Structure and Function of CLCA Proteins

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Loewen, Matthew E., and George W. Forsyth. Structure and Function of CLCA Proteins. Physiol Rev 85: 1061–1092, 2005; doi:10.1152/physrev.00016.2004.—CLCA proteins were discovered in bovine trachea and named for a calcium-dependent chloride conductance found in trachea and in other secretory epithelial tissues. At least four closely located gene loci in the mouse and the human code for independent isoforms of CLCA proteins. Full-length CLCA proteins have an unprocessed mass ratio of ~100 kDa. Three of the four human loci code for the synthesis of membrane-associated proteins. CLCA proteins affect chloride conductance, epithelial secretion, cell-cell adhesion, apoptosis, cell cycle control, mucus production in asthma, and blood pressure. There is a structural and probable functional divergence between CLCA isoforms containing or not containing β1-integrin binding domains. Cell cycle control and tumor metastasis are affected by isoforms with the binding domains. These isoforms are expressed prominently in smooth muscle, in some endothelial cells, in the central nervous system, and also in secretory epithelial cells. The isoform with disrupted β1-integrin binding (hCLCA1, pCLCA1, mCLCA3) alters epithelial mucus secretion and ion transport processes. It is preferentially expressed in secretory epithelial tissues.
including trachea and small intestine. Chloride conductance is affected by the expression of several CLCA proteins. However, the dependence of the resulting electrical signature on the expression system rather than the CLCA protein suggests that these proteins are not independent Ca\(^{2+}\)-dependent chloride channels, but may contribute to the activity of chloride channels formed by, or in conjunction with, other proteins.

I. INTRODUCTION TO CLCA PROTEINS

Secretory epithelial cells possess ion channels that are asymmetrically distributed between the basolateral and apical membrane. Vectorial ion movement through a subset of these channels produces osmotic gradients that drive net fluid movement into the lumen of secretory tissues. Conductive chloride channels located on the apical membrane of secretory epithelial cells are of special interest due to their role as regulatory targets. Regulation of chloride conductance channels by intracellular second messengers has been considered to be the primary level of control for epithelial fluid secretion.

The predominant chloride conductance of most secretory tissues occurs via the cAMP-dependent cystic fibrosis transmembrane regulator protein (CFTR). However, many tissues also have an independent chloride conductance that can be activated by elevating the concentration of intracellular Ca\(^{2+}\). Activity of such a protein has been functionally linked to fluid secretion in the trachea and in the small intestine. Upon their discovery in 1995, the members of the CLCA protein family were thought to be this elusive calcium-dependent chloride conductance. Nearly a decade later, research into CLCA protein biology is proceeding in interesting and unexpected directions.

Attempts to understand CLCA protein function have been complicated by uncertainty about a unifying molecular basis for the actions of family members. Different CLCA proteins have several apparently unrelated, and sometimes conflicting, functional roles. The first discovered member of the family was implicated with chloride conductance in secretory epithelial tissues (39). However, another original CLCA protein, LuECAM-1, functions in tumor cell adhesion and metastasis (1–3, 50). This role for LuECAM-1 in tumor adhesion appears to conflict with significant tumor suppressor activity reported for other CLCA proteins (49, 79, 112). CLCA proteins have also been connected recently to the regulation of mucus production in the asthmatic airway (88, 135), and to ion transport in the airway of cystic fibrosis patients (83, 84). Other roles include CLCA involvement in chloride transport affecting vascular smooth muscle tone and blood pressure, and in retinal functions that are disrupted in Best’s vitelliform macular dystrophy (9, 73, 94, 124).

The history of CLCA cloning will be surveyed, followed by physiological effects observed upon heterologous CLCA isoform expression. Predictions for protein structure and function will connect some basic biophysical results of CLCA isoform expression with the pathophysiology of associated disease processes. The targeting of CLCA proteins for pharmacological modulation of related disease conditions will also be discussed.

II. CLONING THE CLCA GENES

The CLCA gene family members are named for the species from which they were cloned, and numbered within each species in chronological order of discovery. The history of the cloning of the CLCA gene family will be summarized, and cross-species homologs with probable structural and functional similarities will be identified. The phylogenetic relationship between some of the better understood CLCA proteins is presented in Figure 1.

A. Bovine CLCAs

The first CLCA cDNA sequence (bCLCA1) was reported in 1995 (39). The authors were pursuing the gene coding for a 140-kDa bovine tracheal protein that had been identified with by Ca\(^{2+}\)/calmodulin-dependent protein kinase-regulated chloride conductance. The 140-kDa protein was thought to be a homotetramer of identical 38-kDa subunits, linked together by disulfide bonds. Reduction of the 140-kDa
protein produced a 36- to 38-kDa polypeptide lacking chloride channel activity in lipid bilayers (39, 155). A candidate cDNA was identified by screening a bovine tracheal cDNA library with polyclonal antibody to this polypeptide. The identified cDNA was described as coding for a calcium-activated chloride channel (CaCC), but is now called bCLCA1 (39, 50, 60, 146).

A second bovine CLCA sequence (Lu ECAM-1 or bCLCA2) was identified by screening a gene expression library with monoclonal antibody that blocked an adhesion-receptor/ligand pair interaction between lung-metastatic melanoma cells and lung matrix-modulated bovine aortic endothelial cells (50, 211–214). Expression of the Lu ECAM-1 cDNA produced a protein with properties of a lung endothelial cell adhesion molecule (50). Lu ECAM-1 is now termed bCLCA2 (59, 146).

B. Human and Mouse CLCAs

Clones similar to bCLCA1 and -2 were reported in 1998 from human genome and mouse lung library screening. The human CLCA gene and its putative promoter region span 31,902 bp of chromosome 1 at p22-p31 (75). A promoter region containing a TATA box precedes the predicted site of transcriptional initiation by 22 nucleotides. Fifteen exons from 90 to 604 bp are interspersed with 14 introns of 170 to 5,651 bp. The hCLCA1 cDNA was found independently in 1999 by screening an EST database with bCLCA1 sequence. The EST clone, named hCaCC1, is identical to hCLCA1 (4). The 2,745 bp open reading frame (ORF) of hCLCA1 was amplified in polymerase chain reaction (PCR) to clone a full-length cDNA for functional expression.

A bCLCA2 probe was used to identify mCLCA1 in a mouse lung cDNA library (67). mCLCA1 was also identified in an EST database using a probe based on bovine bCLCA1 sequence (160). This group localized mCLCA1 to mouse chromosome 3 at the H2-H3 band. mCLCA1 is the murine ortholog of truncated hCLCA3 (Fig. 1) (158). Several CLCA isoforms were reported in 1999. mCLCA2 was cloned from mouse mammary gland in a suppression subtractive hybridization on the involuting mammary gland (107). mCLCA2 maps closely to mCLCA1 in the mouse genome and may even be a splice variant of mCLCA1 (158). The hCLCA2 cDNA was cloned from a human lung library using a bCLCA1 cDNA probe (78). As with mCLCA2, hCLCA2 was expressed at high levels in the mammary gland, but the murine counterpart of hCLCA2 is the hypothetical mouse protein 4732440A06 (158), now called mCLCA5 (54). hCaCC2 and hCaCC3 (now hCLCA4 and hCLCA3) were discovered independently at the same time (4).

hCLCA3 is a short CLCA protein with a premature stop codon (78). It is most similar to mCLCA1/2 and to bCLCA1 and bCLCA2, although the mouse and bovine isoforms are full-length membrane-associated proteins. hCLCA3 was cloned from human spleen cDNA and has been found in several tissues. Gob-5 or mCLCA3, the mouse ortholog to hCLCA1, was cloned from mouse gut cDNA (103). This mCLCA3 ortholog, as well as mCLCA1/2, mCLCA4, and related mouse ESTs 4732440A06, AI747448 and AI504701 (mCLCA5, 6, and ?) colocalize on mouse chromosome 3 H2-H3 (108, 158, 160).

The human hCLCA2, hCLCA1, hCLCA4, and hCLCA3 genes align consecutively in the same orientation, encompassing 232 kb of chromosome 1 with no other genes interspersed (76). Gene clustering can be a mechanism for controlling temporal gene expression by internal intron or bidirectional promoters (95, 208). An area 1,617 bp upstream of the first intron codes for 24 different transcription factor binding sites.

C. Porcine CLCAs

A monoclonal antibody selected to inhibit chloride conductance in ileal brush-border vesicles was used to identify pCLCA1 cDNA in a porcine ileal gene expression library (61, 63). Cloning and expression of the full-length cDNA was reported in 2000 (68). The screening strategy used to identify this isoform connects pCLCA1 to chloride conductance.

D. Miscellaneous

Reports of a calcium-activated chloride conductance in smooth muscle (85, 137) led to the discovery of mCLCA4 (48). mCLCA4 is most similar to the prematurely truncated hCLCA3, but mCLCA4 is a full-length integral membrane protein.

Incomplete segments of CLCA cDNA have been cloned from other species. A portion of a rat ortholog rCLCA1 has been cloned from rat pancreas cDNA (179). A partial canine CLCA, cCLCA1, cloned from dog retinal pigment epithelium with primers specific for the porcine pCLCA1 has also been reported, and expression levels have been investigated (117). An hCLCA5 cDNA has been reported recently (1). However, little information on its cloning or function is available.

III. CLCA PROTEIN STRUCTURE

A. Human CLCAs

1. hCLCA1

The cDNA for hCLCA1 encodes a 914-amino acid protein with a calculated molecular mass of 100.9 kDa (75). A
signal sequence and four putative transmembrane domains are predicted from hydropathy data. There are 9 potential sites for asparagine-linked glycosylation, 13 consensus sites for protein kinase C (PKC) phosphorylation, and 3 consensus sites for phosphorylation by Ca$^{2+}$/calmodulin-dependent kinase II. There are no PKA or tyrosine phosphorylation consensus sequences (75). In vitro translation of the human CLCA1 resulted in a 100-kDa product that increased in size to 125 kDa after glycosylation.

Membrane topology predictions have been based on detection of the c-myc epitope (EQKLISEEDL) inserted at different regions within the protein. However, epitope insertion may be affecting protein conformation, as CLCA proteins are cleaved by monobasic proteases with conformation-dependent cleavage specificity, and several of the tagged constructs were not processed normally (44, 164). Insertion of the epitope between amino acids 366/367 or 492/493 resulted in a 90-kDa polypeptide, apparently interfering with the ability of the smaller cleaved ~40-kDa product to interact with the larger 90-kDa subunit. Other epitope placements blocked proteolytic cleavage (75). A model based on the c-myc epitope insertion and cell surface biotinylation has an extracellular NH$_2$ and COOH terminus and four transmembrane domains (80, 146) (Fig. 2).

An alternative model for hCLCA1 structure and relationship to the membrane has also been predicted without direct supporting data (Fig. 2A) (197). Using SMART predictions based on sequence homology to the $\alpha_2$-integrin collagen receptor domain, the CLCA family members are suggested to consist of a central (extracellular) von Willebrand factor domain A (VWA domain), leaving only a single transmembrane domain near the COOH terminus (197). The significant disparity between the alternate models involves predictions about the folding and topography of the NH$_2$-terminal half of the protein. This region of the protein is predicted to contain either two helical transmembrane domains (TM1 and TM2), or to be extracellular with secondary and tertiary structure forming a VWA domain. The TM3 and TM4 domains and the cytoplasmic loop between these domains containing protein kinase consensus sites are not excluded by either conformation of the NH$_2$-terminal folding.

VWA domains are recognized for involvement in protein-protein interactions. The voltage-gated calcium channel is a precedent for VWA domain involvement in ion channel subunit interactions. The properties of this channel are modulated by interaction of the pore-forming $\alpha_1$ subunit with the $\alpha_2$ VWA domain-containing subunit complex (86, 197). There is parallel evidence for CLCA interaction with other proteins. Hetero-oligomeric CLCA interactions affect cell binding and mitogenic signaling via integrins (1–3), as well as ion channel activity of the $\alpha_1$-subunit of the large-conductance potassium channel (73). CLCA expression also modulates chloride conductance, leading to predictions that some chloride conductance channels may be hetero-oligomeric structures.

