Molecular and Functional Characterization of a Calcium-sensitive Chloride Channel from Mouse Lung*

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A protein (mCLCA1) has been cloned from a mouse lung cDNA library that bears strong sequence homology with the recently described bovine tracheal, Ca2+-sensitive chloride channel protein (bCLCA1), bovine lung endothelial cell adhesion molecule-1 (Lu-ECAM-1), and the human intestinal Ca2+-sensitive chloride channel protein (hCLCA1). In vitro, its 3.1-kilobase message translates into a 100-kDa protein that can be glycosylated to an approximately 125-kDa product. SDS-polyacrylamide gel electrophoresis from lysates of mCLCA1 cDNA-transformed human embryonic kidney cells (HEK293) reveals proteins of 130, 125, and 90 kDa as well as a protein triplet in the 32–38 kDa size range. Western analyses with antisera raised against Lu-ECAM-1 peptides show that the N-terminal region of the predicted open reading frame is present only in the larger size proteins (i.e. 130, 125, and 90 kDa), whereas the C-terminal region of the open reading frame is observed in the 32–38 kDa size proteins, suggesting a posttranslational, proteolytic processing of a precursor protein (125/130 kDa) into 90 kDa and 32–38 kDa components similar to that reported for Lu-ECAM-1. Hydrophobicity analyses predict four transmembrane domains for the 90-kDa protein. The mCLCA1 mRNA is readily detected by Northern analysis and by in situ hybridization in the respiratory epithelia of trachea and bronchi. Transient expression of mCLCA1 in HEK293 cells was associated with an increase in whole cell Cl− current that could be activated by Ca2+ and ionomycin and inhibited by 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid, dithiothreitol, and niflumic acid. The discovery of mCLCA1 opens the door for further investigating the possible contribution of a Ca2+-sensitive chloride conductance to the pathogenesis of cystic fibrosis.

Cystic fibrosis (CF)1 is an autosomally recessive genetic dis-

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† The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF047838.

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§ The abbreviations used are: CF, cystic fibrosis; CLCA, calcium-sensitive chloride channel; mCLCA1, murine CLCA; bCLCA1, bovine tracheal CLCA (previously named bCaCC); Lu-ECAM-1, lung endothelial cell adhesion molecule-1; hCLCA1, human intestinal CLCA; CFTR, CF transmembrane conductance regulator; kb, kilobase(s); ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; DIDS, 4,4′-diisothiocyanostilbene disulfonate; DTT, dithiothreitol; NFA, niflumic acid; HEK293, transformed human embryonic kidney cells; RACE, rapid amplification of cDNA ends.
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hCLCA1 (17, 21, 23). The open reading frame encodes a 100-kDa protein product that upon expression in HEK293 cells produces glycosylated protein products of 130 and 125 kDa. These protein products are posttranslationally processed into 90- and 38/32-kDa glycosylated components in a manner identical to Lu-ECAM-1 (21). Transfection of mCLCA1 into HEK293 cells combined with whole cell patch-clamp recording confirmed expression of a novel Ca\(^{2+}\)-sensitive Cl\(^{-}\) conductance. The resemblance between the tissue expression patterns of mCLCA1 and CFTR supports previous electrophysiological data for the existence of two (and possibly more) distinct independently regulated chloride channels in the same cell type and underscores the potential importance of CLCAs in CF (24).

MATERIALS AND METHODS

Screening of a Mouse cDNA Library and Sequencing—A mouse lung cDNA library (Stratagene) was screened with the EcoRI-BglII fragment of the Lu-ECAM-1 cDNA (2.4 kb) (21). Hybridization was performed at 65 °C in 5× SSC, 5× Denhardt’s solution, and 0.2% SDS solution overnight with agitation. Blots were washed in 2× SSC, followed by several washes in 0.2× SSC, 0.2% SDS at room temperature for a total time of 30 min. Subclones of each positive clone were isolated by the in vitro exonuclease protocol provided by the manufacturer of the cDNA library (Stratagene). DNA sequencing was performed by University DNA sequencing facility using dRhodamine Terminator cycle sequencing on an ABI Prism 377 DNA sequencer (PE Applied Biosystems). The BLAST program was used for homology searches in existing data bases (25), and the Megalign of the DNAStar package (Lasergene) for multiple sequence alignment.

