Cloning a chloride conductance mediator from the apical membrane of porcine ileal enterocytes

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Gaspar, K. J., K. J. Racette, J. R. Gordon, M. E. Loewen, and G. W. Forsyth. Cloning a chloride conductance mediator from the apical membrane of porcine ileal enterocytes. Physiol Genomics 3: 101–111, 2000.—Attempts to attribute ileal brush-border chloride conductance to specific proteins were pursued by screening a porcine intestinal cDNA library. A 0.94-kb clone was identified on expression screening with a monoclonal antibody that inhibited enterocyte brush-border chloride conductance. Further screening approaches led to the isolation of a 3.1-kb full-length sequence called pCLCA1, consistent with the identification of a 2.9-kb transcript through Northern analysis. This sequence had significant homology to the CLCA gene family of calcium-regulated chloride channels, especially to hCLCA1. However, a strong A-kinase consensus phosphorylation site in a predicted cytoplasmic loop of the protein was a notable difference from the hCLCA1 gene product. Several porcine exocrine epithelial tissues, including ileum, trachea, and the major salivary glands express pCLCA1 mRNA. In situ hybridization studies localized the expression of pCLCA1 mRNA to the crypt and villus epithelia of porcine ileum, whereas tracheal expression was observed in both surface epithelium and submucosal glands. In situ expression of pCLCA1 in mouse 3T3 cells induces an ionomycin-dependent chloride conductance activity in these cells.

Fluid secretion by the intestinal crypts is controlled primarily by transepithelial electrolyte transport, especially the secretion of chloride ions into the gut lumen via apical chloride channels. The importance of controlling chloride secretion into the intestine is illustrated by common secretory disorders. In cystic fibrosis patients, the absence of the chloride conductance channel activity or regulatory activity of the cystic fibrosis transmembrane conductance regulator (CFTR) causes a severe chronic intestinal hyposecretory disorder. At the other extreme, cholera toxin stimulates the production of intracellular second messengers, including cAMP, that elevate the intracellular chloride concentration beyond its equilibrium point and increase the open probability of intestinal chloride conductance channels. This activation causes a life-threatening acute intestinal hypersecretory disorder.

Although it has been clear for some time that multiple intracellular signaling mechanisms control the release of intracellular chloride ions, the identity of the channel or channels responsible for the majority of the chloride secretory response has been actively debated. The activities of CFTR, the outwardly rectifying chloride channel (ORCC), and the ClC exocrine epithelial chloride channels have been studied. However, the number of apical membrane chloride channels and the mechanisms for coordinating channel activity remain problems of current interest.

Calcium-regulated chloride secretion is an important alternative pathway to the activation of chloride secretion via cAMP-dependent agonists. The calcium-dependent pathway accounts for the majority of agonist-induced increases in chloride secretion in human sweat glands (21). The magnitude of the two responses are more nearly equal in human airways and murine pancreas (2).

The activity of the calcium-regulated pathway may increase under circumstances where cAMP-dependent chloride secretion is absent or defective. The cfrtm1Unc2/2 mouse, for example, has an increased nasal epithelial response to ionomycin compared with normal littermates, indicating an upregulation of the calcium-activated pathway (11). A protective role has therefore been proposed for this alternative conductance in tissues affected by CF. Tissues in which this conductance can be demonstrated, such as pancreas and airways, are only mildly affected by the absence of functional CFTR in cfrtm1Unc2/2 mice (2). In the small and large intestine of these mice, where this alternative conductance is absent, a much more severe disease phenotype manifests (2). Multiple chloride conductive pathways, then, are at the very least useful in preserving transepithelial chloride secretion, and may in some circumstances be essential.

The isolation of Ca-CC (or bCLCA1, for chloride channel, calcium-activated), a calcium-regulated chloride channel from bovine tracheal epithelium, was reported in 1992 (19). The subsequent cloning of the gene...
encoding this channel identified it as a member of a new gene family (3). Recent additions to this gene family include a lung-endothelial cell adhesion molecule (Lu-ECAM-1) involved in organ-specific tumor metastasis (8), a murine Ca2+-regulated channel (mCLCA1) (10), and the human homologs hCLCA1, hCLCA2, and hCLCA3 (12, 13, 15).