A metal ion-dependent adhesion site (MIDAS) motif is a noncontiguous amino acid sequence that organizes into a divalent cation binding structure through normal VWA domain folding. MIDAS motifs bind magnesium or calcium and participate in stabilizing protein-protein interactions. CLCA folding, based on sequence homology to a collagen receptor, could form a VWA domain with MIDAS function (197). The MIDAS motif could be a site for calcium binding in CLCA proteins to interact with and alter the conductivity of chloride ion channels. However, MIDAS motif precedents in other proteins involve extra-

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**FIG. 2. Proposed membrane topology of human CLCA orthologs. A:**

1) hCLCA1 membrane topology, with four transmembrane domains and small COOH-terminal secreted cleavage product predicted from epitope insertion (75).

2) Alternative single transmembrane topology to accommodate von Willebrand A domain folding, as proposed by Whitaker et al. (197).

**B:** five transmembrane domains of hCLCA2 as proposed by Gruber et al. (80). Three of the five transmembrane domains locate to the NH$_2$-terminal fragment.

**C:** prematurely truncated hCLCA3 is a secreted protein with stop codon insertion before first transmembrane domain (78).
cellular polypeptide structure, while effects of Ca\(^{2+}\) ionophores on chloride conductance associated with CLCA expression invoke changes in intracellular Ca\(^{2+}\) concentration as the basis for CLCA regulation of chloride conductance. Without structural data, there is no direct evidence for VWA domain formation and function in CLCA proteins in spite of significant amino acid sequence identity and modeling compatibility (see Fig. 3).

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**FIG. 3.** Alignment of the closest mammalian orthologs mCLCA3 (gob-5) and hCLCA1 with pCLCA1. The amino acid sequence identity for pCLCA1:hCLCA1 was 78%, pCLCA1:mCLCA3 was 73%, and hCLCA1:mCLCA3 was 75% as determined by Clustalw software. The von Willebrand A domain, the predicted major transmembrane domains, and phosphorylation consensus sequences on the major cytoplasmic loop are identified.
2. *hCLCA2*

The *hCLCA2* ortholog is predicted to form a 943-amino acid polypeptide (80). It contains a canonical signal sequence with a peptidase cleavage site. A primary translation product of 105 kDa from an in vitro translation assay increases to 120 kDa after glycosylation. Proteolytic processing at the monobasic proteolytic 673/674 cleavage site leaves an NH$_2$-terminal c-myc epitope tag in an 86-kDa protein and a 54-kDa COOH terminus. Deglycosylation reduced the size corresponding to four glycosylation sites on the 86-kDa fragment and one on the smaller 34-kDa fragment. Loss of glycosylation in mutants N150Q and N522Q indicated that these regions are extracellular (80). However, these results are compatible with either multiple transmembrane domains or an extracellular NH$_2$-terminal VWA domain. A N822Q mutation indicated an extracellular loop in the smaller cleaved product between transmembrane domains 4 and 5.

Findings from a microsomal protein protection assay digesting all internal loops gave results in agreement with the interpreted five transmembrane domain structure of *hCLCA2*. It should also be noted that both cleavage products were detected in surface-labeled, nonpermeabilized HEK293 cells, suggesting that both the larger and the smaller products localize to the cell surface. In this model the internal loops contained seven PKC phosphorylation sites, but there were no consensus sites for Ca$^{2+}$/calmodulin protein kinase II or cAMP-dependent protein kinase (80). *hCLCA2*, as well as several other CLCA proteins, contain a 4-integrin binding domain (1) whose significance is discussed below.

3. *hCLCA3*

The *hCLCA3* cDNA contains two internal stop codons (78). Sequential ORFs code for the NH$_2$-terminal 262 amino acids, and a second polypeptide from amino acid 266 to 461. Together, the two ORFs code for approximately the first one-third of the full-length CLCA protein. The NH$_2$-terminal polypeptide terminates before potential transmembrane domains, and the second fragment lacks a signal sequence, although its amino acid sequence includes the first two predicted transmembrane domains of other CLCA family members. In vitro synthesis produced 30- and 22-kDa products corresponding to calculated size for ORF1 and ORF2, respectively, but only the NH$_2$-terminal polypeptide was detected in transfected HEK293 cells. The secreted ORF1 product was also detected upon immunoblotting the supernatant from transfected cells (78). Mouse homologs *mCLCA1/2* and *mCLCA4* lack the internal stop codons, implying that there may be no function for the truncated, secreted *hCLCA3* product.

4. *hCLCA4*

There are no current structural data for the *hCLCA4* clone.

B. Mouse CLCAs

1. *mCLCA1/2*

The in vitro translated size of 100 kDa for this 902-amino acid protein increases to 125 or 130 kDa with glycosylation. Posttranslational processing gives NH$_2$-terminal 90-kDa and COOH-terminal 38/32-kDa components. A general four transmembrane domain structure is proposed for this protein, leaving the COOH terminus without a transmembrane domain (67). There is little structural data available for *mCLCA2*. The *mCLCA2* protein may be a splice variant of *mCLCA1* (158), sharing significant cDNA sequence identity with *mCLCA1* and *hCLCA3*. It is reported to produce similar processed products as found for *mCLCA1* when expressed in HEK293 cells (49).

2. *mCLCA3*

This mouse CLCA is the murine counterpart of *hCLCA1*. The *mCLCA3* cDNA codes for a 100-kDa protein in an in vitro translation assay, and increases to 110 kDa after glycosylation with microsomal membranes (109). Microsomal protease treatment produced a 35-kDa product corresponding to an extracellular NH$_2$ terminus. An NH$_2$-terminal 90-kDa product was identified in transiently transfected HEK293 and COS-7 cells and in the mouse large and small intestine, with the expression being more intense in the small intestine. An unexplained 45-kDa product which may be a fragment of a truncated portion of the amino terminus of the protein with similarities to the secreted 37 kDa amino terminus of *hCLCA3* was also detected in the large, but not small intestine (109).

3. *mCLCA4*

The primary structure of *mCLCA4* is similar to *mCLCA1* and 2. It is 909 amino acids in length, with a conserved cleaved NH$_2$-terminal signal and a second monobasic cleavage site which results in a 90- and 30- to 40-kDa product (48). The primary structure also contains most of the calcium/calmodulin kinase II (CaMKII) sites found in *mCLCA1* and *mCLCA2*.

4. *mCLCA5* and *mCLCA6*

The two proteins have predicted mass ratios of 103.6 and 101.9 kDa, respectively (54). Uncleaved glycosylated proteins expressed in tsA201 cells were 125 kDa. Process-
ing by monobasic cleavage produced COOH-terminal glycosylated fragments of 35 kDa after subtraction of the EGFP tag (54). Several consensus acceptor sites for protein kinase A (PKA), PKC and CaMKII occur in the amino acid sequence, but the significance of these sites is uncertain without more information on the transmembrane orientation of the expressed proteins. Conserved β2-integrin binding motifs on NH2- and COOH-fragments of mCLCA5 are altered so as to be nonfunctional in mCLCA6.

C. Bovine CLCA1

1. bCLCA1

Cloned bovine isoforms are homologs of hCLCA3 and mCLCA1/2. The bCLCA1 cDNA codes for 903 amino acids, giving a predicted product of 100 kDa which shifts to 140 kDa upon glycosylation (39). Motif analysis predicts 15 PKC acceptor sites, 10 Ca2+/calmodulin-dependent protein kinase sites, and 3 tyrosine kinase sites. Four transmembrane domains and an NH2-terminal signal sequence are predicted from hydropathy plots. Polyclonal antibodies generated against an NH2-terminal fragment of the bCLCA1 clone recognized a reduced 36/38-kDa bovine tracheal lysate product similar to the antigen identified by the original antibodies that were used to clone bCLCA1 (39, 155). The original purified protein of 140 kDa was thought to consist of four identical 38-kDa subunits linked together by disulfide bonds (155). However, the sequence data and the identification of the 100-kDa unglycosylated form of bCLCA1 indicate that the 38-kDa protein is a posttranslational cleavage product that remains associated with its larger counterpart (39). This model has now been promoted for the other cloned isoforms and orthologs.

2. bCLCA2

The cDNA sequence codes for a predicted hydrophobic NH2-terminal signal sequence and cleavage site for membrane insertion. In vitro translation produced a 101-kDa protein that increased to 120 kDa with glycosylation. Expression of bCLCA2 in HEK293 cells produced similar 90- and 38-kDa products. Native expression in bovine endothelial cells produced 130- and 32-kDa glycoforms (50). As for bCLCA1, a hydropathy plot predicted four transmembrane domains. Deglycosylation of native bCLCA2 with N-glycosidase F from endothelial cells reduced the 38- and 32-kDa polypeptides to 22 kDa, and the 90-kDa band to 77 kDa, giving the predicted deglycosylated size of bCLCA2 (50). There is good evidence that the primary 101-kDa protein is cleaved into two nonidentical proteins (50), and earlier suggestions of a homotetrameric structure for this protein (155) are incorrect.

D. Porcine pCLCA1

The pCLCA1 protein is the porcine homolog of hCLCA1 and mCLCA3. The cDNA sequence contains 3,079 bp and a 2,751-base ORF that is predicted to encode a 917-amino acid protein with a molecular mass of 100.7 kDa (68). It shares 78% amino acid sequence identity with hCLCA1, its closest ortholog. Similarities of amino acid sequence and hydrophobicity between pCLCA1 and hCLCA1 suggested a similar transmembrane topology for the two proteins. Modeling this structure according to the suggestions of Gruber et al. (80) would give an extracellular NH2 terminus followed by four transmembrane domains and a hydrophobic COOH terminus (Fig. 2) (68).

The predicted pCLCA1 protein shares a signal sequence for membrane targeting and potential proteolytic cleavage sites with hCLCA1. There is an amidation cleavage site at residue 140 and monobasic proteolytic cleavage sites at R602 and K720. This could account for the multiple sizes of protein (~130, 90, and 60 kDa) detected by Western blotting or immunoprecipitation of brush-border vesicle protein with an inhibitory antibody (63, 154).

Expression of exogenous pCLCA1 in transfected Caco-2 cells produced a ~60-kDa product upon Western blotting with polyclonal antibody raised to an NH2-terminal peptide (C250-K266) of pCLCA1. The processing of CLCA isoforms is known to vary with expression in different tissues (60, 146). Examples include an ~60-kDa bCLCA1 fragment and ~60-kDa pCLCA1 product expressed in epithelial cells, as well as a preliminary report of a ~60-kDa protein contributing to anion conductance in the rabbit ileum (147, 155).

Similar to other CLCA clones, pCLCA1 amino acid sequence from V307 to Q462 has extensive homology with sequences that form a VWA domain (Fig. 3). There is a perfect noncontiguous consensus sequence for a MIDAS motif (D313-x-S315-x-S317… T384… D416) within the putative pCLCA1 VWA domain, a motif shared with hCLCA1. Structural modeling of the pCLCA1 MIDAS site reveals that appropriate folding is permitted, although the -SH of C387 replaces -OH of T384 as one of the divalent cation ligands (Fig. 4). Loewen et al. (113) have presented evidence for a direct regulatory role of calcium in the modulation of chloride conductance by pCLCA1, but there is no evidence connecting regulation of pCLCA1 to Ca2+ binding to the MIDAS motif.

Four of the C kinase consensus sequences found within the predicted cytosolic loop between transmembrane domains 3 and 4 are shared with hCLCA1, while pCLCA1 has a fifth unique site in this same loop. Also found in this loop is a strong A-kinase consensus sequence that is unique to pCLCA1 (68). The effect of mutagenesis of this highly phosphorylated loop in the mod-
ulation of chloride conductance by CLCA proteins has not been reported.

