Identification of a Novel Chloride Channel Expressed in the Endoplasmic Reticulum, Golgi Apparatus, and Nucleus*  

Masahiro Nagasawa‡‡, Makoto Kanzaki‡, Yuichi Iino‡, Yasuo Morishita§, and Itaru Kojima‡‡‖  
From the ‡Institute for Molecular and Cellular Regulation, Gunma University and the ††Second Department of Surgery and the ‡‡Department of Emergency and Critical Care Medicine, Gunma University School of Medicine, Maebashi 371-8512, Japan

MID-1 is a Saccharomyces cerevisiae gene encoding a stretch-activated channel. Using MID-1 as a molecular probe, we isolated rat cDNA encoding a protein with four putative transmembrane domains. This gene encoded a protein of 541 amino acids. We also cloned the human homologue, which encoded 551 amino acids. Messenger RNA for this gene was expressed abundantly in the testis and moderately in the spleen, liver, kidney, heart, brain, and lung. In the testis, immunoreactivity of the gene product was detected both in the cytoplasm and the nucleus. When expressed in Chinese hamster ovary cells, the gene product was located in intracellular compartments including endoplasmic reticulum and the Golgi apparatus. When microsome fraction obtained from the transfected cells, but not from mock-transfected cells, was incorporated into the lipid bilayer, an anion channel activity was detected. Unitary conductance was 70 picosiemens in symmetric 150 mM KCl solution. We designated this gene Mid-1-related chloride channel (MCLC). MCLC encodes a new class of chloride channel expressed in intracellular compartments.

Various types of chloride channels modulate diverse cellular functions. Chloride channels expressed on the plasma membrane regulate various cellular functions including control of cell volume, transepithelial ion transport, modulation of membrane potential, neurotransmission, and bone resorption (1). Chloride channels are also expressed in intracellular membranes and may regulate acidification of intracellular compartments and vesicle trafficking (2). Mutations in the chloride channel genes cause various human diseases such as cystic fibrosis (3), kidney stone disease (4), and congenital myotonias (5).

Recently, the molecular nature of chloride channels was revealed by gene cloning, and the functions of chloride channels have been extensively studied at the molecular level. Four major types of chloride channels have been identified to date: the cystic fibrosis transmembrane conductance regulator (6), the ligand-gated receptor channels (7), the ClC family (8), and the CLIC family (9). Among them, members of the CLIC family and some of the CIC family are expressed in intracellular organelles, including endoplasmic reticulum, the nuclear envelope, and endosomes, and regulate ion fluxes across the intracellular membranes.

The MID-1 gene of yeast Saccharomyces cerevisiae (10) was originally identified as a gene mutation that induces cell death during the treatment of yeast with the mating pheromone. Loss-of-function mutation in the MID-1 gene results in the reduction of calcium entry into the yeast and thereby induces cell death (10). Functional analysis of the gene product of MID-1 (Mid-1) revealed that the Mid-1 was a stretch-activated calcium-permeable cation channel (11); Mid-1 functions as a non-selective calcium-permeable channel, the opening of which is regulated by stretch of the plasma membrane. Mid-1 is the first eukaryotic stretch-activated channel whose primary structure was identified by molecular cloning (11). Because stretch-activated channels regulate diverse cellular functions in mammalian cells (12), we attempted to identify mammalian stretch-activated channels using Mid-1 as a molecular probe. During the course of the present study, we identified a new class of ion channel molecule expressed in intracellular compartments. This protein functions as a chloride channel when incorporated in the planar lipid bilayer and, therefore, we designated it the Mid-1-related chloride channel (MCLC).

