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Identification of a Novel Calcium (Ca^{2+})-Activated Chloride Channel-like Membrane Protein 1 Gene that Encodes the Metalloprotease Motif in *Xenopus laevis*

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Abstract

Here, we report cloning and identification of a calcium (Ca^{2+})-activated chloride channel (CLCA)-like membrane protein 1 (CMP1) gene from the *Xenopus laevis* colon, which encodes a metalloprotease motif. CMP1 was identified by *in silico* analysis, rapid amplification of cDNA ends (RACE), and tissue distribution analysis. It was revealed that the CMP1 amino acid sequence showed 49.8% overall identity with rat brain (rb) CLCA1 using *in silico* analysis. In particular, 5′- and 3′-RACE methods were adapted to obtain whole cDNA sequence with an open reading frame (ORF) encoding 936 amino acids. CMP1 also had the following features of CLCA family members: predicted signal sequence (ss), N-linked glycosylation, N-myristoylation, phosphorylation by PKC, and casein kinase II with at least five putative transmembrane (TM) domains. Additionally, it was revealed that CMP1 is ubiquitously expressed, especially in the brain, colon, heart, kidney, oocytes, and spleen with both semi-quantitative reverse transcription (RT) polymerase chain reaction (PCR) and real-time PCR analysis. Tissues except for the brain, heart, and spleen had relatively low CMP1 expression, and expression was almost undetectable in the lung and small intestine. In particular, we could not detect CMP1 in the liver. Collectively, these results enhance the amount of genetic information that is available for *X. laevis* and were useful for characterizing CMP1 as a possible metalloprotease rather than an ion channel.

Keywords: Ca^{2+}-activated chloride channel; *Xenopus laevis;* Gene expression; Real-Time PCR; Rapid amplification of cDNA ends (RACE); Metalloprotease motif

Abbreviations:

CLCA or CaCC: Calcium (Ca^{2+})-Activated Chloride Channel; CMP1: CLCA-like Membrane Protein 1; rbCLCA1: rat brain CLCA1; ss: Signal Sequence; ORF: Open Reading Frame; TM: Transmembrane

Introduction

Many researchers have conducted studies to understand the diverse kinds of ion channels that exist, especially anion channels. It is known that anion channels have diverse functions and physiological roles as well as tissue expression patterns. Among them, the Ca^{2+}-activated chloride channel (CLCA, also called CaCC) was reported and characterized as an anion channel family candidate [1]. Even though it is known that the CLCA gene exists as an anion channel in higher animals, such as humans, mice, and rats, many animals are also assumed to have CLCA members [2-4]. So far, researchers have tried to understand the molecular characteristics of CLCAs for *Xenopus laevis* (*X. laevis*). However, CLCAs from *X. laevis have* not been thoroughly studied even though the demand for genetic information of this species is increasing.

Meanwhile, CLCAs have been known to respond to a Ca^{2+} signal to mostly produce chloride current in humans and rodents [2,5]. Additionally, there is no structural relevance between CLCAs and recently studied TMEM16 families in *X. laevis*, despite their similar functional features such as producing Ca^{2+}-activated chloride conductance [6]. There have been limitations in determining species boundaries of bovines, humans, mice, rats, and pigs, despite the many attempts at characterization of CLCA family members [1-4,7]. In addition, CLCA expression patterns vary in smooth muscle cells, endothelial cells, the central nervous system, and secretory epithelial cells [8-12] as well as in important physiological roles such as olfactory transduction, neuronal excitability, and cell-to-cell adhesion [12,13].

Moreover, recent studies have focused more on the structural rather than the functional aspect of generating anion currents [14-16]. In several CLCA structure studies, it was shown that there is a specific motif that is shared among diverse species [17-19]. This motif is called the “metalloprotease family,” which is dependent on zinc, copper, or other cations and is characterized by sharing the HExxH motif [20]. It is also expected to assign specific characteristics to CLCAs as a possible metalloprotease family [18,19]. Several reports of possible metalloprotease motifs were mentioned among human and mouse CLCAs as well as in a previously reported rat brain (rb) CLCA1 study, which was 82% identical to mCLCA1, as determined by our lab [17,19,21].

The purpose of this study was to identify molecular characteristics of the CLCA-like membrane protein 1 (CMP1) gene of *X. laevis*. Here we report a putative CLCA-like membrane protein 1 (CMP1) gene in *X. laevis* based on its primary structure and organ distribution profiles to understand the similarity and differences between original CLCA and CMP1.