IV. ENDOGENOUS TISSUE EXPRESSION SITES

Combining information about sites of tissue expression and structurally related groups of CLCA proteins (Fig. 1) could help to clarify CLCA protein function, and differences in function for each structural group within the CLCA gene family. However, it is important to note that some early studies of tissue localization may have been less than ortholog-specific. The accuracy of specific CLCA mRNA or protein localization depends on probe specificity and the stringency of the reaction conditions. Current studies addressing temporal ortholog expression

Fig. 4. Predicted folding of CLCA amino acid sequence with homology to the von Willebrand A domain including the metal ion dependent adhesion site (MIDAS) of the α2-integrin collagen receptor. Predicted folding of pCLCA1 (A) and hCLCA1 (B), based on the high-resolution crystal structure of the von Willebrand A domain of the human α2-integrin collagen receptor I domain (C), designated 1DZIA (51, 52). Structures were created in Swiss Model by replacing amino acids 5 to 133 of the 185-amino acid sequence of 1DZIA by amino acids 310 to 428 of pCLCA1 or hCLCA1. 1DZIA sequence added to CLCA protein was removed after modeling so that A and B show only CLCA polypeptide backbone folding. Note the classical Rossmann fold with the open twisted β-sheet surrounded by α-helices on both sides. The surface metal ion coordination site defines the MIDAS motif. Water molecules which may contribute to divalent cation binding were excluded in the modeling for this figure. Replacement of threonine with cysteine in the pCLCA1 MIDAS motif indicates a higher divalent cation binding potential affinity for this isoform.
are dealing more carefully with issues of probe specificity as awareness of CLCA isoforms increases.

A. Human CLCAs

1. hCLCA1

The strongest expression of hCLCA1 occurs in mucus-secreting cells of large and small intestine (75). With Northern blotting the highest expression was in the small intestine, appendix, and colon, with much lower expression levels in the uterus, stomach, testis, and kidney (4). Another extensive study using highly stringent in situ hybridization conditions only detected expression in the small intestine and colon (75). The majority of the mRNA signal was in cells at the base of the crypts, with the goblet cells having the highest expression. Induction of hCLCA1 expression in the airways has been reported more recently under pathophysiological conditions (83, 88, 135, 181, 209).

2. hCLCA2

By Northern blotting the highest expression of hCLCA2 mRNA occurs in the trachea and mammary gland (80). This result was confirmed independently, and the confirming group also reported expression in the testis, prostate, and uterus (4). It has also been found in nasal epithelium (122). Strong expression occurs in most basal cells in stratified epithelia (35). Although isolated from a lung cDNA library, hCLCA2 was not detected in the lung by Northern blot hybridization, and only a weak RT-PCR signal for hCLCA2 was obtained from the lung. Others could not confirm expression in the lung (4).

The hCLCA2 mRNA was highly expressed in a non-malignant transformed human mammary epithelial cell line MCF10A using both Northern blotting and RT-PCR. However, hCLCA2 expression could not be detected by Northern blotting in tumorigenic cell lines MDA-MB-231, MDA-MD-468, and MCF7. These findings were in agreement with an in situ hybridization staining of acini and small ducts in normal mammary tissue, but there was no staining in breast cancer samples (80).

3. hCLCA3

The third human ortholog, hCLCA3, was originally cloned from spleen. The mRNA transcript is expressed in the lung, trachea, mammary gland, and thymus (78). Its presence has also been reported in nasal epithelium (122).

4. hCLCA4

Unlike the other human orthologs, hCLCA4 is highly expressed in neural tissue (4). On Northern blot analysis it is found in the amygdala, caudate nucleus, cerebral cortex, frontal lobe, hippocampus, medulla oblongata, occipital lobe, putamen, substantia nigra, temporal lobe, thalamus, and accumbens expressed hCLCA4. The cerebellum and spinal cord did not show any evidence of hCLCA4 expression. The strongest signal from hCLCA4 came from the colon, with twice the intensity found in neural tissue. Expression also occurred in the bladder, uterus, prostate, stomach, testis, salivary gland, mammary gland, small intestine, appendix, and trachea (4).

B. Murine CLCAs

1. mCLCA1

The strongest expression of mCLCA1 was detected in the lung, aorta, spleen, and bone marrow using real-time RT-PCR quantitation normalized against expression of elongation factor 1a (110). High levels were also reported in the kidney and skin by Romio et al. (160). Lower levels occur in many tissues, but expression was not detected in the prostate or in the pregnant, lactating, and involuting mammary gland. However, mCLCA1 expression was found in virgin, pregnant, and lactating gland in an independent study (49). There is agreement that mCLCA1 expression is lost during mammary gland involution.

There is a dynamic change in mCLCA1 expression over gestation (49, 110) pointing to an undefined role of mCLCA1 in cell maturation and tissue differentiation. mCLCA1 is the only CLCA reported in the cerebellum and brain stem (110). This differs significantly from hCLCA4, which is expressed extensively in other areas of the nervous system (4). In the same study, mCLCA2 was undetectable in cerebellum and brain stem (77). These reports of temporal and tissue-specific expression patterns within the same physiological system may be hints of diverse roles for CLCA proteins in tissue development and functional differentiation.

Given the importance of a calcium-activated chloride conductance in renal epithelium, it was expected that CLCA cDNAs related to mCLCA1 and mCLCA2 would be expressed in a mouse inner medullary collecting tubule cell line (19). RT-PCR of mouse nephron RNA found mCLCA1 mRNA in the glomeruli and thick ascending limb, but not in the proximal tubule and cortical collecting duct (19). Interestingly, a study using a nonspecific mCLCA1/mCLCA2 probe identified CLCA in the tubular epithelial cells but not in the glomeruli. The proximal tubule had the most intense staining with weaker staining in the loop of Henle and distal tubuli. The collecting duct was negative (77). These contradictory findings may reflect probe specificity and primers used in the studies, as much as they show a difference in the CLCA form expressed at different areas within the kidney.
2. mCLCA2

mCLCA1 and mCLCA2 may be splice variants of the same gene locus (158). High mCLCA2 expression was reported from pregnancy to involution as determined in two studies by RT-PCR (49, 115), but the strongest mCLCA2 expression was detected in the involuting mammary gland (48, 107, 110). mCLCA1/2 expression was detected in both alveolar and ductal epithelial cells by in situ hybridization in normal murine mammary tissue (77). Lower mCLCA2 levels were detected in most tissues tested except for the brain stem, cerebellum, and prostate (110). In a real time quantitative RT-PCR study, the involuting mammary gland seemed to express purely mCLCA2. This was the only tissue screen with absolutely no mCLCA1 signal (110). The dynamic interplay between the mCLCA1 and mCLCA2 isoforms suggests that they are involved in different physiological roles. It is interesting to note that the highest expression of mCLCA2 seems to be in those tissues with the high rate of cell division and cell death (49). A possible connection to cell cycle control or an apoptotic role for mCLCA2 could be considered for these tissues.

3. mCLCA3

Mucus-secreting cells are the primary site for expression of mCLCA3, the mouse homolog of hCLCA1 and pCLCA1 (103, 109). The mCLCA3 was originally cloned from, and found to have highest expression in, the crypts of the large intestine (103). An extensive immunohistochemical investigation of mCLCA3 showed exclusive expression in the digestive and respiratory tracts and uterus (109). The mCLCA3 antigen was not found in the gallbladder, kidney, pancreas, sublingual salivary glands, oviduct, mammary gland, or prostate (109). Concurrent staining for mucin and immunohistochemical localization of mCLCA3 antigen indicated that mCLCA3 was expressed only in mucin-producing cells, but not in all mucin-producing cells.

A dynamic expression pattern was seen in the crypt goblet cells in both the large and small intestine, with expression only in approximately the upper two-thirds of the crypt, whereas weak, or no, staining was observed in the basal third (109). It is interesting that the distribution pattern for gastrointestinal mCLCA3 correlates with the crypt-villus axis (Fig. 5). CLCA proteins have been promoted as proapoptotic (49), but it is the cell group directed toward apoptotic removal at the base of the crypts that loses mCLCA3 expression. However, mCLCA1/2 expression may increase as mCLCA3 levels decline and play some role in apoptosis at the base of the crypts.

The development of a suitable antibody allowed intracellular localization of the mCLCA3 antigen (109). In simple parafin-embedded tissue sections, the cytosol was stained positive with a diffuse granular pattern. Goblet cells often had an intensely stained apical membrane, with obvious labeling around the mucin granules. An electron microscopic study localized mCLCA3 antigen to the peripheral membrane of mucin granules in positively staining cells. Antigen was not found in the center of the granule, cytosol, nucleus, other organelles, or along the basolateral membrane of goblet cells (109). These findings support a connection between mCLCA3 expression and mucin production.

4. mCLCA4

Cloned from smooth muscle, mCLCA4 was detected in the gastrointestinal tract, uterus, lung, and heart in a multiple cDNA array using gene-specific primers. A large component of mCLCA4 cDNA expression was detected by RT-PCR in the smooth muscle of the dissected tunica muscularis of the bladder and stomach (49). In situ hybridization with a mCLCA4-specific probe was performed to determine cell-specific expression in these mixed organs. This probe produced a very strong signal in the pulmonary vein, aorta, and atrioventricular bundle with a much lower level in cardiac muscle, coronary artery, and endothelium. Both the bronchioles and blood vessels were labeled in the lung. In the gastrointestinal tract, labeling was associated more with the mucosa than the muscularis tunica. This mucosal labeling was most intense towards the villus tip (48). It should be noted that this is the first CLCA with a reported increase in expression towards the villus tip. mCLCA1 and possibly mCLCA2 are thought to be primarily expressed in the crypt (77), and mCLCA3 is associated with upper crypt and midshaft of the villus (109). These differing expression patterns of each isoform within a tissue apparently associate with major differences in cell physiology. Adipose and connective tissue were consistently negative (48).

5. mCLCA5 and mCLCA6

Mouse homologs of hCLCA2 and hCLCA4 have been designated as mCLCA5 and mCLCA6 (54). Like its human
counterpart, mCLCA5 is widely expressed, with eye, spleen, and lactating mammary tissue notable for strong expression (14, 54). However, mCLCA6 is expressed in intestine and stomach, but not in whole brain. In this respect the human and mouse homologs appear to be functionally distinct.

C. Bovine CLCAs

1. bCLCA1

The bCLCA1 ortholog is primarily expressed on the brush border of ciliated tracheal epithelial cells (50), the tissue from which it was originally cloned (39). This expression site is similar to that found for mCLCA1/2 (77). The expression in other tissues has not been reported.

2. bCLCA2

The bCLCA2 ortholog is reported to be predominantly a luminal membrane protein of the venular endothelia of the lungs and the spleen. However, antibodies to bCLCA2 located a strong antigen signal in endothelia of small to medium size venules as well as in the respiratory epithelial of the bronchi and trachea (50).

D. Porcine pCLCA1

Immunohistochemistry with the IgM monoclonal antibody used to clone pCLCA1 identified antigen on the enterocyte border of the villi. Labeling intensity was distributed over the mucosal surface, with the most intense staining in the mid to upper crypt region (154). In the trachea, pCLCA1 mRNA expression was localized to surface epithelium and the underlying submucosal glands, with the most intense staining found in a subset of the submucosal glands (68). These tissues were also positive for pCLCA1 expression when tested by RT-PCR. pCLCA1 expression was not detected in the colon. RT-PCR identified pCLCA1 mRNA in the parotid, sublingual, and submandibular salivary glands (68). Other tissues that express a variety of chloride channels including the exocrine pancreas, cardiac and skeletal muscle, liver, and kidney had no detectable pCLCA1 mRNA.

E. Other CLCAs

Antibodies against a pCLCA1 peptide were used in immunohistochemistry to identify a CLCA epitope in canine retinal pigment epithelial cells (117). Intense staining was seen on the apical secretory side of the epithelium. The Muller cells, which are reported to maintain appropriate extracellular environment for retinal neurons, had significant cCLCA1 expression. CLCA epitope was also prominent in the corneal epithelium and in the ciliary body (M. E. Loewen, B. H. Grahn, and G. W. Forsyth, unpublished data).

Antibodies generated to rCLCA1 reacted extensively in the rat pancreas. The subcellular location of the antigen was mainly on the zymogen granules (179). In an interesting aside, hCLCA1 expression was not detected by RT-PCR in a human pancreatic duct adenocarcinoma cell line that had an active calcium-dependent chloride conductance (56). This negative finding is consistent with observations by others that CLCA expression does not always correlate with the presence of calcium-activated chloride currents (114).

F. Correlating Tissue Expression Sites to CLCA Groups

Figure 1 illustrates a connection between two structurally related CLCA groups. The first “group” contains the cross species homologs, hCLCA1, mCLCA3, and pCLCA1. The divergent connecting branch contains hCLCA2 and hCLCA4 and now also mCLCA5 and -6 corresponding to these human forms (14, 54). The second group contains two subgroups. The first of these includes mCLCA1/2, which may be alternate splice products from a single locus (158) and mCLCA4. The second subgroup contains hCLCA3, bCLCA1, and bCLCA2. Functional similarities within these structural subgroups are probable, and similarities in cross-species tissue expression sites within CLCA groups support this hypothesis.