MATERIALS AND METHODS

Cloning of Rat MCLC cDNA—A BLAST search using the partial sequence of Mid-1 led to the identification of a Xenopus unknown transmembrane protein (GenBankTM accession number X92871). To obtain mammalian homologues, the total sequence of X92871 was used to search again, and this led to the identification of an expressed sequence tag (EST accession number H51262). A rat brain oligo(dT)-primed ZAP (Stratagene, La Jolla, CA) cDNA library was screened using cDNA probes derived from H51262. Replicate filters were prehybridized for 3 h at 37 °C in the following solution: 5 × SSPE (standard saline phosphate-EDTA solution), 10 × Denhardt’s solution, 50% formamide, 0.1% SDS, and 0.1 mg/ml denatured herring sperm DNA. The filters were then hybridized in the same solution plus 5 × 107 cpn 32P-labeled randomly primed probe (Ready to Go DNA labeling kit, Amersham Pharmacia Biotech). The hybridized filters were washed three times at room temperature with 0.1× SSC plus 0.1% SDS and then washed for 1 h at 42 °C with the same buffer. The cDNA was excised from the ZAP vector with the use of a helper phage according to the manufacturer’s instructions (Stratagene). The entire sequence of both strands was determined using an ABI PRISM dye terminator cycle sequencing FS ready reaction kit and an Applied Biosystems DNA sequencer 373S (Applied Biosystems, Cambridge, MA).

Cloning of Human MCLC cDNA—A BLAST search using the total sequence of rat MCLC led to the identification of expressed sequence tags of the human homologue of MCLC (EST accession numbers AA375206, AA906589, and AB018304). The cDNA template was synthesized from MCF7 and HepG2 cells using a gene-specific primer

PCR, polymerase chain reaction; MOPS, 4-morpholinepropanesulfonic acid; CHO, Chinese hamster ovary; ER, endoplasmic reticulum.
and a 5' end-specific forward primer (5'-ATTCAACAGTATCGTGCTGCTCTTTC) and a 3' end-specific reverse primer (5'-GCTGGTGTTCCTCTAGCCACA) were utilized for the amplification of cDNA. The PCR products were cloned and sequenced as described above. DNA Recombination Procedures and Transfection—The rat cDNA clone was subcloned into the BamHI and XhoI sites of the vector pcDNA3 (Invitrogen). To introduce the FLAG epitope tag into the carboxyl terminus of MCLC, we amplified a fragment of MCLC (nucleotides 1128–1623) by PCR using the sense primer (5'-AGATGACAGAAGACGACAGAAGGAACTTG) and the antisense primer (5'-TTATTATCAGTGCTACATCGCTTCTTTTGATGAAGCCAAGCGGCTGCTGAC- CAAG); the PCR products were then exchanged for native MCLC cDNA. The PCR was carried out using high fidelity enzyme Ultima (Roche Molecular Biochemicals), and the PCR-generated constructs were verified by sequencing the amplified region. The MCLC expression vectors were purified using a concert high purity plasmid maxiprep system (Life Technologies, Inc.) according to the manufacturer's instructions. Cells were transfected using LipofectAMINE Plus reagent (Life Technologies, Inc.). To establish stable cell lines, the cells were replaced at a lower concentration in fresh medium containing 10% fetal calf serum at 24 h after transfection. 48 h after transfection, the transfected cells were selected using 1 mg/ml neomycin analog G418. After 10–14 days, independent colonies were picked up, grown in 35-mm
Chloride Channel in Intracellular Membranes

![Image](61x61 to 256x729)

**Fig. 2. Expression of mRNA for MCLC in various rat tissues.** RNA was extracted from various rat organs, and Northern blotting was performed. Ribosomal RNA (28 S) is shown in the lower panel.

dishes, and screened for a high level of expression of MCLC and monooestony by immunostaining.

**Northern Blot Analysis**—RNA from various tissues was isolated using a TRIZOL Reagent (Life Technologies, Inc.). 40 μg of total RNA was electrophoresed on a 1.0% agarose gel containing 2.2 M formaldehyde, 40 mM MOPS (pH 7.0) and transferred to a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech) using a capillary blotting technique with 20× SSC (sodium citrate buffer). Northern blots were performed as described elsewhere (15).

**Cell Culture**—Chinese hamster ovary (CHO) cells provided by the Riken Center bank (Tsukuba, Japan) were cultured in Ham’s F12 medium and the lysate was cleared by centrifugation at 1000 g. Rabbit IgG antibody or anti-FLAG M2 antibody at room temperature for 1 h. Washed two times with phosphate-buffered saline and two times with 10 μM EDTA, 10 mM MOPS-Tris (pH 7.0). Ion channels were incorporated into the bilayer by flushing 9 μl of the microsome suspension (containing 0.5 to ~1 μg of protein) directly toward the bilayer from the cis side.