A porcine ileal conductive chloride pathway has been under continued investigation in this laboratory. The kinetics of this pathway have been shown to be saturable (4), and the activity of the putative channel is sensitive to inhibition by α-phenylcinnamate (5), although it is relatively insensitive to typical chloride channel blockers such as SITS or anthracene-9-carboxylate (4). The conductance activity could be isolated in the constitutively open state, or in a closed state where it was activated by A-kinase stimulation (6). The development of an antibody that inhibited the chloride conductance activity (8) led to the identification of a 90-kDa brush-border protein as a candidate protein for this chloride channel (18).

The inhibitory monoclonal antibody developed by Racette et al. (18) was used to screen a porcine small intestinal cDNA expression library. This manuscript reports the detection of an antigen-producing clone and the elucidation of the complete cDNA coding for the antigenic species. The tissue expression of this gene was investigated to compare its expression with that of known chloride channels and to correlate this expression with the location of the antigen against which the antichloride conductive antibody had been raised.

MATERIALS AND METHODS

Materials. Restriction nucleases enzymes were obtained from New England Biolabs. Sequenase enzyme and reagents were from US Biochemicals (Amersham). Taq DNA polymerase was purchased from Promega, and Taq extender (Pfu polymerase) was from Stratagene. Spin columns for cleaning PCR products were from Qiagen. The vectors pcDNA2 and pcDNA 3.1 were Invitrogen products. Oligonucleotide primers for sequencing and PCR were synthesized by University Core DNA Services, University of Calgary. Other reagents and materials were of molecular biology grade. Tissue samples were obtained from 10- to 15-wk-old weanling pigs of mixed commercial breeding (Prairie Swine Center, University of Calgary). Bovine trachea and other tissues were obtained from undiseased freshly killed specimens (WCVM Prairie Diagnostic Laboratories). The pig ileal expression library was a gift from Dr. Brad Bosworth, National Animal Disease Center, Ames, IA.

Cloning. Antichloride conductance monoclonal antibody (18) was used to screen a porcine small intestinal cDNA library in the plasmid vector pcDNA2. Standard dideoxy DNA sequencing was carried out according to protocols supplied with USB Sequenase enzyme. Difficult-to-sequence GC-rich segments were cycle-sequenced by University Core DNA Services, University of Calgary. Sequence derived from a 943-bp initial positive clone was used to design a digoxigenin-labeled cDNA probe, which was then used to identify two slightly longer clones (1,031 and 1,164 bp) from the porcine library. No full-length copy was detectable in screening >106 colony-forming units (cfu) from human gut or pig lung cDNA libraries. A PCR-based cloning strategy subsequently identified a fourth clone from the porcine library. Sequences between a gene-specific antisense primer (5′-CTCCCAGGC-CCACACTTGTAC-3′) and the flanking Sp6 sense promoter primer (5′-GATTTAACCTATGACT-3′) were amplified with 2.5 U of Taq polymerase (Promega) and 2.5 U of Taq extender (Stratagene), under the following reaction conditions: denaturation, 94°C, 45 s; annealing, 52°C, 45 s; extension, 72°C, 1 min 30 s, for 40 cycles. Products obtained from PCR using a total of 5 × 105 cfu of porcine small intestinal library as template were then subcloned into an EcoRI V-cut pDK101 vector. The 5′ sequence of the gene was obtained by cloning a PCR product based on the pCLCA1-specific antisense primer (5′-CTCCCAGGCACCACACTTGTAC-3′) and a sense primer sequence from the 5′ end of hCLCA1 (5′-AGGAAGCTTAGGAGA-TGCAAGCAATG-3′) into pcDNA3.1 (12).

Northern blotting. Poly(A)+ RNA was purified from surgically derived porcine parotid salivary gland tissue samples using the QuickPrep Micro mRNA purification system (Pharmacia). Denatured mRNA was electrophoresed on a 1% formaldehyde-agarose gel, then transferred to Hybond N+ nylon membranes (Amersham) and prehybridized and hybridized at 65°C in 6× SSC, 5× Denhardt solution. Hybridization took place overnight under the same conditions, with the addition of 15 ng/ml digoxigenin-labeled probe (17).