Construction of Full-length Mouse mCLCA1 cDNA—5′ rapid amplification of cDNA ends (5′ RACE kit; Life Technologies, Inc.) was used to clone the 5′ end of the mouse cDNA from a pool of mouse lung poly(A)⁺ RNA (CLONTECH). A gene-specific primer 5′-GAA CCT TGC CAG GGG CCG-3′ (nucleotides 2367 to 2350) was employed to reversely transcribe the cDNA from mouse lung mRNA. A second nested gene-specific primer 5′-CCA CGT CCT TCT GGG ATT GCA C-3′ (nucleotides 836 to 875) and a primer recognizing the 5′-terminal tag served to polymerase chain reaction amplify the 5′ end of the cDNA. Polymerase chain reaction products were cloned into the pGEM-T vector (Promega). A full-length chimera was generated by fusing the RACE product clone with the longest cDNA clone in plBluescript (Stratagene), using the unique PstI restriction site in the shared overlapping region. To confirm the existence of the resulting contiguous ORF in lung, primers corresponding to its ends were used to amplify the entire ORF for direct sequencing.

Northern Analysis and in Situ Hybridization—A mouse multiple tissue Northern blot (CLONTECH) was probed with a radioactively labeled HindIII fragment from the mCLCA1 ORF (2.2 kb). Hybridization was done at 65 °C in 5× SSC, 5× Denhardt’s solution, and 0.2% SDS solution overnight with agitation. Blots were washed in 2× SSC followed by several washes in 0.2× SSC, 0.2% SDS solution at room temperature for a total time of 30 min.

In situ hybridization was performed on formalin-fixed murine lung and trachea sections with single-stranded digoxigenin-labeled sense or antisense RNA probes (24). Bound probe was detected by alkaline phosphatase-conjugated antidigoxigenin antibodies and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as substrates (Boehringer Mannheim). Sections were counterstained with methyl green.

RESULTS

Cloning and Sequence Analysis of mCLCA1—Low stringency hybridization conditions were employed in the screening of a mouse lung cDNA library with the ORF of the bovine Lu-ECAM-1 cDNA as probe. Positive phages were purified and analyzed by Southern blot hybridizations to verify specificity. The largest of the isolated cDNA fragments was 2.2 kb in length. It lacked the 5′ end, as determined from sequence comparison with the bovine homolog. The missing 0.9 kb of the mCLCA1 cDNA was obtained using 5′-RACE. The full-length mouse mCLCA1 cDNA was assembled by fusing the RACE product to the 2.2-kb cDNA insert. It is 3.1-kb long and encodes a polypeptide of 902 amino acids (Fig. 1). The mCLCA1 amino acid sequence is 71% identical to both of the previously described bCLCA1 (17) and Lu-ECAM-1 (21) protein sequences and 53% identical to human intestinal hCLCA1 protein (23). Moreover, the mCLCA1 sequence between amino acids 308 and 365 is 57% identical to an expressed sequence tag from porcine small intestine (27). Hydropathy analysis of the mCLCA1 ORF predicts it to be a transmembrane protein with at least two and possibly four membrane-spanning domains (data not shown). A four-transmembrane structure is consistent with the extracellular location of five putative glycosylation sites of mCLCA1 and has been suggested for bCLCA1, Lu-ECAM-1, and hCLCA1 (17, 21, 23).

The sequence alignment of the four members of the CLCA gene family isolated so far indicates conservation throughout the entire length of the sequence, without the compartmentalization of more conserved domains (Fig. 1). However, the N-terminal extracellular domain contains a highly conserved spacing of five cysteine residues, i.e., C\(_{X12-13}\)C\(_X4\), C\(_{X12-13}\)C\(_{X4}\) (amino acids 187–223). No significant homology to any other chloride channel proteins were detected using the BLAST program with either the entire mCLCA1 ORF or parts thereof.

