream intron (71%). Three possible branch point sequences (BPS), CTCTGAC from -5 to -11, CTATAAC from -33 to -39 and CAATGAC from -134 to -140 (in reference to the first base of

the 60 bp exon), were observed in the upstream intron based on sequence similarity to the consensus YNCTGAC (where Y represents pyrimidine residues and N represents either purine or pyrimidine residues) (18,19), while no obvious BPS was seen in the downstream intron. In addition, A is the nucleotide preceding the 3' splice site AG in the upstream intron, which has been previously demonstrated to be less efficient in intron splicing (20).

Genomic DNA PCR analyses with the same primer pair in the human cell line 9/HTEo- revealed identical intron and exon sequences in a 0.7 kb PCR fragment from NHBE cDNA, which may contain some pre-mRNA. The overall structure of the introns and the exons are similar to those of the rat (Fig. 2C). The exon sequences are identical to the published human CIC-2. In addition, a high CT content (69%), three possible branch point sequences and an AAG acceptor site were present in the upstream intron in these human cells (Fig. 2B). Although the downstream intron of human and rat is also similar in size, 112 and 134 bp respectively, none of them have a high CT content nor AAG 3' splice sites.

Table 2. Ratios of long/short form CIC-2 mRNA transcripts in rat tissues

Tissues	Ratio L/S ^a
L2 cells	1.87 ± 0.90
Adult rat lung	1.78 ± 0.66
Fetal rat lung	2.04 ± 0.69
Adult rat brain	6.67 ± 1.70 ^b
Adult rat kidney	3.33 ± 0.44
Adult rat intestine	3.26 ± 1.60

^aMean ± SEM, *n* = 3.

^bSignificant *f* prob. 0.0013, one way ANOVA, *P* < 0.05, Bonferroni.

Adult rat lung has a decreased long/short form ratio of CIC-2 transcripts

Initial agarose gel electrophoresis of the PCR products using HRC5' and HRC3' suggested that the brain had the highest ratio of long/short form transcript compared with other tissues, especially the lung and the L2 cells. To quantify the ratio of these long and short transcripts, RT-PCR was performed with a fluorescent-labeled oligonucleotide (16). As only one pair of primers was used in the PCR reactions and two fragments were amplified, each fragment acted as the internal standard for the other. PCR products were collected at different cycle numbers in each reaction, so that fractions during exponential amplification could be selected for further analysis. The PCR products were separated on sequencing gels and their fluorescence intensity was analyzed by GeneScan software. Examples of the RT-PCR plots are shown in Figure 3. Adult rat brain was found to have the highest ratio of long/short form CIC-2 transcripts, while the lowest ratios were found in adult and fetal rat lungs (Table 2). This difference between adult brain and all other tissues that we studied here is statistically significant (*P* < 0.05, by one way ANOVA, followed by the Bonferroni). The lower long/short ratios in adult and fetal rat lungs are consistent with a similar ratio in the L2 cell line (type II-like rat alveolar pneumocytes) (Table 2). Although the ratios of the two forms were estimated within each tissue and compared between tissues, this experiment did not intend to compare quantitatively the amount of each form alone between

Figure 3. Examples of RT-PCR plot from adult rat tissues and cells. Primers HRC5' and HRC3' were used in the amplification. Number of cycles are labeled. Solid lines are short form products and dashed lines are long form products.

tissues. Our results suggest that CIC-2S is favored relative to CIC-2 in cells of lung-derivation compared with that of brain.

DISCUSSION

Rat CIC-2 transcript contains a truncated form produced by alternative splicing

A number of CIC channels have been described among which CIC-2 is ubiquitously expressed in rat, rabbit and human tissues based on Northern hybridization (8,10,11). CIC channel protein short forms, however, have been demonstrated only in a few cases. In rat CIC-K2, a 55 amino acid residue fragment in the second membrane spanning domain has been found truncated in a short form, CIC-K2S (3). In rabbit, a short form of CIC-2G has been reported with a 68 amino acid residue truncation at the very N-terminal end (12). In both cases, the authors speculated that different RNA processing might be the cause of the truncation. CIC-2 is different from CIC-K2 and CIC-2G in that there is a deleted portion within the cytosolic domain, which is less conserved among CIC channel family members compared with other regions of the protein. This makes non-specific PCR amplification less likely. The possibility of non-specific PCR amplification of other CIC channel genes is further eliminated by using three different primer pairs in our experiments, including using the full-length cDNA clone pRK as the PCR template, size determination of the PCR products by GeneScan, and ultimately, cloning and sequencing of the two PCR fragments.