RT-PCR analysis of porcine pCLCA1 expression. Total RNA was isolated by the standard acid guanidium thiocyanate-phenol-chloroform method (Trizol, GIBCO, Life Technologies) from surgically derived porcine tissue samples, then treated with DNase I. Following a denaturation for 5 min at 90°C, 5 μg of total RNA from each tissue were reverse transcribed for 30 min at 42°C with Moloney murine leukemia virus (MMLV) reverse transcriptase and the antisense primer 5′-AATGCCAGGAATGGT-3′. PCR amplification of the sequences between the antisense primer and the sense primer 5′-GGTGGATAACGGCAGTA-3′ was carried out for 30 cycles under the conditions described above for amplifying a cDNA fragment from the library, with the use of 1 μl of the reverse transcriptase reaction as a template.

Relative pCLCA1 abundance in pig ileal mRNA. Five micromgs of total RNA from porcine ileal mucosa was reverse transcribed with oligo(dT) primers for 30 min at 50°C using SuperScript II reverse transcriptase. pCLCA1 sequences were amplified from an amount of the reverse transcriptase reaction equivalent to 250 ng of total RNA using the sense primer 5′-GTAACCCGGATGTCTAGCTGTCCTAG-3′ and the antisense primer 5′-CGAGTTTGCTCTTATCAGAC-3′ (94°C, 45 s; 52°C, 45 s; 72°C, 1 min). Villin internal control sequences were PCR amplified from this template with 26 cycles using the antisense primer 5′-CTGAGTCTCACC CATGCGGT-3′ and the sense primer 5′-CAGCTGCCATCTCATGCGA-3′. PCR was carried out in parallel reactions using the cloned pCLCA1 cDNA as template. The amount of cDNA template used in the PCR reaction was adjusted to give a product of equal intensity to that obtained from the cDNA produced by reverse transcribing 250 ng of total RNA. DNA bands were quantitated using Gel Doc hardware and Quantity One software (Bio-Rad) after separation on 10% polyacrylamide gel electrophoresis and staining with ethidium bromide.

In situ hybridization. Formalin-fixed, paraffin-embedded tissue sections were thin-sliced onto 3-aminopropyltriethoxysilane (TESPA)-treated glass slides and dried overnight at 42°C. The samples were dewaxed in xylene and dehydrated in a graded series of ethanol baths before prehybridization. Protein hydrolysis was carried out through a 20-min incubation in 0.2 M HCl, followed by a 15-min incubation at 37°C in 20 μg/ml proteinase K. Tissues were postfixed for 20 min in 4% paraformaldehyde in PBS, then blocked for 10 min in a

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0.1 M triethanolamine in a 0.5% acetic anhydride solution before equilibration in 2× SSC.

Hybridization took place overnight at 50°C in 10 mM Tris-HCl (pH 7.6), 0.3 M NaCl, 5 mM EDTA, 10 mM DTT, 10% dextran sulfate, 50% formamide, 100 μg/ml yeast tRNA, 500 μM uridine 5′-a-thio)triphosphate containing 0.25 ng/ml 35S-labeled RNA probe. Antisense and sense probes were prepared from the 3′ 1,158 bp of pCLCA1 cDNA in a linearized plasmid vector, in an in vitro transcription reaction using the Sp6 RNA polymerase (Promega) and T7 RNA polymerase (GIBCO, Life Technologies).

Following hybridization, slides were washed twice for 30 min in 50% formamide, 2× SSC, and 10 mM 2-mercaptoethanol at 50°C, then exposed for 30 min to 20 μg/ml RNase A in 4× SSC/TE (TE is Tris-EDTA) at 37°C. Slides were washed once for 30 min in 4× SSC/TE, then once for 60 min in 50% formamide, 2× SSC, and 10 mM 2-mercaptoethanol at 50°C.

Finally, slides were dehydrated in a graded series of ethanol/35S-labeled cRNA probe. Antisense and sense probes were prepared from the 3′ 1,158 bp of pCLCA1 cDNA in a linearized plasmid vector, in an in vitro transcription reaction using the Sp6 RNA polymerase (Promega) and T7 RNA polymerase (GIBCO, Life Technologies).

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Comparative analysis of pCLCA1 and hCLCA1 expression.