The hCLCA1, mCLCA3, and pCLCA1 forms have a similar distribution pattern in the three species (Table 1), concentrated in mucus-producing epithelium in the gastrointestinal and respiratory tracts. This group of CLCA proteins may participate in mucin secretion and modulation of chloride conductance in secretory epithelial tissues. The members are conspicuously absent from pancreas, smooth muscle, and endothelial cells.

The intermediate hCLCA2 and hCLCA4 forms and mCLCA5 are more widely distributed than the first group. The extensive expression of hCLCA4 in neural tissue, with the exception of the cerebellum, is noteworthy. The absence of expression in the mucosal surfaces of the small intestine is another prominent difference between this and the hCLCA1, mCLCA3, pCLCA1 group. In this respect, mCLCA6 is more like its mCLCA3 counterpart. Functions related to tumor cell metastasis and chloride channel activity have been proposed.

The most striking property of the second cluster of structures, including mCLCA1/2, mCLCA4, hCLCA3, bCLCA1, and bCLCA2, is the wide distribution of all of these forms. Their presence has been reported in most tissues where their expression has been investigated, although there is limited data for hCLCA3, bCLCA1, and bCLCA2. Disparate functional roles for this third group
### TABLE 1. Tissue sites of expression of related CLCA proteins

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Reference numbers in parentheses identify the source of the positive expression data. Negative data (unattributed) are designated with a hyphen. Tests not performed or reported are indicated with unmarked cells.
include suggestions of smooth muscle chloride channel modulation (mCLCA4, and by extrapolation, the human isofrom hCLCA3), and possible endothelial adhesion factors hCLCA2, hCLCA2, and mCLCA1. A third function is suggested by the surface epithelial expression of endothelial adhesion CLCAs in the gastrointestinal tract.

V. FUNCTIONAL RESPONSES TO EXPRESSION OF CLCA PROTEINS

A. Human CLCAs

1. hCLCA1

Transient expression of hCLCA1 in HEK293 human embryonic kidney cells increased calcium-activated whole cell currents from 1.57 ± 0.72 pA/pF in control cells to 11.06 pA/pF in transfected cells, producing a time-independent outwardly rectifying current (75). This current was said to be “electrically isolated” for chloride, based on the blockage of cation current using large bulky cations, but the chloride dependence of the current was not determined. These putative chloride currents were inhibited by DIDS, dithiothreitol (DTT), and niflumic acid, although DIDS is the only one of these compounds known to be a specific blocker of chloride channels.

This study also presented the only single-channel patch-clamp study of the CLCA proteins. Single channels of hCLCA1-transfected cells had a slope conductance of 13.4 pS in cell-attached conformation after the addition of ionomycin in the presence of 1 mM Ca\(^{2+}\) (75). There was no mention in the report of endogenous HEK293 channels responsible for the background of nonselective and anion currents previously reported in these cells (206, 215).

2. hCLCA2

Transient transfection with hCLCA2 produced a calcium-activated, non time-dependent, outwardly rectifying increase in whole cell current in HEK293 cells. The whole cell current increased from 1.52 ± 1.83 pA/pF in control to 10.77 ± 3.8 pA/pF in transfected cells. Once again, neither the background current in untransfected cells nor the chloride dependence of the current produced in transfected cells were reported. However, the “electrically isolated” anion current was blocked by DIDS, DTT, niflumic acid, and tamoxifen (80).

3. hCLCA3 and hCLCA4

There are no current reports of functional ion transport data for hCLCA3 and hCLCA4.

B. Mouse CLCAs

1. mCLCA1

mCLCA1 expression in HEK293 cells caused the appearance of a calcium-activated chloride current. The current was outwardly rectifying and not time dependent (67). The current increased from 2.05 ± 1.09 to 10.23 pA/pF upon addition of ionomycin. Background chloride currents were apparently insignificant in this study, but the anionic dependence of the current was not determined. Niflumic acid, DTT, and DIDS inhibited the inferred chloride current.

Romio et al. (160) concurrently cloned and expressed mCLCA1 in *Xenopus* oocytes. Injection of mCLCA1 cRNA into oocytes caused a significant increase in current without added calcium ionophore. At −80 mV, the current in mCLCA1 mRNA-injected oocytes was −398 ± 136 nA compared with −97 ± 12 nA for water-injected oocytes. The current in the mCLCA1-injected oocytes, but not in the water-injected oocytes, was chloride dependent. The lack of a reversal potential \((E_{\text{rev}})\) shift when bath solution was changed to low NaCl indicated that the background oocyte current was a combination of both anion and cation currents. DIDS and niflumic acid inhibited chloride current in mCLCA1-injected oocytes and in water-injected controls. The application of a Ca\(^{2+}\) ionophore (ionomycin) caused a transient, statistically insignificant increase in current to 1,805 ± 473 nA in mCLCA1-expressing oocytes compared with 1,109 ± 389 nA in control oocytes (160). It was difficult to determine any effect of Ca\(^{2+}\) on the chloride conductance associated with CLCA expression due to the large background currents in *Xenopus* oocytes.

Generally mCLCA1 expression in HEK293 cells resulted in a mildly outwardly rectified, time-independent current (25, 73). This current requires 2 mM Ca\(^{2+}\) in the pipette and does not increase above control values with 500 nM Ca\(^{2+}\). The stimulated mCLCA1 current was most permeable to SCN\(^−\) > Cl\(^−\) > isethionate (25), as were the native Ca\(^{2+}\)-activated chloride channels in smooth muscle from which mCLCA1 was cloned. The anion dependence of the whole cell current in control HEK293 cells was not assessed.

The mCLCA1-dependent calcium-stimulated current changed significantly from time independent to time dependent, as well as increasing the total whole cell conductance when coexpressed with a potassium channel \(\beta\)-subunit (73). Coexpression also resulted in a greater sensitivity to activation by calcium. Addition of 500 nM Ca\(^{2+}\) in the pipette solution could stimulate the anion current in \(\beta\)-subunit mCLCA1 expressing cells. This difference in current and agonist sensitivity was shown by a mammalian two-hybrid system to be the result of a direct interaction between the potassium channel \(\beta\)-subunit and
mCLCA1. Unfortunately, the anionic dependence of the current was only assessed through the permeability ratios for SCN⁻ and Cl⁻, which were consistent with those found in smooth muscle. Again, as with several other CLCA studies, the background currents were not reported (73). This study by Greenwood et al. was the first report suggesting that a CLCA protein requires coexpression with other proteins to produce functional chloride channels and that the currents induced on CLCA1 expression could be modified by an accessory protein.

2. mCLCA2

There are no published electrophysiological data on expressed mCLCA2.

3. mCLCA3

The effects of mCLCA3 or Gob 5 expression have been characterized briefly in HEK293 cells (203). mCLCA3 expression increased an outwardly rectifying current without agonist addition. Current densities at +60 mV increased from 59 ± 17 to 230 ± 47 pA/pF. Addition of 10 mM EGTA to the pipette solution significantly decreased these currents (203).

4. mCLCA4

Treating transient mCLCA4-transfected HEK293 cells held at an undefined controlled voltage with ionomycin or methacholine increased intracellular calcium and evoked transient inward currents (48). This effect would be consistent with the inward movement of cations or the outward movement of anions. In this case, a strong anionic dependence to the stimulated current was shown by a shift in the current-voltage relationship to the equilibrium potential of chloride when bath solution was switched to low chloride after ionomycin addition. Unlike previously characterized isoforms, the current was found to be nonrectifying. The transient nature of the current was thought to be analogous to the Ca²⁺-independent inactivation of the smooth muscle Ca²⁺-activated chloride channel. This inward current was found to be spontaneous, similar to those in smooth muscle cells that are induced by a “calcium spark” and are involved in buffering the membrane potential, relaxation, and spontaneous rhythmic contraction of smooth muscle (92, 136). Current-voltage relationships in nontransfected cells were not examined, as voltage-clamped inward currents were not seen (48).

5. mCLCA5 and 6

Expression of these isoforms caused an ionomycin-dependent increase in whole cell current in HEK293 cells (54). Chloride involvement was verified by reversal potential measurements. A requirement for 2 mM calcium in the pipette to induce these increases in chloride current was consistent with other reports that are difficult to reconcile with channel regulation at intracellular calcium concentrations. In contrast to normal mammary epithelial cells, metastatic mammary tumor cells express little or no mCLCA5 upon starvation or detachment. Transfection of a mammary tumor cell line with mCLCA5 caused a dramatic reduction in colony formation by transfected tumor cells (14).

C. Bovine CLCAs

1. bCLCA1

Oocytes transfected with bCLCA1 cRNA had a significantly larger current than water-injected control oocytes (39). bCLCA1 expression apparently increased an endogenous outwardly rectifying current. This current was sensitive to DIDS and DTT but insensitive to niflumic acid. Addition of 1 µM ionomycin to oocytes resulted in the activation of endogenous chloride channels which were inhibited by niflumic acid. Unfortunately, data showing the effect of the ionophore on bCLCA1-injected oocytes were not presented or discussed (39).

A nonrectifying, Ca²⁺-activated, DTT-inhibitable current was produced when bCLCA1 was expressed in COS-7 cells (39). This linear current observed after bCLCA1 expression was unlike the outwardly rectified currents seen with expression of most of the other CLCA family members. However, no other family members have been characterized using COS-7 cells. It is interesting to note that the currents induced in the oocytes had a both time-dependent and rectifying quality, whereas those induced in the COS-7 cells had neither (39).

Lipid bilayers constructed of membranes from oocytes that had been injected with bCLCA1 cRNA had a unit channel conductance of 21 pS (39). The open probability increased from 0.41 ± 0.07 to 0.60 ± 0.08 in the presence of Ca²⁺ added only to the side opposite to that where DIDS was added. The channel was inhibited by DIDS and DTT, but insensitive to niflumic acid. The channel had an 8:1 anion to cation selectivity ratio and 3:1 selectivity for iodide over chloride under bionic conditions. The biophysical properties were similar to those found for a purified chloride channel from bovine trachea. However, some bilayers had a Ca²⁺-activated chloride conductance that was inhibited by niflumic acid. This was said to be the endogenous Ca²⁺-activated chloride channel of the oocyte. Others using bCLCA1 as a control for mCLCA1 characterization found the bCLCA1-induced current was inhibited by 78% with niflumic acid. Subsequent addition of DIDS (100 µM) caused a further 33% inhibition of the anion current (160). The basis for these differences is not clear.
D. Porcine pCLCA1

pCLCA1 expression in NIH/3T3 cells increased a calcium-activated $^{36}\text{Cl}^-$ efflux (68, 115, 117). Agonists for calcium-dependent protein kinase (PKC) or cAMP-dependent protein kinase (PKA) had no effect. However, application of 10 μM ionomycin significantly increased $^{36}\text{Cl}^-$ efflux from transfected cells. Inhibition of this effect by membrane-permeable Ca$^{2+}$ chelator, combined with the lack of inhibition by the CaMKII inhibitor KN-93, suggested that Ca$^{2+}$ may directly regulate a pCLCA1 effect on chloride transport. The pCLCA1 effect on chloride efflux was inhibited by 5-nitro-2-(3-phenylpropyl-amino)benzoate (NPPB), glibenclamide, diphenylamine carboxylate (DPC), and α-phenylcyclamate (α-PC). NPPB and DPC inhibited the Ca$^{2+}$-activated chloride efflux at much lower concentrations than were required for inhibition of CFTR. NPPB was effective at a concentration similar to that which was required to inhibit Ca$^{2+}$-activated chloride conductance in Xenopus oocytes (115).