Biochemical Analysis of mCLCA1 Protein—mCLCA1 cDNA was translated in vitro, and proteins analyzed by SDS-PAGE (Fig. 2A). The unglycosylated translation product resolved as a 100-kDa protein, which corresponded to the size predicted by the mCLCA1 ORF. Using canine microsomes, glycosylation
of this protein yielded a product of approximately 125 kDa. SDS-PAGE band patterns were consistent with that observed for the in vitro transcribed/translated Lu-ECAM-1 cDNA (21). The translation products of mCLCA1 cDNA-transfected HEK293 cells were further analyzed in Western blots using crossreacting antisera generated against various Lu-ECAM-1 peptides. Protein bands of 130, 125, 90 kDa and a triplet of bands in the 32–38 kDa size range were specific to the mCLCA1 transfected cells (Fig. 2B). Antisera raised against the N-terminal region of Lu-ECAM-1 (CU8) reacted exclusively with the large sized bands of 90, 125, and 130 kDa (Fig. 4A), whereas antisera raised against the C-terminal region of Lu-ECAM-1 (CU21) recognized only the triplet of smaller protein bands (Fig. 2B). This recognition pattern is similar to that observed for Lu-ECAM-1 (21) and suggests that the ORF of mCLCA1 cDNA encodes a precursor protein, represented by alternate glycoforms of 125 and 130 kDa, that is posttranslationally processed into 90-kDa and 38/32-kDa components.

The tissue expression pattern was analyzed by probing a multiple tissue Northern blot (CLONTECH) with mCLCA1 cDNA (Fig. 3). Unlike bCLCA1, mCLCA1 seemed to be expressed in a wide variety of tissues. There was strong expression in spleen, kidney, lung, and liver and weak expression in the brain. The signal in liver probably represented gall bladder, which was strongly positive by in situ hybridization (24). Two transcripts of 3.1 kb and 5 kb were detected in most tissues, whereas only the 3.1-kb transcript was present in brain and spleen. The isolated full-length ORF corresponded in size to the smaller 3.1-kb transcript (Fig. 3). The relationship of the two transcripts detected is unclear. The larger transcripts may represent a precursor mRNA species or an unknown highly related homolog.
Expression of mCLCA1 in HEK293 Cells—Expression of mCLCA1 in HEK293 cells was associated with the appearance of a novel Ca$^{2+}$-sensitive Cl$^{-}$ conductance as determined from whole cell recordings in the presence and absence of the Ca$^{2+}$-ionophore ionomycin (2 mM) (Fig. 5A). At low intracellular free Ca$^{2+}$ concentrations, the basal current at +100 mV in mCLCA1-transfected cells was 2.05 ± 1.09 pA/pF (mean ± S.D., n = 5). Upon perfusion of the bath with a solution containing 2 mM ionomycin, the current increased to 10.23 ± 3.46 pA/pF (n = 5) (Fig. 6). This experimental manipulation had no significant effect on membrane currents recorded from either nontransfected cells, or cells transfected with the pEGFP1 reporter vector alone (Fig. 6). When subjected to whole cell recordings in the presence of 2 mM Ca$^{2+}$ in the pipette, basal currents in mCLCA1-transfected HEK293 cells averaged 12.01 ± 6.31 pA/pF (n = 5). Perfusion of 300 mM DIDS through the bath reduced the current to 1.84 ± 0.96 pA/pF (n = 5). Similarly, NFA (100 mM) and DTT (2 mM) reduced the whole cell current to 2.58 ± 1.19 pA/pF and to 2.59 ± 1.02 pA/pF (n = 3), respectively (Figs. 5B and 6).

DISCUSSION

Electrophysiological studies and, more recently, molecular cloning have identified a bewildering variety of chloride channels (reviewed in Refs. 16 and 28). These channels differ in their structure (e.g. transmembrane topology), biophysical properties (e.g. ion selectivity, voltage dependence), mode of regulation (e.g. by ligands, calcium, G-proteins, CFTR, etc.), tissue expression patterns, and associated diseases. The significance of Ca$^{2+}$-sensitive chloride channels in CF and mouse models of the disease is well established, but their molecular nature has been unknown until quite recently (17, 21, 23, 24). Here we disclose molecular, biochemical, and functional characteristics of such a protein derived from mouse lung (mCLCA1). This protein is homologous to the recently cloned bovine tracheal epithelial bCLCA1 (17), the bovine endothelial Lu-ECAM-1 (21), and the human intestinal hCLCA1 (23). Sequence alignment and comparison between these four proteins show a high level of homology throughout the open reading frame, but especially in the pattern of cysteines (CX$\text{X}_{17}$-15CX$\text{X}_{4}$CX$\text{X}_{3}$CX$\text{X}_{12}$C) in the N-terminal domain of the protein. A similar pattern of conserved cysteine residues is observed in members of the ligand-gated chloride channel family, albeit the functional significance of this motif has not yet been established (29).