Two micrograms total RNA purified from porcine postmortem tissue samples and 2 μg total RNA from human sources (Clontech) was reverse transcribed with oligo-dT for 30 min at 42°C using SuperScript II reverse transcriptase (GIBCO, Life Technologies). pCLCA1 sequences were PCR-amplified at 42°C using SuperScript II reverse transcriptase (GIBCO, Life Technologies). pCLCA1 sequences were PCR-amplified using the pCLCA1-specific antisense primer (5′-AGCAGTCCTTTGAGATTTAGC-3′) and the sense primer (5′-ACGATGCAAATGGTCGATACAG-3′) for two rounds of PCR (94°C, 45 s; 45°C, 45 s; 72°C, 1 min). hCLCA1 was amplified using the same sense primer as pCLCA1, paired with the hCLCA1-specific antisense primer (5′-CAAAATTCAGCCCTAGGC-3′) for two rounds of PCR (94°C, 45 s; 45°C, 45 s; 72°C, 1 min) in which a 1-μl volume of the first round reaction was taken after 30 cycles and used as a template for a further 25 cycles.

β-Actin control sequences were amplified from tissue total RNA reverse transcribed with oligo dT, then PCR amplified with the β-actin-specific antisense primer (5′-TAGATGGGCACAGTGTGGG-3′) and the sense primer (5′-GGCGT-GATGGGTGGCATGG-3′) for 30 cycles (94°C, 45 s; 54°C, 45 s; 72°C, 1 min) in which a 1-μl volume of the first round reaction was taken after 30 cycles and used as a template for a further 25 cycles.

Stable pCLCA1 transfectants. Mouse fibroblast 3T3 cells were transfected with the pCLCA1 clone in pcDNA3.1 (Invitrogen) by electroporation, and stable transfectants were selected by growth in genetin (400 μg/ml). pCLCA1 expression in transfected cell lines was verified by RT-PCR.

Chloride efflux studies. Transfected and control 3T3 cells cultured at 37°C with 5% CO2 in DMEM with 5% fetal calf serum were grown to confluence in 35-mm dishes. Efflux media contained 120 mM NaCl, 4 mM KCl, 1 mM KH2PO4, 2 mM MgCl2, 1 mM CaCl2, 10 mM HEPES, pH 7.4, and 5 mM glucose. Cells were prepared for chloride efflux studies by replacing normal growth medium with efflux media containing 36Cl2, 2 μCi/ml, and incubating at 37°C for 2 h. Efflux of 36Cl was measured at 24°C. Cells were washed four times to remove extracellular 36Cl, followed by changes in efflux medium at 2-min intervals. Ionomycin (10 μM) was added to efflux medium to study the Ca2+ dependence of chloride efflux.

RESULTS

Cloning of pCLCA1. Initial screening of the porcine intestinal expression library with monoclonal antibod-

ies 1C2 and 2H6 (18) resulted in the isolation of a partial cDNA clone expressing polypeptide that reacted with these antibodies in a Western blot assay. Complete sequencing indicated that the size of the cDNA insert in this clone was 943 bp, with a continu-
ous open reading frame for the first 700 bases. Re-
screening of the porcine library with a digoxigenin-
labeled DNA probe resulted in the isolation of an overlapping 1,146-bp clone containing the same 3′ end of the cDNA.

A 918-bp fragment comprising the middle segment of the cDNA sequence was obtained by a PCR-based cloning strategy using mixed Taq and high-fidelity Pfu DNA polymerases. Inserts were amplified from the porcine intestinal cDNA library using a vector-specific sense primer and a gene-specific antisense primer that gave 250 bp of overlap with the 5′ end of the longer library clone. The PCR product was ligated into a pGEM-5ZF plasmid and sequenced, showing that it was 918 bp in length, and provided 600 bp of new sequence at its 5′ end.

The extreme 5′ end of pCLCA1 coding sequence was cloned in a parallel project by reverse transcribing porcine ileal mRNA with a gene-specific antisense primer positioned 196 bp from the 5′ end of the middle segment of cDNA. The reverse transcriptase cDNA was

Fig. 1. Northern blot demonstrating hybridization of digoxigenin-labeled probe to porcine parotid salivary gland mRNA. Samples electrophoresed on a denaturing gel were transferred to Hybond N+ nylon membranes and probed. A: RNA standards (9.49, 7.46, 4.40, 2.37, 1.35, and 0.24 kb). B: Northern blot. Lane 1, 20 μg porcine parotid mRNA. Lane 2, linearized pCLCA1c clone in pcDNA2. Lane 3, PCR-amplified DNA product from pCLCA1c clone corresponding to the probe sequences (positive control).
amplified in PCR using a sense primer spanning the initiation codon in the human hCLCA1 sequence (12). The resulting 5′ segment of the cDNA was then cloned into pcDNA3.1. Sequencing showed that it was 1,514 bp in length, with the predicted 196-bp overlap with the middle segment. Independent sequencing of three clones of each of the two cDNA segments generated by high-fidelity PCR was used to confirm the complete coding sequence for the pCLCA1 protein.