Materials and Methods

Materials

Molecular reagents were purchased from Takara Korea (Seoul, Republic of Korea), Finnzymes (Espoo, Finland), Promega (Madison, WI, USA), Toyobo (Osaka, Japan), Invitrogen™ (San Diego, CA, USA), iNtRON Biotechnology (Seongnam, Republic of Korea), Amersham Biosciences (Buckinghamshire, England), and BD Science (Franklin Lakes, NJ, USA) as indicated. pGEM®-T Easy vector, DyNAmoColorFlash SYBR Green qPCR kit, a peltier thermal cycler PTC-200 cycler, and CHROMO4™ real-time PCR system were purchased from Promega, Finnzymes, and BIO-RAD (Hercules, CA, USA) through PharmaTech Co. All other chemical reagents used were purchased from BioPure (Burlington, Ontario, Canada). Female *X. laevis* (Xenopus I, Ann Arbor, MI, USA) were kindly provided by Dr. S.Y. Nah (Konkuk University, Seoul, Republic of Korea).

RNA preparation

First, *X. laevis* was surgically dissected to obtain various tissues to extract total RNA. Total RNA was isolated from the tissues with easy-BLUE (iNtRON Biotechnology) following the manufacturer's instructions and then digested with RNase-free DNase I (Invitrogen™) to avoid any genomic DNA contamination.

In silico Search of the *X. laevis* Expressed Sequence Tags (EST) Database and Analysis

We queried the rbCLCA1 cDNA sequence using the National Center for Biotechnology Information (NCBI)-Basic Local Alignment Search Tool (BLAST) server to determine whether the cDNA was homologous to CLCAs that exist in the *X. laevis* EST database to identify the putative target sequence. *X. laevis* cDNA sequences showing appropriate homology (40–60%) were selected as candidates for the new CLCA gene family. We found a partial cDNA sequence (DC_066238) in EST that was regarded as a putative CLCA family member compared with rat CLCAs. The Simple Modular Architecture Research Tool (SMART) was used to identify and analyze CMP1 protein domain architectures. Sequence analysis, including multiple alignment and homology, was conducted by GENETYX® software (GENETYX, Tokyo, Japan).

Gene cloning of *X. laevis* CMP1

To synthesize the first-strand cDNA, RT of *X. laevis* total RNA was performed as described in our previous report using an oligo-d (T) [22]. DC_066238-specific primers were generated to complete full-length cDNA and summarized in Table 1. In short, primers used are as follows: first round with S106 and a 3′-linker primer, second round with a 5′-linker primer and S108, and final round with a 5′-linker primer and S110. Three rounds of rapid amplification of cDNA ends (RACE) are shown in Figure 1A. All described PCR procedures were carried out using KOD-Plus *Taq* polymerase (Toyobo) with the proofreading function to avoid the errors. The first PCR was performed under the following conditions: pre-denaturation at 94°C for 2 min, 25–35 repeats of 94°C for 30 sec, 55–62°C for 30 sec, and 68°C for 1–3 min. A sephacryl S-400 spin column (Amersham Biosciences) was used to remove the extra oligo-primers from the first PCR products. To obtain complete 3′- and 5′-RACE products, nested PCR was performed with $1/50^{th}$ the volume of the first PCR products as follows: denaturation at 94°C for 2 min, 25–35 repeats of 94°C for 30 sec, 55.5–61°C for 30 sec, and 68°C for 1–3 min. The pGEM-T Easy vector (Promega) carrying the sub-cloned cDNA was transformed into the *E. coli* DH5 strain.

The nucleotide sequence was determined using an ABI PRISM system (model 377) after plasmid DNA extraction, and then sequence homology search and analysis were conducted on the NCBI-BLAST server.

Finally, full-length CMP1 was amplified using the primer set S116 and S117 as follows: pre-denaturation at 94°C for 2 min, denaturation at 94°C 30 repeats for 30 sec, annealing at 56°C for 30 sec, and extension at 68°C for 3 min. A nested PCR procedure with the primer set S118 and S117 was conducted as follows: pre-denaturation at 94°C for 2 min, denaturation at 94°C, 30 repeats for 30 sec, annealing at 60°C for 30 sec, and extension at 68°C for 3 min.