In whole cell patch clamp, pCLCA1 increased an endogenous Ca$^{2+}$-activated chloride conductance, making the current more anion dependent (115). The pCLCA1 chloride current was outwardly rectifying and time dependent. This time dependence of chloride currents in NIH/3T3 fibroblasts has not been reported for other CLCA proteins, suggesting contributions by the expression system to the chloride current. As with $^{36}\text{Cl}^-$ efflux, DIDS did not inhibit whole cell chloride current, but NPPB, DPC, and α-PC were inhibitory (115). Both DTT and DIDS were reported to inhibit chloride current activity in previous studies with different CLCA proteins and different expression systems (39, 67, 75), but did not inhibit effects of pCLCA1 in 3T3 cells. In contrast to the fibroblast expression system (113), transfection of an epithelial Caco-2 human colon carcinoma cells with pCLCA1 produced a cAMP-activated chloride conductance, possibly through effects on CFTR (113). The pCLCA1 and PKA-dependent currents were anion dependent, time independent, and nonrectifying. The effects of pCLCA1 expression on PKA-dependent chloride conductance were evident in freshly passaged and in differentiated epithelial cells, both of which have endogenous CFTR-mediated chloride conductance. This modulation of chloride conductance by pCLCA1 expression was also seen in nonepithelial NIH/3T3 fibroblasts coexpressing CFTR and pCLCA1 (117).

Expression of pCLCA1 changed the chloride conductance activated by calcium ionophore or PKA agonists in Caco-2 epithelial cells to a time-dependent outwardly rectifying current (114). However, the endogenous Ca$^{2+}$-activated chloride conductance is lost as Caco-2 cells mature, and the ability of pCLCA1 to activate Ca$^{2+}$-dependent chloride conductance also disappears (114). Again, the endogenous chloride channel activity in the expression system influences the properties of the chloride channels observed upon CLCA expression.

E. Other CLCAs

A rCLCA1 isoform indirectly implicated in bicarbonate transport and vesicle exocytosis in rat pancreas and a canine cCLCA1 isoform (117) produced in secretory retinal pigment epithelium have not been fully cloned or functionally characterized.

VI. PATHOPHYSIOLOGICAL CONNECTIONS TO CLCA EXPRESSION

A. CLCA in Asthma

Orthologs of the CLCA gene family are overexpressed in bronchial allergic asthmatic responses that overproduce mucus (88, 135, 181). There is significant evidence that the CLCA proteins have a causal role in this condition, suggesting that these proteins may have some interest as targets for pharmacological intervention in allergic asthma.

I. Asthma and the genesis of its mediators

Bronchial allergic asthma is a serious inflammatory condition of the airways resulting in bronchial narrowing, constriction, and overproduction of mucus (27, 181). Simplistically, the uncontrolled inflammatory response, accompanied by the overexpression of CLCA protein, is thought to occur through a dysregulation between type 1 helper T (Th1) and type 2 helper T (Th2) cell immune responses to allergens (10, 27, 47, 90, 134, 159, 192). The Th1-immune response involving interleukin (IL)-2 and interferon (IFN)-γ cytokines is seen as a cell-mediated response associated with disease in situations of poor hygiene or infection with Mycobacterium tuberculosis, measles virus, or hepatitis A virus (21, 127, 130, 167, 171). The Th2 disease response involving a different set of cytokines (IL-4, -5, -6, -9, and -13) is considered to be humoral, involving the stimulation of B cells and the production of IgE. Released IgE binds to IgE receptors on mast cells, lymphocytes, eosinophils, platelets, and macrophages. Binding of allergen to the IgE results in the release of inflammatory mediators from mast cells and activation and potentiation of an inflammatory response (27).

Proper priming of the immune system to different immunological challenges is essential to develop a normal Th1 response. The Th1 response then has a role in modulating the Th2 response. The asthma disease state is dominated by an uncontrolled Th2 response, and blockade of the Th2 cytokine pathways significantly dampens the asthmatic response (26, 57).
2. Th2 cytokines mediate CLCA expression

The Th2 response and the associated cytokines IL-9, IL-4 and IL-13 appear to be directly linked to CLCA overexpression in the asthmatic patient, and in mouse models of asthma (209). However, instilled Th1 cytokine INF-γ did not induce expression of mCLCA3. mCLCA3 synthesis was induced in the lung and bronchi in an allergic asthma mouse model of ovalbumin sensitization, and transfection with an adenoviral mCLCA3 antisense construct prevented airway hyperresponsiveness (AHR) and mucus production after ovalbumin challenge (135). Application of a sense mCLCA3 mRNA construct to this same model produced severe AHR and mucus secretion (135). In vitro overexpression of mCLCA3 or its closest human ortholog hCLCA1 in human mucoepidermoid cells (NCI-H292) increased mucus secretion and overexpression of the major secretory mucin gene of asthma, MUC5AC (88). An increase in hCLCA1 correlated with an increase in mucus and IL-9 receptor in asthmatic patients (Fig. 6). This relationship suggests that hCLCA1 might be a therapeutic target in asthma patients.

Exposure of human bronchial epithelial cells to Th2 cytokines, IL-13 or -4, greatly increases the magnitude and duration of activated Ca²⁺-dependent chloride conductance (12, 40). This group of cytokines tends to switch epithelial transport from net absorption to a secretory phenotype by decreasing sodium absorption and increasing apical chloride conductance. Inflammation may trigger this phenotypic switch to help flush particulates and secreted mucus out of the airway. Pseudohypoaldosteronism is an excellent model for this strategy, where increasing net secretion by decreasing absorption significantly increases the rate of mucociliary clearance (18, 40, 97).

Epithelial Ca²⁺-activated chloride conductance increases in AHR. The pharmacological sensitivity of this induced chloride conductance is similar to that reported for CLCA-stimulated conductance (11, 40). There is no evidence that CLCA induction is responsible for the Th2 cytokine-mediated increase in Ca²⁺-activated chloride conductance, but CLCA could have a dual role to connect and coordinate fluid and mucus secretion.

CLCA proteins increase the activity of Ca²⁺-dependent chloride channels, and inhibiting CLCA expression inhibits mucus production (135), but it is not clear how these proteins increase mucus secretion. The identification of mCLCA3 expression in mucin granule membranes of the respiratory and gastrointestinal tract implicates this protein in mucin storage or release (109). It was suggested that mCLCA3 is needed for mucin maturation. Negatively charged mucus glycoproteins require strong acidification for mucin condensation in the granules. Granule acidification could occur via a H⁺-ATPase with electroneutrality maintained by a parallel CLCA-modulated chloride conductance (53, 109, 178, 190). Potassium release and Ca²⁺ entry into the vesicles may also be important in the condensation process (53, 178).

CLCA proteins could also participate in mucus secretion by promoting rehydration of condensed mucins or by

![FIG. 6. Hypothetical roles for CLCA protein in the asthmatic airway. Increased expression of CLCA is presumably cytokine-driven. CLCA could then 1) increase the transcription or translation of MUC genes, 2) increase the transport of mucin to the plasma membrane by increasing the condensation (packaging process) by acidification of the mucin granule, 3) take part in the membrane fusing process by interaction with the SNARE apparatus, and 4) facilitate the ion transport component of the exocytosis process. CLCA protein may also have a role in increasing net ion/fluid transport (increasing chloride conductance and decreasing sodium absorption) to the inflamed epithelium to increase mucus transport up the mucociliary ladder.](http://physrev.physiology.org/)
connecting synaptobrevin mucin granule docking proteins with the T-SNARE (syntaxin and SNAP-25) plasma membrane proteins of exocytosis (131, 145). SNARE machinery permits fusing granules to form a pore through the cytoplasmic membrane. Rehydration of stored mucus by influx of extracellular solution through the grulate membrane from the cytosol, is required for mucin release through this pore (178). CLCA protein could control the ion transport required for mucin rehydration through the vesicle membrane during exocytosis (Fig. 6).

Putative inhibitors of chloride conductance associated with CLCA protein expression can inhibit mucus secretion (210). However, on closer examination, niflumic acid inhibited Ca\(^{2+}\) entry into the epithelial cells, independent of a chloride channel blockage effect (16). Calcium ion entry is central to the process of mucin exocytosis, triggering activation of the SNARE complex (16). The role of CLCA protein in controlling mucin secretion remains obscure, but answers may come from a better understanding of chloride channel modulation by CLCA proteins.

**B. CLCA Function in Cystic Fibrosis**

Cystic fibrosis (CF) disease arising from problems with the CFTR protein leads to dysregulation of epithelial ion transport affecting most secretory epithelial tissues in the body (128, 129). CLCA expression in secretory epithelial cells and its function as a modulator of chloride conductance makes it a secondary focal point, after CFTR, in investigating possible compensatory mechanisms for defective epithelial ion transport (68, 113–115, 117).

**C. CLCA in the Normal Epithelium**

Ion gradients produced by the basolateral 3Na\(^{+}\)-2K\(^{+}\)-ATPase provide the electrical driving force for the chloride transport that is modulated by CLCA proteins (101, 102, 111). The inwardly directed Na\(^{+}\) gradient produced by this vectorial cation transport drives the coupled electroneutral entry of Na\(^{+}\), K\(^{+}\), and Cl\(^{-}\) into the cell through the basolaterally located Na\(^{+}\)-K\(^{-}\)-2Cl\(^{-}\) cotransporter (141, 142). The charge separation provided by the stoichiometry of the 3Na\(^{+}\)-2K\(^{+}\)-ATPase and the basolateral K\(^{+}\) conductance lowers the resting membrane potential, creating a driving force for chloride to exit the epithelial cell through CLCA-modulated anion conductance channels in the apical membrane (36, 111, 113–117). Sodium exit through the tight junctions maintains electrical neutrality, and water follows, resulting in a secretory epithelium. However, a decrease in apical chloride conductance can cause sodium to be drawn back into the cell by the electrochemical gradient created by the 3Na\(^{+}\)-2K\(^{+}\)-ATPase, making the epithelium absorptive. Chloride then follows the absorptive sodium movement across the cell (200, 201).

**D. CLCA in CF Epithelium**

The CLCA orthologs have a modulatory role on the normal transepithelial sodium chloride transport, which is defective in CF disease (113–117, 128, 129). The genetic abnormality in transport is caused by mutations in the CFTR. CFTR is the major apical chloride transporting channel of epithelium (Fig. 7), and it downregulates the activity of the apical absorptive sodium channel in epithelium (ENaC) (105, 172). The most common mutation in CFTR, the deletion of phenylalanine at position 508 (F508) (96, 157), can function normally, but ΔF508 CFTR transport through the endoplasmic reticulum (ER) and Golgi to the cytoplasmic membrane is interrupted (30). Fewer CFTR molecules at the apical membrane of secretory tracheal epithelial cells decrease fluid secretion and also indirectly increase salt and fluid absorption via the loss of normal negative effects of CFTR on sodium absorption by ENaC. The resulting reductions in the airway surface liquid volume (37, 129) increase the viscosity of airway mucus, decrease mucociliary clearance, and favor bacterial colonization of the airway surface (119, 150).

FIG. 7. Possible roles by which a CLCA protein (pCLCA1) could contribute to epithelial ion transport in the cystic fibrosis (CF) patient. Modulation of apical chloride conductance by CLCA protein interactions may have beneficial effects on ion transport in the CF patient. Beneficial mechanisms would occur through increasing the activity of CaCC or promoting proper CF transmembrane conductance regulator trafficking or function.
The activity of alternative chloride channels in the apical membrane of CF epithelium could compensate for the lack of CFTR. CLCA could increase the activity of such an alternative channel given its effects on both cAMP- and Ca\(^{2+}\)-activated chloride conductance (75, 113–115, 117). This situation may occur in the CFTR knockout mouse where upregulation of a Ca\(^{2+}\)-activated chloride channel appears to protect against lung pathology (62, 74, 169, 177, 180). Human CF patients also have this Ca\(^{2+}\)-activated chloride channel, but it is apparently unable to adapt sufficiently to maintain normal airway surface layer (ASL) hydration and prevent disease (9, 20, 199). Activation of this channel through purinergic receptors has been explored in CF patients (34, 207). There may be a degree of natural compensation for defective CFTR, as both hCLCA1 (83, 84) and Ca\(^{2+}\) of natural compensation for defective CFTR, as both explored in CF patients (34, 207). There may be a degree of this channel through purinergic receptors has been explored in CF patients (34, 207). There may be a degree of natural compensation for defective CFTR, as both hCLCA1 (83, 84) and Ca\(^{2+}\)-activated chloride conductance (9, 20, 34, 199) increase in CF airway. CLCA proteins have been shown to increase both endogenous Ca\(^{2+}\)-activated and cAMP-activated chloride conductance (113, 114), although the cAMP-dependent response may require functional CFTR (Fig. 7). However, activation of a Ca\(^{2+}\)-dependent chloride conductance does not replace the inhibitory effects of CFTR on ENaC, and persistent sodium absorption aggravates ASL dehydration (37, 129).