A comparison of the in vitro and in vivo translation products of mCLCA1 reveals a complex pattern of protein processing. While the in vitro translation of the full-length mCLCA1 cDNA yields a 100-kDa protein, which can be glycosylated to a 125-kDa size product, in vivo overexpression of the mCLCA1 cDNA in HEK293 cells shows SDS-PAGE bands of 125 and 130 kDa. These two bands most likely represent two alternatively glycosylated forms of the full-length protein (21). The transfected cells also produce a 90-kDa protein band as well as a triplet of bands in the 32–38-kDa size range. Immunoblots show that the N terminus of the ORF is contained in the 90-, 125-, and 130-kDa protein products, whereas the C-terminal region of the ORF is detectable only in the smaller proteins of the 32–38-kDa size range. The absence of expected immunostaining of the 125- and 130-kDa components by the C-terminal-specific antibody presumably reflects the low relative abundance of these components and the poor conservation of the C-terminal region between bovine and mouse. The appearance of three smaller protein components at 32–38 kDa is similar to those observed for Lu-ECAM-1 and reflects differences in glycosylation of the same peptide backbone (21). Taken together, these data show an identical posttranslational processing pattern of mCLCA1 to that reported for Lu-ECAM-1 and hCLCA1, generating peptide products of similar sizes and glycosylation (21, 23).

Our present results show that expression of mCLCA1 in HEK293 cells is associated with the appearance of a Ca$^{2+}$-sensitive chloride conductance. Under whole cell conditions, the current was outwardly rectified and inhibited by the anion...
channel blockers DIDS and NFA as well as the reducing agent DTT. The NFA sensitivity was identical to that of the human intestinal homolog hCLCA1 (23) but in contrast to the pharmacological profile previously reported for bCLCA1 studied in a similar eukaryotic expression system (COS-7 cells) (17). These differences between members of the CLCA family of proteins could therefore provide important insights into the regions of the protein required for drug binding and/or channel gating.

The tissue expression patterns of bCLCA1, Lu-ECAM-1, hCLCA1, and mCLCA1 are quite different. bCLCA1 is expressed exclusively in the respiratory epithelia of trachea and bronchi (17, 21), Lu-ECAM-1 predominantly in bovine aortic endothelial cells and endothelia of pulmonary venules (22, 30), hCLCA1 in intestinal epithelia (23), and mCLCA1 in many tissues (24). Strong expression of mCLCA1 is recorded in tissues with secretory or ion regulatory functions including epithelia of the mammary gland, the respiratory system, gall bladder, pancreas, kidney, uterus, and epididymis (24). Expression of mCLCA1 is also observed in germinal centers of lymphatic tissues, spermatids, and keratinocytes of the skin, esophagus, and cornea. A precedent for differences in the tissue expression patterns of members of the same chloride channel family exists for the ClC gene family. For example, ClC-1 has been reported to be expressed predominantly in skeletal muscles where its disruption is linked to muscle myotonies (31). In contrast, ClC-Ka and ClC-Kb are expressed only in the kidney, ClC-3, ClC-4, and ClC-5 mostly in kidney and brain, and ClC-2, ClC-6, and ClC-7 are ubiquitously expressed (reviewed in Ref. 16). This heterogeneity in tissue expression suggests differences in function, regulation, and associated disease among the various members of a structurally distinct family of chloride channels.

The reported tissue expression pattern of mCLCA1 overlaps with that of CFTR, suggesting that both might contribute to the pathogenesis of CF (24). In accordance, Boucher and associates (11, 13) recently reported that a calcium-mediated chloride secretory pathway was up-regulated in upper respiratory epithelia isolated from CFTR (−/−) mice and that this alternate chloride secretion pathway effectively compensated for the lack of CFTR. Correspondingly, transduction of wild-type CFTR into nasal epithelial cells from CF patients suppressed Ca²⁺-mediated chloride secretion in these cells, suggesting that the Ca²⁺-mediated pathway of chloride secretion is switched off once functional CFTR becomes available (32).
These experiments clearly indicate that the Ca$^{2+}$-dependent chloride secretory pathway is of importance in human cystic fibrosis and may prove amenable to pharmaceutical manipulation and amelioration of the degree of CF disease in specific tissues (17), e.g. the respiratory system. Cloning and molecular characterization of a murine Ca$^{2+}$-sensitive chloride conductance protein provides a tool to further clarify the complementary roles of Ca$^{2+}$-sensitive chloride channels with CFTR in CF.

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