Name	Sequence
S106	5' AAACCTGCAACTGCATCCGTG 3'
S108	5' TTTGTGGGTCACGGATGCAG 3'
S110	5' GGTCATCTCCAAACTTGAGATGG 3'
S116	5' ATATACATTCATCAGGTAGATT 3'
S117	5' GATCATGTTCAGTTTATTGTAA 3'
S118	5' CATCAGGTAGATTAACACAGGG 3'
XHRMbA1	5' ATGCCATCCTGCGTCTGGACCTG 3'
xbA3	5' CCAATGATGAAGAAGAGGCAG 3'
C204	5' TTTGTAGAAAATGGGCAAGTAGGGT 3'
C210	5' CAGTTGCTGTCCAAGTCAACAC 3'

Table 1: Primer sequence and name (5'-RACE; S108, S110, 3'-RACE; S106, β-actin; XHRMbA1, xbA3, RT-PCR and real-time PCR; C204, C210, 5'- and 3'-UTR; S116 and S118, S117).

Semi-quantitative RT and real-time PCR

Total RNA from the dissected *X. laevis* tissues were used in RT-PCR to determine the expression level of each sample. Nine types of tissues were employed as follows: brain, heart, lung, liver, small intestine, colon, kidney, spleen, and oocytes. The nine independent cDNAs that were mentioned were also used to analyze expression level of each tissue and confirm the RT-PCR results. PCR was performed as follows: pre-denaturation at 94°C for 1 min, 20 repeats of denaturation at 94°C for 15 sec, annealing at 55°C for 20 sec, extension at 72°C for 20 sec; and for the second PCR, 94°C for 1 min, 25 repeats of 94°C for 15 sec, 57°C for 20 sec, and 72°C for 1 min. The intensity of each product was measured with the ImageJ program and analyzed using the GraphPad Prism5 program. To maintain consistency, nine RT-PCR products from were used for the subsequent part of the

experiment. A CHROMO4™ real-time PCR system was employed for analysis. DyNAmoColorFlash SYBR Green qPCR kit (Finnzymes) was used and the procedures were conducted as follows: 95°C for 7 min, 35 repeats of denaturation at 95°C for 10 sec, at 60°C for 20 sec, at 72°C for 20 sec, and final extension step at 72°C for 7 min, and detection of melting curve from 65°C to 90°C. Expression levels were calculated using the GeneXpression Macro CHROMO4™ Opticon4 program, which was provided by the supplier. RT-PCR and real-time PCR were performed at least three times.

Results

CMP1 gene cloning in *X. laevis*

A partial cDNA (DC_066238) was selected and screened as described in our previous study [22]. The first-strand cDNA was constructed by performing RT-PCR (Figure 1A). We have obtained a full-length CMP1 cDNA using RACE as mentioned in the Materials and Methods (Figure 1B and 1C). After performing RACE, partial cDNAs were combined to obtain and clone the entire 2,959 bp CMP1 coding sequence including a large open reading frame (ORF) (Figure 1A). As shown in Figure 1B, CMP1 contains three fragments; S106-3′-linker primer, 5′-linker primer-S108, and 5′-linker primer-S110 primer sets were used to obtain these fragments, and the fragment sizes were 2,406 bp, 490 bp, and 410 bp, respectively. After sub-cloning into pGEM-T Easy Vector (Promega), each sequence showed no missing or changed bases. The full-length cDNA also contains a short 5′- and a 3′-untranslated region (UTR) as well as a poly-adenylated tail. This sequence was registered as the xCLCA2 gene in the DNA data bank of Japan (DDBJ) (GenBank, Accession No. AB591376).

CMP1 primary structure

Next, primary structure analysis using an *in silico* approach revealed that the full-length CMP1 has a single ORF with 5′- and 3′-UTRs. It was also shown that the CMP1 consisted of 936 amino acids (aa). The aa sequence was compared with rbCLCA1 to determine whether they were significantly homologous. It showed about 50% of identity to that of rbCLCA1 within the ORF of CMP1 (Figure 2A and Table 2). The overall homology search revealed CMP1 as a possible new CLCA-like family member from *X. laevis* (Table 2). We found putative characteristic sites including N-linked glycosylation, phosphorylation by protein kinase C (PKC), the signal cleavage site, cAMP- dependent protein kinase recognition, and casein kinase II interaction (Figure 2A). Even though CMP1 showed significant homology to rbCLCA1, the carboxyl terminus was not identical (Figure 2A). This as analysis was interesting because the unique carboxyl sequence of CMP1 was compared to that of rbCLCA1. Domain analysis by SMART (http://smart.embl-heidelberg.de/) predicted that the following CLCA domains: N-terminal, vWA (von Willebrand factor A) and DUF1973 (Domain of unknown function 1973) (Figure 2B). In hydropathy analysis, the Kyte-Doolittle method was applied to show that the ORF in CMP1 has at least five potential transmembrane (TM)-spanning domains using the GENETYX program. The result of hydropathy analysis for CMP1 is overall consistent with other CLCA families that we previously reported including CMP6 (Figure 2C).