In addition to the observed exon skipping in the cytosolic domain, other CIC-2 transcripts in which exon-skipping occurs in other regions of the gene may also exist. We have detected a full length CIC-2 cDNA from rat kidney by RT-PCR differing from the published rat brain sequence by only the 60 bp deletion, indicating the presence of the short form transcript CIC-2S without any other variations. The putative amino acid sequence of the deleted region is highly positively charged, and the genomic structure is conserved in rat and human. Both of these may suggest a functional or structural importance of this alternative splicing and possible participation of the short form in the rat CIC-2 regulation or function. When the human cell lines 9/HTEo- and NHBE were analyzed by RT-PCR, CIC-2S was not detected. The conservation of the upstream intron in human and rat predicts that CIC-2S may be produced in human tissues *in vivo*.

Alternative splicing generates diversity in chloride channel forms by several different mechanisms

Alternative RNA splicing is one of the most common strategies that cells use to generate further diversity (21). The mechanisms involved in exon skipping are diverse and not well understood. Small exon size itself may facilitate the aberrant splicing (22). However, a number of examples of exon skipping involve changes in some basic elements in their DNA sequences such as branch point sequences, polypyrimidine tracts and 3' splice sites in their upstream introns. Mutations introduced at these points led to exon skipping, and return of these elements to their respective consensus sequences appeared to correct the skipping (23,24). While the structure of these conserved DNA elements is necessary for normal RNA splicing in many cases, only a few studies showed unusual amounts of pyrimidine residues in the introns upstream from deleted exons. In the case of CFTR (cystic fibrosis transmembrane conductance regulator) exon 9, an inverse relationship has been demonstrated between the length of a polythymidine tract in its intron 8 acceptor site and the proportion of exon 9[−] CFTR transcript, i.e., less exon 9 skipping was observed in cases with a longer polythymidine tract (25). High pyrimidine content is also evident in the rat β -tropomyosin gene intron 6 (68%) where exon 7 is skipped in skeletal muscle (26). The high pyrimidine content of the upstream intron in the CIC-2 gene may be one of the basic *cis*-acting elements which participate in the alternative splicing, however further evidence is needed to confirm its role. Another possible mechanism which could lead to exon skipping is the failure to recognize the 3'-splice site of the upstream intron. Competition between 3'-splice sites for recognition is largely dependent on their structure. AAG in the upstream intron 3'-splice site is unique to all of the CIC-2 introns sequenced to date (data not shown), others being either TAG or CAG, both preferred for recognition over AAG (20). The high pyrimidine content and the 3'-splice site sequence may constitute basic regulatory elements present in the rat genomic DNA. Since lung has a lower ratio of long form/short form transcripts than the brain, there might be other *trans*-acting regulatory factors with different abundance or activity in either lung or brain that interact with the upstream intron or splice sites and either promote the exon skipping as seen in the lung or help retain the exon as seen in the brain.

CIC-2 and CFTR are both expressed in organs affected in cystic fibrosis

CFTR is a cAMP-regulated chloride channel (27) expressed in many secretory epithelia. In cystic fibrosis patients, mutant CFTR expression results in abnormal chloride permeability and significant respiratory and digestive tract symptoms. Our PCR results in rat gave lower ratios of long form/short form CIC-2 transcripts in the lung and intestine than in brain. These results, in addition to our previous observation that CIC-2 expression in rat lung is down-regulated after birth at both RNA and protein levels (1),

may suggest that lower amounts of alternative chloride channels like CIC-2, or relatively lower amounts of certain forms, may contribute to disease pathogenesis in CF airways.

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