The solution on the cis and trans sides of the bilayer were connected to a patch clamp amplifier (EPC-7, List, Darmstadt, Germany) via 0.5 μKCl-agar bridges in series with Ag-AgCl electrodes. To shield from electromagnetic and mechanical interference, the bilayer chamber and the head stage of the patch clamp amplifier were placed in a Faraday cage mounted on an antivibration table. The electrical current across the bilayer was through an 8-pole low pass Bessel filter and was visualized on a storage oscilloscope.

**Immunohistochemistry**—For immunostaining, cells were grown on coverslips, fixed for 5 min in 3% paraformaldehyde, washed twice with phosphate-buffered saline, and incubated with blocking Ace (Snow Brand, Tokyo, Japan), and incubated with anti-MCLC anti-rabbit IgG antibody or anti-FLAG M2 antibody at room temperature for 1 h. For colocalization experiments, MCLC-expressing cells were transfected with PEYFP-ER vector (CLONTECH), and anti-green fluorescent protein (GFP) antibody was used. Anti-calnexin (Santa Cruz Biotechnology) or anti-Golgi 58K antibody (Sigma) was used in other experiments. After washing with phosphate-buffered saline, the coverslips were incubated with secondary antibodies obtained from Jackson ImmunoResearch (West Grove, PA) conjugated with indocarbocyanine (Cy3), fluorescein isothiocyanate, or tetramethyl rhodamine isothiocyanate (15).

**Results**

**Cloning of Rat MCLC**—A BLAST search using the partial sequence of Mid-1 led to the identification of a Xenopus unknown transmembrane protein (GenBank accession number X92871). To obtain a mammalian homologue, the total sequence of X92871 was used to search again, and this led to the identification of an expressed sequence tag (EST accession number H51262). A rat brain cDNA library was screened using the cDNA probe derived from H51262. The full-length rat MCLC cDNA was obtained and sequenced (Fig. 1A). The rat MCLC cDNA contained an open reading frame of 1623 base pairs and coded for a protein of 541 amino acids with a calculated molecular mass of 61 kDa. There was no overall sequence similarity between MCLC and other members of the CLIC family. There was, however, similarity in the sequence of the third transmembrane domain (Fig. 1B). The amino acid sequence of this domain resembled those of members of the CLIC family.

**Cloning of Human MCLC**—A BLAST search was performed on EST databases with the rat MCLC sequence. Three human sequence tags were identified corresponding to the partial sequence of 5′ and 3′ regions. A cDNA template was synthesized from MCP7 and HepG2 cells using gene-specific primers, and PCR was performed to clone the complete coding sequence of human MCLC (Fig. 1A). Fig. 1D depicts the phylogenetic tree of rat, human, and Xenopus MCLC.

**Tissue Distribution of Rat MCLC mRNA**—Northern blot analyses were carried out to examine the tissue distribution of MCLC in the rat. Two hybridization band sizes were found in the rat tissues, including the spleen, liver, testis, kidney, heart, aorta, brain, and lung (Fig. 2). A strong expression was found in the testis.
Expression of MCLC in CHO Cells—To clarify subcellular localization of MCLC protein, expression vector rat MCLC–pcDNA3, a FLAG tagged-rat MCLC–pcDNA3, was constructed. Localization of MCLC was studied by indirect immunofluorescence with anti-MCLC antibody or anti-FLAG antibody in CHO cells stably expressing MCLC. A strong fluorescence staining was detected at the perinuclear region as well as in a fine reticular network extending through the cytoplasm (Fig. 3A). This pattern was similar to that seen with known endoplasmic reticulum (ER) markers such as calnexin (16) (Fig. 3B). Localization of MCLC in ER was confirmed by colocalization of MCLC with ER-targeted enhanced yellow fluorescent protein (Fig. 3C). MCLC is also localized in the Golgi apparatus. The colonization of a Golgi marker, anti-Golgi 58K (17) with MCLC was observed in the transfected CHO cells (Fig. 3D). MCLC was not localized in mitochondria, as assessed by using MitoTracker (data not shown). We also studied the localization of endogenous MCLC. Endogenous MCLC was detected in PC12 cells and A10 smooth muscle cells. The pattern of immunoreactive MCLC was similar to that of transfected cells mentioned above (data not shown). Western blot analysis was carried out to verify the expression of MCLC protein in transfected cells. Under nonreducing conditions, anti-FLAG antibodies detected a major band with a molecular mass of 120 kDa from CHO cells transfected with a tagged MCLC (Fig. 4A). Under reducing conditions, a molecular mass of 60 kDa was detected. A similar blot profile was obtained by using affinity-purified anti-MCLC antibody (Fig. 4B). No signal was obtained from control CHO cells. The mass of 60 kDa was in good agreement with the predicted molecular mass of MCLC.