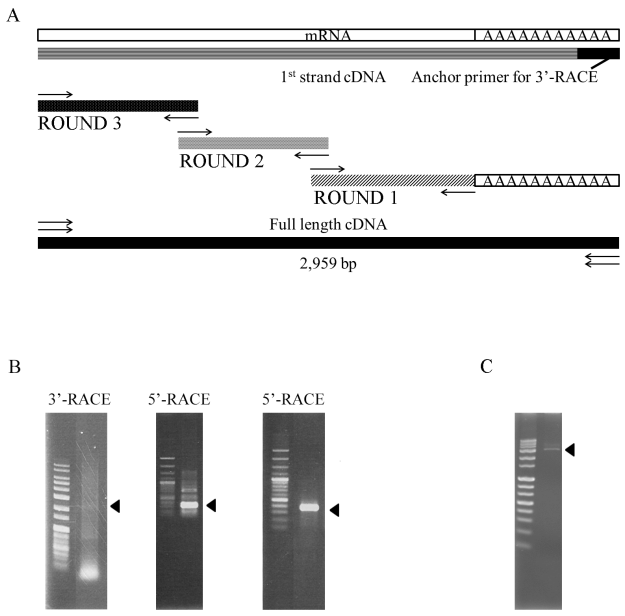

Figure 1: The strategy of constructing CMP1 and full-length amplification. (A) Three rounds of PCR were conducted to obtain the full length of CMP1 cDNA. First-strand cDNA (grey line) and PCR products from round 1 to round 3 are depicted with a diagonal line, dense dotted line, and short black line, respectively. (B) PCR products of 3′-RACE (left: Round1) and 5′-RACE (middle: Round2, and right: Round3) were electrophoresed in a 1% (3′-RACE) or 1.5% (5′-RACE) agarose gel. Black arrowheads indicate the 2,406-bp, 490-bp, and 410-bp target products. (C) Full-length PCR product of *X. laevis* CMP1. The 2,959-bp cDNA was amplified by gene-specific primers (S118 and S117).

Gene	Homology (%)
mCLCA1	49.66
mCLCA2	49.21
mCLCA3	46.65
mCLCA4	50.34
mCLCA5	43.76
mCLCA6	46.44
hCLCA1	47.49
hCLCA2	44.25
hCLCA4	47.65
rbCLCA1	49.83
rbCLCA2	50.05

Table 2: Overall percentages of amino acid homology for CMP1 (Mouse, human and rat brain are indicated as m, h and rb, respectively).