The involvement of hCLCA1 in mucus secretion is a concern when considering attempts to upregulate CLCA activity in CF patients. Elevations in hCLCA1 expression in CF patients could contribute to mucus overproduction and airway obstruction (84). Parallels have been drawn between elevated levels of hCLCA1 expression in AHR in asthmatic patients and significantly increased hCLCA1 levels associated with the inflammatory processes of CF (83). Agents such as niflumic acid may be clinically beneficial through reduction of mucus production (94), although these effects of niflumic acid may not be to inhibit the chloride conductance of hCLCA1. Future efforts could be directed toward differentiating beneficial CLCA effects on net fluid secretion from less desirable stimulation of mucus secretion (16).

VII. CLCA PROTEINS IN ONCOLOGY

A. Cell Cycle Control

Orthologs of the CLCA gene family apparently play two seemingly contradictory roles in cell division and metastasis. CLCA functions as a tumor suppressor within the transformed cell, possibly by modulating ion transport. Expression of CLCA protein is frequently lost in tumor cell lines (49, 79, 112). However, tumor cells that have lost CLCA expression can bind endothelial-expressed CLCA via a novel endothelial CLCA/β\(_3\)-integrin interaction. This tumor cell adhesion to endothelium can lead to intravascular arrest, promoting a mitogenic signal for intravascular tumor growth followed by metastatic tissue invasion (1–3, 211–213).

1. Effects of CLCA overexpression on the cell cycle

Aberrant morphology of cells overexpressing mCLCA2 has been interpreted as evidence for modulation of the cell cycle by CLCA. This morphologic effect is reported to require relatively high levels of mCLCA2 expression (49). Inappropriate amounts or timing of CLCA expression affecting chloride channel activity were assumed to disrupt the intricate depolarization and polarization accompanying the cell cycle. The overexpressing cells tended to be larger and multinucleated, as a probable consequence of abnormalities in cell cycle control (49). Blockage or partial arrest at various points in the cell cycle can disrupt cellular pathways leading to the production of multinuclear giant cells (133). However, specific cell cycle studies with mCLCA2 expression have not been reported.

2. CLCA suppression of tumor cell cycling

There is a growing body of evidence showing that the activity of anion channels that are modulated by CLCA, as well as independent cation channels, have a significant impact on cell cycle control (98, 99, 113, 115, 121, 204). Blockage of potassium conductance reduces cellular proliferation and causes synchronization at G\(_s\)/G\(_1\) of the cell cycle in several cell types (43, 121, 139, 193, 204, 205). Decreased chloride conductance causes the opposite effect, increasing cell proliferation and protecting cells from apoptotic death (42, 99, 202, 204). Cell cycle-specific chloride currents are highest during G\(_1\) and lowest during S phase (188, 191). mCLCA2 could contribute to this current by stimulating chloride conductance. Then down-regulation or loss of mCLCA2 expression or activity could be a feature of tumorigenic cell lines, and restoring mCLCA2 expression or activity could be a novel strategic approach to suppressing the growth of transformed cells (49, 79, 98, 99).

3. Apoptosis in CLCA tumor suppression

The mechanism for these proposed tumor suppressive effects of CLCA proteins is not understood, but it has been suggested that mCLCA2 could suppress tumor growth through proapoptotic effects (79). Transfection of the mammary epithelial cell line HC11 with mCLCA2 significantly increased the rate of apoptosis in cultures subjected to serum starvation compared with serum-starved control cells (49). Selection for resistance to detachment-induced apoptosis (anoikis) in the normally nontumorigenic HC11 cell line was accompanied by the loss of mCLCA1 message and mCLCA2 was assumed to be aberrantly spliced and not functional. These selected cells
were then tumorigenic when injected into mice (49). Apparently mCLCA2 expression does not change growth rate or anchorage-independent growth in culture conditions where the cellular environment does not favor apoptosis, but the presence of hCLCA2 may create a permissive environment or tip the balance towards apoptosis in cells receiving an apoptotic signal.

Stable mCLCA1 or mCLCA2 transfectants selected and maintained with G418 grew normally (79), but transfected cells selected by resistance to Zeocin grew slowly, and died within a 4-wk period (49). This difference may be due to the distinct mechanisms of the selective drugs. Geneticin (G418) binds to the 80S ribosome and prevents protein synthesis in eukaryotic cells without directly inducing apoptosis. Zeocin causes cell death by intercalating into, and cleaving, DNA (69, 132). This type of stress activates the p53 apoptotic pathway and, at higher doses, independent pathways. This means that Zeocin-selected but not G418-selected cells are always fighting apoptosis. Combining this stress with proapoptotic effects of hCLCA2 could account for poor growth and increased apoptosis seen with Zeocin and not G418.

4. A mechanism for CLCA proapoptotic effects

The preceding information suggests that CLCA protein expression can slow cell division through effects on anion conduction. A number of reports have shown that blockage of chloride conductance increases cell proliferation and confers protection from apoptotic death (71, 99, 174, 204). Generally, there is an influx of calcium in the initial stages of apoptosis which activates both potassium and chloride channels (143). One possible apoptotic scenario would have increasing potassium conductance hyperpolarizing the cell, increasing the driving force for anion conductance through the already open Ca$$^{2+}$$-activated chloride channel. Then intracellular pH could be lowered by bicarbonate exiting through the anion-conducting channel. A reduction in the intracellular pH would increase the activity of caspase 3, cause destruction of DNA, and trigger an apoptotic cascade (165). Expression of CLCA could activate anion conductance to stimulate the exit of bicarbonate or chloride, resulting in a larger number of cells entering the apoptosis cascade (Fig. 7).

5. Loss of CLCA in tumorigenic cell lines

Expression of mCLCA2, hCLCA2, or its mouse counterpart mCLCA5 has a significant impact on in vivo and in vitro growth of tumor cells (14, 49, 79). Nontumorigenic breast epithelial cells (MCF10 A and MDA-MB-453) express hCLCA2, but transformed tumorigenic cell lines (MDA-MB-231, MDA-MB-435, MDA-MB-468, and MCF7) do not (79). Hypermethylation of the hCLCA2 promoter may cause this transcriptional downregulation in tumorigenic cell lines, inviting speculation that hCLCA2 is the 1p31 breast cancer tumor suppressor gene (112). mCLCA1 is also normally expressed in mammary tissue, and mCLCA1 transcripts were not found in tumor cells, suggesting disrupted expression at the promoter level in the tumor (49, 112). Abnormal RNA processing may also disrupt CLCA2 expression (79, 112). Intron/exon junction information in mCLCA2 transcripts identified PCR products of mCLCA2 cDNA from JC and CSML-0 adenocarcinoma cell lines as splice variants (79).

Transfecting tumorigenic MDA-MB-435 and MDA-MB-231 cells with CLCA2 under the control of a foreign promoter resistant to inhibitory hypermethylation did not change growth rate or anchorage-independent growth of these cells in soft and hard agar. Potential invasiveness, measured as in vitro cell migration through 8-μm pore polycarbonate membranes or Matrigel films, was reduced upon CLCA2 transfection of tumorigenic MDA-MB-435 and MDA-MB-231 cells (79). In either subcutaneous or intravenous injections into nude mice, mCLCA2-transfected MDA-MB1–231 cells produced fewer tumors, reduced tumor size, or no lung tumors compared with control MDA-MB-231 inoculates (79). Without changes in growth rate or anchorage-independent growth, these intriguing in vitro decreases in invasiveness and migration do not totally account for the loss of tumorigenicity in this study. Undetermined phenotypic effects of CLCA in relation of cell differentiation remain to be defined.

The relationship between loss of hCLCA2 expression and tumorigenicity has been confirmed, providing evidence that hCLCA2 is the product of the hitherto unidentified 1p31 breast cancer tumor suppressor gene (112). The importance of the 1p31 locus is illustrated by the loss of heterozygosity in this region in 60% of breast tumors (87).

B. Cell Adhesion and Tumor Metastases

1. CLCA and cell adhesion

The bCLCA2 (Lu-ECAM-1) ortholog was cloned using antibody that blocked the adhesion-receptor/ligand pair that mediates binding of lung metastatic melanoma cells to bovine aortic endothelial cells (50, 70, 212, 214). The origins of the clone identify bCLCA2 as the endothelial adhesion molecule that binds hematogeneous tumor cells, causing vascular arrest before tumor growth and invasion. hCLCA2 and mCLCA5 may be counterparts to the original bCLCA2 (Lu-ECAM-1) clone (2). Like bCLCA2, hCLCA2 is expressed by endothelial cells from different lung vascular compartments (2, 213). This expression is critical for the establishment and colonization of human breast cancer cell lines (2).
2. **CLCA binds lung colonizing cells in vitro**

Only hematogeneous lung colonizing tumorigenic cells were able to bind to hCLCA2-expressing HEK293 cell lines (2). The MDA-MB-231 cell line, which colonizes the lungs of nude mice following intravenous injection, bound to a hCLCA2 expressing monolayer of HEK293 cells at a 75% efficiency. Cell lines that form metastases after orthotopic tumor xenografts but are unable to form metastasis on intravenous injection (MDA-MB-435) or nontumorigenic MCF7 cells only bound at ~4 and 7%, respectively, to hCLCA2-expressing HEK293 monolayers (2). This evidence suggests that binding of MDA-MB-231 cells to endothelial CLCA is necessary for hematogeneous metastasis and lung colonization.

3. **β₄-Integrin expression, CLCA binding, and tumor metastasis**

A coimmunoprecipitation strategy followed by specific antibody screening identified β₄-integrin from the MDA-MB-231 cells as the protein that was coprecipitated with hCLCA2 (2). There was a strong correlation between the level of β₄-integrin expression and the adhesion of HEK293 cells expressing hCLCA2 in studies involving additional cancer cells lines expressing different levels of β₄-integrin. A similar correlation was observed in vivo between β₄-integrin expression in tumorigenic cell lines and the number of lung metastases observed in nude mice given the different cell lines.

Overexpression of β₄-integrin in a Kirsten-Ras-transformed Balb/3T3 oncogenic cell line caused an increase in metastatic performance in syngeneic mice that paralleled an increase in adhesion of these cells to mCLCA1 (2). The locations of the metastases were consistent with reported sites of mCLCA1 expression in the mouse. In addition, cDNA arrays have shown a definitive increase in β₄-integrin with increased metastatic potential (2, 182). These findings support a role for β₄-integrin binding to CLCA protein in hematogeneous metastasis.

Antibodies to either β₄-integrin or hCLCA2 were able to inhibit binding of the hCLCA2-expressing HEK293 cells to the MDA-MB-231 cell line. Antibodies directed to integrins other than β₄ did not reduce binding, but specific cleavage of cell surface β₄-integrin with matrilsyn greatly reduced binding. Concurrent injection of nude mice with antibodies to hCLCA2 and β₄-integrin along with MDA-MB-231 cells reduced lung metastases by 84 and 100% (2).

Nontumorigenic cell lines selected for β₄ expression or transfected to add β₄ expression had increased binding to CLCA but did not increase lung colonization. The metastatically incompetent MDA-MB-468 cell line has a relatively normal growth phenotype in vitro, and slow adenomatous growth in vivo. Moderate levels of β₄ overexpression in this cell line did not correlate with a lack of in vivo metastases for these cells. Apparently, β₄-integrin effects on metastasis are somewhat cell type dependent, and β₄-integrin expression leads to lung metastasis only in those cancer cells that have the genotype of overexpression combined with altered expression of the appropriate transforming genes (2).

Overexpressed β₄-integrin in the nontumorigenic cell line may be interacting with an unidentified membrane protein, as β₄ and α₆-integrins coimmunoprecipitated from the metastatic MDA-MB-231 cell line but not from the nonmetastatic MDA-MB-435 cells. When this cell line was transfected with, and overexpressed β₄-integrin (MDA-MB-435 β₄), it had an increased adherence to hCLCA2 in vivo, but was unable to produce metastatic lung colonies. Failure to coimmunoprecipitate β₄ and α₆-integrins may be due to β₄-integrin interacting with something other than its normal α₆ counterpart in the MDA-MB-435 β₄ cells. The unidentified protein could have a dominant negative effect on metastasis. It appears that the β₄-integrin can cause endothelial binding, but that binding to some target of the α₆- and β₄-interaction pair promotes metastasis (2).