Expression of Endogenous MCLC—Western blot analyses were then carried out to clarify endogenous MCLC protein localization. Endogenous MCLC was detected in PC12 cells and A10 smooth muscle cells, similar to the pattern observed in transfected cells. Western blot analysis under nonreducing conditions showed a major band with a molecular mass of 120 kDa, while under reducing conditions, a band at 60 kDa was observed, consistent with the predicted molecular mass of MCLC.
expression in various rat tissues. The strong expression of MCLC with a molecular mass of 60 kDa was detected in the testis, liver, and lung (Fig. 5). In the testis, a strong immunoreactivity was observed in primary spermatocytes (Fig. 6A). In these cells, staining of the cytoplasm and the nucleus was observed, and distribution of MCLC was different depending upon the stage of the spermatocyte. In some cells, immunoreactivity was found predominantly in the cytoplasm (Fig. 6B), but in other cells MCLC was localized in the nucleus (Fig. 6C). Note that immunoreactivity disappeared when the antibody was preincubated with the antigen peptide. In hepatocytes, cytoplasmic but not nuclear stainings were observed (data not shown).

Channel Activity of MCLC—The above findings indicated that MCLC was predominantly expressed in the intracellular compartments such as the endoplasmic reticulum and the Golgi apparatus. To clarify whether or not MCLC had a channel activity, we incorporated microsomal vesicles obtained from MCLC-transfected and mock-transfected CHO cells into the planar lipid bilayer and measured the channel activities. We confirmed the presence of MCLC protein in the microsomal fraction from MCLC-transfected cells but not from mock-transfected cells by Western blotting (data not shown). Channel activities were consistently detected in microsomal vesicles obtained from MCLC-overexpressing CHO cells but not in those from mock-transfected cells. As shown in Fig. 7A, a bursting current was observed. The burst current showed open and closed states. Single channel conductance was determined by linear regression analysis of the current-voltage relationships in symmetrical 100 mM KCl solutions (Fig. 7B). The slope conductance of the unitary current was 70.2 ± 5.0 pS (mean ± S.D., n = 5). The channel was more permeable to anions than to cations. Based on the conductance data, the permeability ratio $P_{Cl}/P_K$ was calculated to be 3.7 using the Goldman-Hodgkin-Katz current equation. The permeabilities of various anions were also estimated from reversal potentials in asymmetric solutions of 100 mM KCl in the trans compartment and 100 mM KBr, 100 mM KF, or 50 mM K$_2$SO$_4$ in the cis compartment. The reversal potential for Br$^-$ was $-1.8$ mV ($n = 3$) and the $P_{Br}/P_{Cl}$ was 1.1. The reversal potential for F$^-$ and...
the $P_F/P_C$ value were +12.0 mV (n = 4) and 0.54, respectively. The reversal potential for $SO_4^{2-}$ and the $P_{SO_4}/P_{Cl}$ value were +18.3 mV (n = 3) and 0.26, respectively.

We then examined the effect of chloride channel blockers. 4,4'-disothiocyanatostilbene-2,2'-disulfonate (DIDS), known to be a specific inhibitor of various anion channels (18), did not block the channel activity (data not shown). ATP, which blocks the anion channel in platelets (19), did not affect the channel activity. Note that Gd$^{3+}$, a blocker of stretch-activated channels, did not affect the channel activity of MCLC.