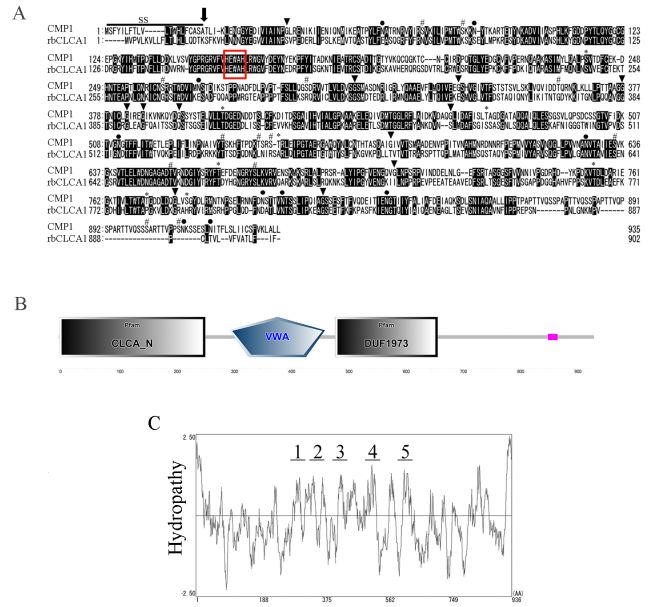

Figure 2: Primary structure analysis of CMP1. (A) CMP1 amino acids were aligned by querying with rbCLCA1 on CLUSTAL W. The homology of amino acids of CMP1 is indicated by a black brick pattern. The red box indicates the putative metalloprotease motif (HExxH). Consensus sites are marked as follows: the predicted signal sequence (SS), N-linked glycosylation (•), N-myristoylation sites (▼), phosphorylation by PKC (#), and casein kinase II (*). An arrow indicates the predicted cleavage site. (B) CMP1 structure analysis using SMART. It revealed that CMP1 contains CLCA_N terminal, vWA, and DUF1973 domains. Low complexity region is shown with pink. (C) Hydropathy analysis of CMP1. The hydropathy plot was analyzed by the Kyte-Doolittle method using the GENETYX program. Hydrophobic domains are given as positive values. There are five segments of the proposed hydrophobic domains.

Quantitative analysis of CMP1

We performed 45 RT-PCR repeats to examine the tissue expression pattern of CMP1 in different *Xenopus* tissue samples, including the brain, heart, lung, liver, small intestine, colon, kidney, spleen, and oocytes. CMP1 is a gene-specific product and β-actin (a housekeeping gene) showed 217-bp and 159-bp products, respectively (Figure 3A). The analysis revealed that CMP1 was expressed in multiple tissues with significant expression levels in the brain, spleen, heart, colon, kidney and oocytes. In contrast, CMP1 did not show any bands in the lung, liver and small intestine (Figure 3A). Considering the individual expression level, CMP1 was expressed in the following order: brain > spleen > heart > colon ≥ oocyte ≥ kidney; however, no bands were found in the lung, liver, and small intestine (Figure 3B).

To confirm the data mentioned above, real-time PCR was performed to determine tissue distribution with the same samples (Figure 4). The exponential graph was converted and shown as a bar graph with numerical values for each product. As a result, we obtained the distinct order of expression: heart > spleen > brain.

This graph also showed that the colon and oocytes had little CMP1 expression. Additionally, CMP1 was undetectable in the lung, liver, small intestine and kidney. Despite few differences in expression order, these results were mostly consistent with the semi-quantification RT-PCR results. Melting curve analysis showed that CMP1 products had unique one PCR band in each tissue (data not shown) compared with the semi-quantitative RT-PCR analysis.

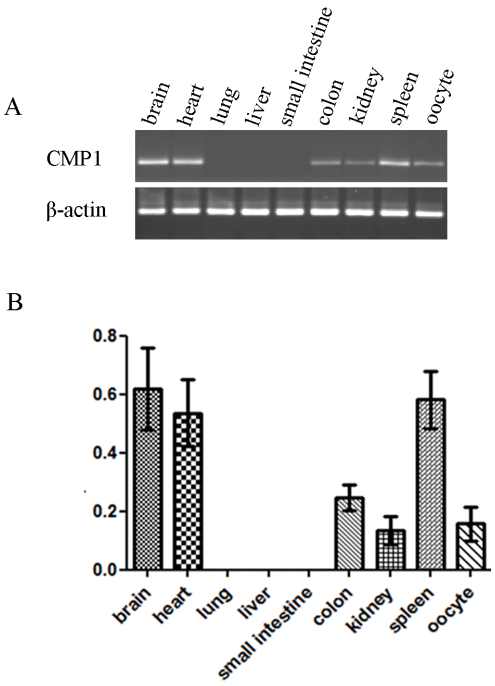

Figure 3: Tissue distribution pattern of CMP1 using reverse transcription (RT) PCR and real-time PCR. (A) RT-PCR products in *Xenopus* tissues. The amplified PCR products were electrophoresed in a 2% agarose gel. The specific RT-PCR products are a 217-bp CMP1 (C204 and C210) and a 159-bp β-actin gene as an internal control (XHRMbA1 and xbA3). Expression ratios of CMP1 are shown as relative calculated values for β-actin. (B) CMP1 showed high expression in the brain, heart, and spleen. Each graph represents at least three independent experiments. Data are expressed as means±s.d. (n=3).

Discussion

Genome studies have been flourishing since extensive DNA genome projects of many species have been completed. According to the the *Xenopus* Gene Collection (XGC), total XGC full ORF clones are varied approximately 10,000 clones with *Xenopus* species (http://xgc.nci.nih.gov/). However, the diversity of *X. laevis* DNA profiling studies is still limited. Thus, several attempts have been made to understand the role of specific *X. laevis* genes in our lab [22,23].