4. **CLCA binding domains**

Specific motifs in both the 90- and 35-kDa fragments of most CLCA family members are involved in the association with β₄-integrin (2). These binding motifs belong to the addressins, which are distinct vascular addresses used by both immune and blood-borne cancer cells to colonize specific organs (162). It is believed that cell binding in lung metastatic cancer is particularly mediated through binding of the β₄-integrin to the CLCA addressin (1).

Antibodies that blocked β₄/CLCA adhesion identified a candidate binding area that would be in the proposed second extracellular domain of hCLCA2, hCLCA2, and mCLCA1 (67, 80). Amino acids 479–488 and 740–749 that could account for the binding activity in hCLCA2 were identified in a computer-aided motif search (1). Motifs with a consensus sequence F(S/N)R(I/L/V)(S/T)S occur in the second extracellular domain of hCLCA2, bCLCA2, and mCLCA1 (1, 54). When expressed and purified, the motifs in the 90-kDa and the 35-kDa fragments bound to β₄-integrin, but not to β₁, β₃, fibronectin, or BSA. Adhesion increased with concentration of ligand and was Mn²⁺ but not Mg²⁺ or Ca²⁺ dependent. These purified binding motifs also bound to lung metastatic MDA-MB-231 cancer cells. Incubation of tumorigenic cells with purified binding motifs before intravenous injection of these cells into SCID/beige mice prevented binding and blocked lung colonization (1).

The β₄-integrin binding domain in hCLCA2 is present in the hCLCA1 35-kDa segment, but is disrupted by the substitution of amino acids G473 A474 for SR in the hCLCA1 90-kDa segment. The FGALSS sequence in hCLCA1 and pCLCA1 or FGALAS in mCLCA6 lacks β₄-integrin recognition.
integrin binding activity (1). Both NH2- and COOH-terminal β4-integrin binding motifs are disrupted in pCLCA1, mCLCA3, and mCLCA6. The COOH-terminal 35-kDa change is to FSRTAS in both pCLCA1 and mCLCA6. The mCLCA3 90-kDa β4-integrin binding domain region is changed to (FAALSS) and the 35-kDa sequence is [FSRT-(deletion)SS]. The loss of these binding domains from the same CLCA homolog in mouse, pig, and human parallels other indications of structural and functional divergence within the CLCA protein family (Table 1, Fig. 1).

The β4-integrin binding motif on both the 90-kDa and the 35-kDa fragments of the CLCA proteins would have extracellular locations in either of the proposed topologic models for membrane insertion (Fig. 2). Hence, the functional data arising from studies of CLCA protein binding to the β4-integrin do not resolve conflicting topology prediction issues.

5. Novel β4-integrin binding domain

The binding of CLCA to the β4-integrin is mediated through a novel domain in the β4-integrin (1, 2). Binding to the specificity-determining loop (SDL) was predicted, as this is a nonconserved loop sequence that has been associated before with ligand binding (151, 175). A short purified fusion protein of β4 segments corresponding to the predicted loop of β1- and β3-integrins, found previously to be important in integrin binding, was constructed and tested to determine the binding domain (175). An area in the β4 SDL sequence (amino acids 184–203) bound to CLCA in ELISA and pull-down assays. The same sequence from the β1-integrin did not bind. The relevance of the binding to hCLCA2 was confirmed by using the synthetic peptide sequence of the β4 loop to block metastatic cell line adhesion of CLCA protein (1).

The binding of the β4-integrin to laminin-5 has been attributed to a binding site at the NH2 terminus of the SDL loop as determined by point mutations K177A and Q182L (186). However, as described above, the β4-integrin to CLCA binding appears to involve the COOH-terminal part of the SDL (1). Hence, it is likely that laminin-5 and hCLCA2 interact with different SDL binding motifs. This difference in interaction may explain the difference in the mitogenic pathways activated by β4-integrin when binding to CLCA or laminin.

6. Response to CLCA/β4-integrin binding

As previously discussed, the binding of β4-integrin to hCLCA2 is important for cell adhesion in the beginning stages of lung metastasis (2, 211). Once adhered, the tumor cell can either extravasate early, resulting in immediate tissue invasion, or it can undergo intravascular growth. Extensive intravascular growth leads to occupation of the entire circumference of the vessel lumen by tumor cells, disintegration of the vessel wall, and invasion of the adjacent tissue by the tumor (106, 189). It is the β4-integrin/CLCA binding that provides the mitogenic stimulation important for intravascular growth (2, 3).

7. A novel β4 mitogenic signaling pathway

A model for the role of β4-integrin in a mitogenic pathway has evolved over the past decade. Initial experiments revealed that laminin or antibody-induced ligation of the α6β4 complex resulted in the adaptor protein Shc associating with a phosphotyrosine-containing β4-subunit (Fig. 8). The Shc is then phosphorylated, recruiting Grb2 and potentially activating the ras mitogen-activated protein (MAP) kinase pathway, through the recruitment of SOS (120, 186). The binding of Shc to β4 activates phosphoinositide-3-OH kinase (PI3K) through an undefined mechanism, which in turn activates Akt kinase resulting in phosphorylation of Bad, activation of oncogenic transcription factors, and cell survival (33, 66, 166, 176, 182). Mutagenic studies of the β4 cytoplasmic tail show that Shc preferentially bound to tyrosines 1440 and 1456 (41). The initial phosphorylation/activation of the β4-integrin for Shc binding is thought to be mediated in part through the Met tyrosine kinase of the receptor for the hepatocyte growth factor (HGF) (182). Similarly, the ErbB-2 receptor oncogene mediates its effects through binding with the cytoplasmic tail of the β4-integrin and activation of the PI3K (66) (Fig. 8). Interestingly, neither of these oncogenic effects required the extracellular domains of β4-subunit. Thus the β4-subunit is acting more as a modulator of oncogenic signals. The events accompanying the initial phosphorylation during laminin or antibody binding of β4 and release of its own mitogenic cascade, is still uncertain.

In comparison with the established activation pathway described above, the binding of CLCA to the β4-subunit is thought to initiate a novel signal transduction phosphorylation sequence (Fig. 9). The CLCA-initiated cascade is described as Met, ErbB-2 independent, and not requiring phosphorylation of the cytoplasmic tail or Shc binding domain of the β4-subunit for downstream signaling (3). In addition, there is no activation of PI3K. Instead, the CLCA pathway proceeds from CLCA binding with β4-integrin to the aggregation of focal adhesion kinase (FAK) with the β4 cytoplasmic tail through a yet undetermined mechanism. When β4-expressing B16-F10 cells were serum deprived and plated onto mCLCA1-coated dishes, only FAK was significantly activated. β4-Integrin ligation to mCLCA1 was required to coimmunoprecipitate FAK. Complex formation between FAK and β4-integrin was assumed to lead to the autophosphorylation of FAK at tyrosine-925, causing the binding of Src protein tyrosine kinase which then phosphorylates FAK at tyrosine-925 (3) (Fig. 9). Phosphorylated FAK binds Grb2 and then
recruits Son of Sevenless (SOS), leading to the activation of the Ras-MAP kinase pathway involving extracellular signal-regulated kinase (ERK) (3). A similar pathway has been described in astrocytoma cells and the transforming growth factor-

\[ \text{h3} \] 

(100). In addition to FAK, downstream Src kinase and ERK were also found to be activated (3). ERK reached maximum activation slightly after FAK. However, Src was constitutively activated in the tumor cell line. Antibodies that blocked the adhesion of mCLCA1 to \[ \text{h3} \] -integrin also blocked FAK and ERK activation.

The activation of FAK by the mCLCA1/\[ \text{h3} \] -integrin complex was somewhat unique, as this activation was not seen when cells were grown on laminin which binds \[ \beta_1 \]-integrin. Transfection of the cell line with the dominant negative FAK (FANK) (a nonkinase FAK) blocked activation of ERK. Transfection with the wild-type FAK significantly increased ERK activation upon ligation with mCLCA1 (3).

The FAK-mediated activation of ERK was found to be Src dependent (3) as the dominant negative FAKY397F did not allow phosphorylation and reduced complex formation between Src and FAK. Impairing Src binding also impaired Src phosphorylation of FAK for growth factor receptor-bound protein (Grb) binding. Antibodies directed against β-Grb coimmunoprecipitated FAK but not FAKY397F, indicating that the activated Src must bind autophosphorylated FAK at 397 and phosphorylate Y925 to allow Grb2 binding, which then allows activation of ERK (3).

Briefly, the binding of Grb2 allows the translocation of SOS to the membrane to increase the guanine nucleotide exchange of Ras (45, 161). GTP Ras has increased activity and activates the downstream MAP kinase pathways or Raf/MEK/ERK (5, 168). In the GTP-bound state, Ras interacts with and activates Raf, which phosphorylates MEK, which in turn phosphorylates ERK, which goes on to phosphorylate a number of transcription factors, activating the cell cycle and causing proliferation.

It is interesting to note that both dominant negative cell lines of B16-F10 melanoma cell produced as described above had greatly reduced lung metastatic potential (3). This is evidence for the importance of CLCA contributions to this pathway of tumor metastasis. In addition, this CLCA-activated pathway may also be important in those metastases that undergo extravasation before growth because the ERK activation can also induce the production of invasive products such as metalloproteinase, and even increase drug resistance (5).
8. Tumor invasion

Despite the activation of a cell proliferation cascade, there is little invasion into the actual organ until the vessel becomes filled with mammary tumor cells (3, 106, 189). There almost seems to be a need to restrict some of the blood supply before tumor cells can pass through the walls of the blood vessels into the tissue. It is also possible that previously discussed β₂/mCLCA1 interactions may activate an endothelial chloride conductance that promotes endothelial cell apoptosis. Tumor cell invasion could start by adhesion to endothelial cells, proliferation to fill the vessel and restrict blood supply to the endothelium, which in turn becomes slightly hypoxic, dropping the intracellular pH. The β₂/mCLCA1 could then participate in opening a channel to allow chloride entry to electrically balance ischemic hydrogen ion production and facilitate a rapid drop in intracellular pH (Fig. 6). The proposed pH drop would activate caspases and the apoptotic cascade (22, 165). The endothelial cells would die, and the tumor would invade.

VIII. CLCA, VASCULAR TONE, AND HYPERTENSION

Orthologs of the CLCA gene family mCLCA1, mCLCA2, mCLCA4, and, by inference, hCLCA3 are expressed in vascular smooth muscle (25, 48, 110). CLCA proteins are known to modulate Ca²⁺-activated chloride conductance as well as to interact with the subunits of the Ca²⁺-activated potassium (BK) channel (73, 114, 115). Ca²⁺-activated chloride conductance and potassium conductance control vascular tone, making a
modulating protein such as CLCA an interesting target for intervention in conditions such as hypertension and erectile dysfunction.

A. Hypertension

Hypertension or an increased vascular tone is defined as the mean arterial blood pressure in humans being greater than 110 mmHg. This usually results from the diastolic pressure greater than 90 mmHg and the systolic pressure greater than 140 mmHg (32, 55, 198). Sustained increases in vascular tone can be very detrimental. Increased blood pressure contributes to the development of congestive heart failure by increasing the work load on the heart, and to chronic renal failure through small renal hemorrhages and scarring. Hypertension also predisposes to coronary artery disease and stroke (32, 156).

Hypertension can result from, or be modulated through, changes to the renin-angiotensin system (32). The regulation of blood pressure begins with the rate of renal glomerular sodium clearance. Sodium clearance determines the amount of chloride presented to the macula densa, which determines the rate of reabsorption from the filtrate via the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter (163). Upon a decrease in the rate of the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter, the macula densa signals the juxtaglomerular cells, via a cyclooxygenase-2 inhibited process, to release renin (24, 29, 72). Renin is converted to angiotensin I and angiotensin II in the lung. Angiotensin II causes vasoconstriction and increases blood pressure through contraction of the smooth muscle of the vasculature, increasing glomerular sodium chloride clearance, and activity of the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter. These effects combine to cause a decrease in renin release (82). The release of aldosterone by angiotensin II also increases blood pressure by volume intake in the distal collecting duct of the kidney, which also increases glomerular sodium chloride clearance and decreases renin release. This system permits modulation of vascular tone, and its malfunction results in hypertension, but the actual etiology of most chronic hypertension is unknown (195).