**DISCUSSION**

In the present study, we identified a gene, MCLC, using the MID-1 gene as a molecular probe. MCLC encoded a transmembrane protein with four putative transmembrane domains, but there was no overall similarity between MCLC and MID-1. MCLC was identical to AK2–8, previously identified as a gene expressed in the suprachiasmatic nucleus of the hypothalamus (14), the expression of which changed according to diurnal rhythm. Fukuhara (14) suggested that AK2–8 was involved in the regulation of circadian rhythm. *Xenopus* unknown transmembrane protein, a *Xenopus* homologue of MCLC, was expressed in the pituitary. Yet the function of *Xenopus* unknown transmembrane protein or AK2–8 has not been identified.

When MCLC was transfected in cultured cells, it localized in intracellular compartments but not in the plasma membrane. In culture cells, MCLC was expressed in the ER and the Golgi apparatus, as assessed by colocalization by the ER and Golgi markers. Although MCLC has a nuclear localization signal, MCLC is not targeted to the nucleus when transfected in various types of culture cells. As shown in Fig. 6A, however, MCLC expressed in the testis was localized in the nuclei as well as in the cytoplasm. In spermatocytes, strong immunoreactivity was found in the nucleus, and immunoreactive MCLC was condensed in the nucleus in some stages of the spermatocytes during maturation. The nuclear localization of MCLC in the nucleus of spermatocytes suggests that MCLC may play a critical role in spermatogenesis.

MCLC has four putative transmembrane domains and is expressed in intracellular organelles. When microsomal vesicles obtained from MCLC-expressing cells were incorporated into the planar lipid bilayer, the chloride channel activity with a unitary conductance of 70 picosiemens was consistently observed. Such activity was not detected in microsomal vesicles obtained from mock-transfected cells. Given the primary structure of MCLC as a transmembrane protein, it is likely that MCLC functions as a chloride channel. We cannot, however, rule out the possibility that MCLC exerts channel activity by interacting with other proteins in microsomal vesicles. Hence, we should await definitive evidence clarifying the function of purified MCLC protein in the lipid bilayer. Electrophysiologically, the MCLC channel permeates anions, and the sequence of permeability ratios was $\text{Br}^->\text{Cl}^-\text{Cl}->\text{SO}_4^{2-}$. MCLC forms dimers when expressed in culture cells. These properties are different from those of members of known chloride channel families, in particular CLIC, which localizes in intracellular compartments. The primary structure of MCLC is also quite different from other chloride channel families so far identified. Hence, rat MCLC and its human and *Xenopus* homologues belong to a new family of chloride channel. In this regard, the MCLC sequence in the third transmembrane domain shares similarity with most of the CLIC channel family (Fig. 1C). Because CLIC family members are expressed in intracellular compartments, including the endoplasmic reticulum, the nuclear envelop, and endosome, the similarity between the two families may be interesting. It is possible that this region plays a critical role in the determination of either the localization or the function of these channels.

During the course of the study to identify a mammalian stretch-activated channel, we obtained the cDNA of MCLC using a sequence taken from a stretch-activated channel, Mid–1. Unexpectedly, MCLC functions as a chloride channel expressed in intracellular membranes. A question then arises whether or not MCLC is activated by stretch of the membrane. Because we analyzed the channel activity of MCLC in the lipid bilayer, a direct answer to this question is not available. In this regard, Gd$^{3+}$, which blocks stretch-activated channels (20), did not affect the activity of MCLC. Consequently, there is no information at present suggesting that MCLC is regulated by stretch. Further studies are necessary to address this issue.

With regard to ion selectivity, unlike Mid-1, MCLC functions as an anion channel. In many instances, ion selectivity is determined by amino acid composition of the channel pore. Although we obtained MCLC using MID-1 as a probe, there is no sequence similarity between the transmembrane domains of the two gene products. It is conceivable that these two channels permeate different ions.

In summary, we identified a new chloride channel family expressed in intracellular compartments, including the endoplasmic reticulum, the Golgi apparatus, and the nucleus. The physiological role of this channel remains to be elucidated.

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