CLCAs are thought to function as an ion channel that responds to Ca^{2+} being released from the endoplasmic reticulum (ER) [12]. Mainly studied in mammals (bovine, mouse, rat, and human), CLCAs have been characterized as a chloride channel with several TM domains [1,24-26].

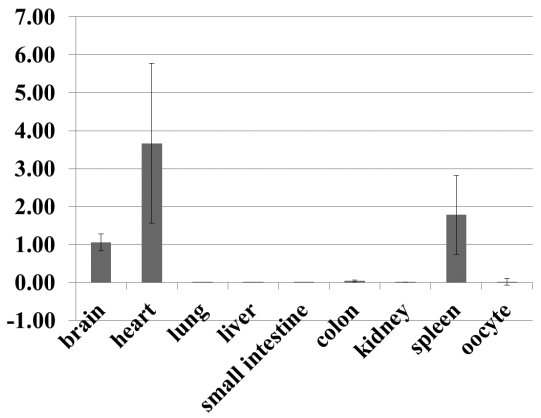

Figure 4: CMP1 quantification by real-time PCR.Quantitative histogram of real-time PCR. Real-time PCR was carried out with 45 repeats for quantitative analysis for CMP1 expression in *X. laevis* tissues. The result confirms that CMP1 is expressed in the heart, spleen, and brain. Data are expressed as means±s.d. (n=3).

However, recent studies have argued that general CLCA characteristics do not satisfy anion channels requirements and in fact reveal opposite evidence [5,18]. In some cases, it was shown that there is a lack of membrane localization [25] and no electrophysiological current present as well as possessing fewer than 5 TM domains [14]. With this evidence, researchers have considered the possibility that CLCAs might be ion channel modulators and not form ion channels themselves [5,24].

After our successful cloning of CMP1 (Figure 1), which originated from a Xenopus EST clone comparing to rbCLCA1, there have been many efforts to understand its roles and functions.

First, we focused on the primary structure of the CMP1 aa sequence to exam if it has the characteristics of general CLCA protein members. It resembles most of the characteristics except for the fact that TM domain varied, which was determined based on several domain search engines (data not shown). Additionally, a previous report of xANO2 (also known as the TMEM16 family) as a new CaCC in *X. laevis* indicate the possibility that there could be a relationship between CMP1 and TMEM16 families; however, there was very low homology even though they were shown to overall be in the same category (data not shown).

In tissue expression pattern analysis, CMP1 was abundantly detected in multiple tissues, especially in the brain, heart, and spleen (Figure 3A). These data conflict with the result of tissue expression pattern in CMP6, which has been previously reported by our lab [22]. In that study, CMP6 showed very high expression levels only in the intestine and colon as well as moderate expression in the liver, while CMP1 showed a slightly opposite pattern. We suggest that there might be a possible compensatory reaction or action between CMP1 and CMP6 in *X. laevis.*

We next searched for functional expression of CMP1 by either co-transfecting CMP1 and EGFP or transfecting EGFP alone as a control using human embryonic kidney cells (HEK293T cells). It was our assumption that the currents would be evoked by adding the Ca2+ containing pipette solution in a voltage-dependent manner in the whole cell patch-clamp system (data not shown).

We also attempted the oocyte electrophysiology technique using Axolotl oocytes with support from Lily Yeh Jan's group but failed to evoke any currents to identify CMP1 as an anion channel (data not shown). These results show another possibility that the CMP1 could not form an ion channel itself as was shown in the mouse CLCA studies [5].

Recently, it was proposed that CLCAs are in potentially in the metalloprotease family with the HExxH domain [20]. Metalloproteases form a very large family of enzymes that require cations such as zinc, copper, and sometimes magnesium for their function [27]. During our CMP1 study, we found the HExxH domain with a search engine suggesting the CMP1 as a possible metalloprotease family member. Further studies could be conducted to determine why CMP1 did not produce CaCC currents. Additionally, future studies could focus on the metalloprotease activity of CMP1 to enhance our understanding of its role.

Acknowledgment

Xenopus laevis was kindly provided by Dr. S.Y. Nah (Konkuk University, Seoul, Republic of Korea). We also thank Dr. Lily Yeh Jan's group for support with the patch clamp experiment.

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