Current therapy for hypertension often targets the renin-angiotensin system, either by blocking angiotensin receptors or by preventing the conversion of renin to angiotensin (32). Direct modulation of vasoconstriction by reducing vascular smooth muscle tone could be an alternative to inhibiting agonists of hypertension. Smooth muscle tone might be reduced by manipulating the interplay of potassium, chloride, as in the case of CLCA, and calcium channels that regulate the membrane potential to control calcium influx into the smooth muscle cell (91).

In smooth muscle, the equilibrium potential for chloride is more positive than the resting membrane potential, while the potassium equilibrium is more negative (31). The net result is that negative charges leave the cell upon chloride channel activation, resulting in membrane depolarization from approximately −60 mV to a chloride equilibrium potential of approximately −30 mV (91). This depolarization results in the activation of L-type calcium channels, permitting an influx of extracellular Ca\(^{2+}\) that causes muscle contraction (89, 138). Simplistically, it is believed that the Ca\(^{2+}\) entering the cell bind to calmodulin, resulting in a complex which activates myosin light-chain kinase (149). The resulting phosphorylation of myosin allows myosin/actin cross-bonding and initiates the “ratchet theory” of contraction through the hydrolysis of ATP (93, 187). Then the Ca\(^{2+}\) which activated contraction also activates Ca\(^{2+}\)-dependent potassium channels, resulting in potassium release, cell hyperpolarization, closing of L-type Ca\(^{2+}\) channels, and an end to the contraction cycle (31, 91, 138).

Thus agonist-induced increases in potassium conductance and decreases in chloride conductance in smooth muscle will result in muscle relaxation, vasodilation, and hypotension. Conversely, an agonist such as angiotensin or norepinephrine that decreases potassium conductance or increases chloride channel conductance will cause muscle contraction, vasoconstriction, and hypertension (Fig. 10). Smooth muscle contraction is significantly reduced by the inhibition of norepinephrine or angiotensin II-induced chloride conductance (81, 194). The presence of CLCA protein in smooth muscle, the effects of CLCA on Ca\(^{2+}\)-activated chloride currents, and CLCA interaction with subunits of the BK potassium channel make it likely that this gene family is directly involved in agonist-induced smooth muscle contraction (25, 48).

The molecular mechanism of basal vascular tone is just beginning to be understood. The basal vascular tone is thought to be maintained by “calcium sparks” (92, 136) which originate from a ryanodine-sensitive Ca\(^{2+}\) release from the sarcoplasmic reticulum (92). This Ca\(^{2+}\) release is local and does not cause contraction. However, it does activate the large-conductance Ca\(^{2+}\)-activated K\(^+\) (BK) channels. This BK channel is made up of a pore-forming \(\alpha\)-subunit and the \(\beta_1\)-subunit that regulates its Ca\(^{2+}\)-sensitive activity (7). Rodent hypertensive models illustrate the importance of this regulatory subunit. \(\beta_1\)-Knockout mice were hypertensive (23), and the \(\beta_1\)-subunit expression is downregulated in genetically hypertensive rats (8). In addition, rats made hypertensive by prolonged exposure to angiotensin II were found to downregulate the \(\beta_1\)-subunit (8). This downregulation correlated with in vivo whole cell and single-channel studies reporting a decrease in the channel sensitivity to Ca\(^{2+}\).

The CLCA gene family has been implicated in vascular tone and in calcium sparks. CLCA expression has been identified in the vascular smooth muscle of the mouse (25, 48). When mCLCA4 was expressed in HEK293 cells...
which do not express endogenous BK potassium channel \( \beta_1 \) regulatory subunit) they produced a nonrectifying and time-independent \( \text{Ca}^{2+} \)-activated inward current, or outward movement of chloride depolarizing the cell (48). Similarly, expression of mCLCA1 from murine portal vein produced a time-independent and only slightly rectifying anion current upon stimulation by \( \text{Ca}^{2+} \) (73). These biophysical properties observed upon mCLCA1 or mCLCA4 expression in HEK293 cells are distinctly different from smooth muscle cell recordings, which show time-dependent currents with strong rectification. However, on cotransfection with the \( \beta_1 \)-subunit of the BK potassium channel the current became very similar to the smooth muscle recordings (73). The effect of \( \beta_1 \)-subunit on CLCA-associated conductance was shown by a mammalian two-hybrid system to involve a direct protein-protein interaction. Unfortunately, these authors did not report the effect of cotransfection on the inward-induced current. The inward current (outward movement of chloride) would likely have been reduced in the dual transfection, given the strong rectification induced by the \( \beta_1 \)-subunit. An interaction of CLCA with \( \beta_1 \)-subunit in smooth muscle would suppress chloride conductance, reduce the depolarization of the membrane, and prevent contraction.

In smooth muscle, the “calcium spark” may occur through the \( \beta_1 \) BK subunit interactions that reduce the stimulatory effects of CLCA proteins on chloride conductance. This makes endogenous chloride channels more rectifying and stops chloride release. In contrast to the “spark,” there is larger \( \text{Ca}^{2+} \) release on agonist addition. At higher \( \text{Ca}^{2+} \) concentration, there is a significantly larger inward current or (movement of chloride out of the cell) in the dual CLCA \( \beta_1 \)-transfected cells (73). Thus, at lower [\( \text{Ca}^{2+} \)] during sparks, the outward flux of chloride may be inhibited by the \( \beta_1 \)-subunit interactions with a CLCA protein. At higher [\( \text{Ca}^{2+} \)] this inhibitory effect is lost. Thus, altering CLCA activity in smooth muscle cells could modulate an endogenous chloride conductance with a significant therapeutic benefit to hypertensive subjects.

IX. CLCA, BESTROPHINS, AND RETINOPATHY

Bestrophin proteins are associated with chloride transport in the retinal pigment epithelium. Mutant forms of these proteins cause the inherited disorder named Best’s vitelliform macular dystrophy. Bestrophins are of particular interest as candidates for the molecular identity of \( \text{Ca}^{2+} \)-activated chloride conductance (152). However, bestrophins may be only one component of a hetero-oligomeric calcium-dependent chloride conductor. Because one major function of CLCA proteins may be to modulate chloride conductor activity, it is important to examine the connection between CLCA and bestrophin proteins to get more insight into the disease condition as well as into the molecular identity of the components that make up the \( \text{Ca}^{2+} \)-activated chloride conductance.

Best’s vitelliform macular dystrophy (BMD) is an autosomal dominant inherited disorder of the eye, with juvenile onset. The disease is characterized by a vitelliform (“egg yolk” or “vitelline”) macular (discolored spots) lesion easily visible on examining the fundus (15). The vitelliform lesion is due to deposition of lipofuscin (fatty pigment)-like material within and below the retinal pigment epithelium (58, 196). This often results in degeneration of the retinal pigment epithelial cells (140). However, the most defining clinical feature of the disease is a light peak to dark trough ratio in the electrooculogram (EOG) of <1.5, without abnormalities in the electroretinogram (ERG) (38). The EOG measures the late response of the eye to light. It is the result of depolarization of the basal plasma membrane of the retinal pigment epithelium.
coupled to the uptake of Cl\textsuperscript{−} to receptors generates a large sodium gradient across the membrane adjacent to the retinal rod and cone photoreceptors. The Na\textsuperscript{+}/H\textsuperscript{+}-ATPase located in the membrane adjacent to the retinal rod and cone photoreceptors generates a large sodium gradient across the cell membrane (17, 183, 184). This sodium gradient is couped to the uptake of Cl\textsuperscript{−} and K\textsuperscript{+} across the membrane by the usual Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporter. The vectorial arrangement of ion transport proteins includes a chloride conductance on the opposite side of the polarized epithelium, adjacent to the choriocapillaris venous drainage. Normal coordinated operation of these ion channels drives net ion and fluid movement from the vitreous to the choroid (17). Chloride conductance is generally under the control of Ca\textsuperscript{2+} (117), and the rate of chloride release increases in the light-evoked EOG (65). These facts lead to the hypothesis that a defect in basolateral Ca\textsuperscript{2+}-activated chloride conductance is part of the pathology of BMD.

The mutation responsible for BMD was found in the gene coding for a 68-kDa protein. This protein, now named bestrophin, has become a molecular candidate for the Ca\textsuperscript{2+}-activated chloride channel (148, 152) and a target for activity modulation by CLCA proteins. At least 79 different bestrophin mutations have been found in BMD patients (125) (summarized at the VMD2 mutation data base, www.uni-wuerzburg.de/humangenetics/vmd2.html). These include missense, single amino acid deletions, as well as splice site and frameshift mutations (6, 13, 28, 46, 104, 118, 123, 126, 144, 148). Bestrophins share homology with the Caenorhabditis elegans RFP gene family, named for the presence of a conserved RFP amino acid sequence motif which is found in 26 transmembrane proteins with related sequences grouped in worm family eight (170).

Four bestrophin isoforms are known in humans (126, 148, 185). hBest1 or VMD2, the mutated gene in BMD, is expressed in RPE, retina, and testes. hBest2 or VMD2L1 protein is found in RPE, colon, and testes; hBest3 or VMD2L3 is expressed in skeletal muscle, brain, spinal cord, bone marrow, retina, thymus, and testes; and hBest4 or VMD2L2 was found predominantly in the colon and weakly in fetal brain, spinal cord, retina, lung, trachea, testes, and placenta (170, 185). It is interesting that hBest1, hBest2, and hBest4 are highly expressed in the RPE and colon, two tissues with very active ion and fluid transport (185). The membrane localization of hBest1 in the RPE indicates that bestrophins are properly situated to be calcium-activated chloride channels (124).

Electrophysiological experiments with expressed Best1 and -2 as well as C. elegans RFP proteins in HEK293 cells generated chloride currents that increased with micromolar intracellular Ca\textsuperscript{2+} concentration (173, 185). However, hBest1 was unable to produce a detectable chloride current when expressed in Xenopus oocytes (185). In a subsequent study, Xenopus bestrophin produced a Ca\textsuperscript{2+}-activated chloride current when expressed in HEK293 cells (153). Apparently HEK293 cells contain an accessary subunit that may be required for bestrophin to function. Introduction of a nonconducting mutation produces a dominate negative effect in wild-type oocyte channels (153). It seems likely that bestrophins may have a direct impact on Ca\textsuperscript{2+}-activated chloride conductance, but that these proteins are not working alone. The hCLCA1 protein is a good candidate to associate with and affect the activity of a bestrophin chloride channel. CLCA proteins have been localized to the same RPE membrane as Best1, adjacent to the choriocapillaris (117) where Ca\textsuperscript{2+}-activated chloride conductance occurs (65).

X. SUMMARY

The CLCA gene family clusters together on a small portion of the chordate genome and codes for the production of a structurally similar, but functionally diverse, group of proteins. The functions associated with members of this gene family implicate the CLCA proteins in several interesting physiological and pathological processes. They have intriguing roles in cell cycle control and in apoptosis, which may be associated with tumor suppressor activity. Expression of CLCA protein in different systems produces major differences in calcium-dependent whole cell chloride currents, cAMP-dependent chloride currents are modulated by CLCA expression. CLCA-induced currents are altered significantly by coexpression with a potassium channel β1-subunit. With the exception of function as cell-surface adhesion molecules that can anchor metastasizing tumor cells, these other processes associated with CLCA expression all have some connection to chloride channel activity. However, the underlying question of whether CLCA proteins function directly as chloride channels, or somehow interact with and affect the conductance of other ion channels has not been resolved.

There may be some convergence in connecting CLCA proteins to bestrophins, and to Ca\textsuperscript{2+}-activated chloride conductance, as neither protein appears to be a fully functional chloride conductor without expression in a proper complimentary background. It is an intriguing possibility that some combination of CLCA and bestrophin proteins may constitute the molecular identity of a Ca\textsuperscript{2+}-activated chloride channel. However, the upregulation of CLCA proteins in the inflammatory process, and the di-
verse interactions of CLCA proteins with other chloride channels and with other ion conductances invite speculation that CLCA proteins may have a broader involvement in determining the balance between epithelial absorption and secretion.

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