Attempts to obtain an estimate of transcript size by Northern analysis of ileal poly(A)1 RNA were unsuccessful, probably because of the difficulties of completely inhibiting pancreatic ribonuclease activity on the intestinal mucosal surface. Poly(A)+ RNA was therefore purified from porcine parotid salivary gland to estimate the size of the full-length pCLCA1 transcript. Based on this Northern blot analysis, the size of the pCLCA1 message was 2.9 ± 0.3 kb (Fig. 1).

**Predicted protein structure.** The full pCLCA1 sequence includes a 3,079-bp cDNA, of which a 2,751-base open reading frame encodes a 917-amino acid protein product with a predicted molecular mass of 100.7 kDa. There were no database homologues when the original pCLCA1a clone was obtained. With the release of the bCLCa1 sequence it became apparent that the clone was related to the gene product from bovine tracheal epithelium encoding a calcium-regulated chloride channel (3). Subsequent identification of ECAM-1 and the human hCLCA1 and hCLCA2 sequences revealed shared homology with the porcine intestinal pCLCA1 protein (7, 12, 13). A phylogenetic alignment diagram constructed from the predicted amino acid sequence of the CLCA family members indicates that pCLCA1 is most closely related to hCLCA1 (Fig. 2), a relationship that is supported by a comparison of the hydrophathy plots of the two predicted proteins.

Predicted pCLCA1 and hCLCA1 proteins share 78% amino acid identity, and have similar cleavable signal sequences (Fig. 3). The regions with the largest number of amino acid differences include the amino terminus area from K181 to S232, and the proposed cytosolic loop lying between transmembrane domains 3 and 4. The hydrophobic similarities between pCLCA1 and hCLCA1 (Fig. 2) indicate a significant probability that these proteins share a common transmembrane topology. Based on localization of epitope tags in hCLCA1, this topology has been suggested to consist of an extracellular NH2 terminus for the protein, followed by four transmembrane domains and a hydrophobic COOH terminus (12).

The predicted amino acid sequence contains a potential amidation cleavage site at residue 140 that is conserved between pCLCA1 and hCLCA1, but is not present in other currently known CLCA family sequences. The pCLCA1 sequence contains 14 potential C-kinase phosphorylation sites, with 10 of the 14 sites conserved between pCLCA1 and hCLCA1. Four of the conserved sites, at residues T68, S84, T98 and S232 lie in the predicted extracellular amino terminus of the...
protein. Four more conserved C-kinase sites, plus two sites unique to pCLCA1, are found in the predicted cytosolic loop that lies between TM domains 3 and 4.

There are two potential phosphorylation sites for protein kinase A and cGMP-dependent protein kinase in pCLCA1. The conserved site at residue T286 with the weak consensus sequence KKTT is located in the predicted extracellular amino terminus of the protein. The strong A-kinase consensus site at residue S587 is unique to pCLCA1. It is located in the predicted TM3-TM4 cytosolic loop that also contains the highest localized concentration of C-kinase sites. There are four consensus sites for calcium/calmodulin protein kinase II (Ca/MK II). Two of these sites are conserved between pCLCA1 and hCLCA1, although one of the conserved sites overlaps with the predicted monobasic proteolytic cleavage site at residue R662. The other conserved monobasic proteolytic cleavage site is present at K720; both of these sites are located in the predicted COOH-terminal extracellular loop. The predicted pCLCA1 protein also contains 6 consensus sites for N-linked glycosylation. These six sites are conserved between pCLCA1 and hCLCA1, and all six lie in predicted extracellular regions of the protein [hCLCA1 contains two additional N-linked glycosylation sites at N587 (cytosolic) and N835].

Porcine tissue expression of pCLCA1. The presence of pCLCA1 mRNA in pig ileal total RNA samples was demonstrated by RT-PCR, confirming the expression of this gene in the tissue originally used as a source of antigen for the antichloride conductance antibody (Fig. 4). Specific amplification of pCLCA1 cDNA was obtained through the use of a reverse transcriptase primer with sequence specific to a unique region of the pCLCA1 3’ untranslated sequence. Unlike the broader expression of hCLCA1 and the murine gene gob-5, expression of pCLCA1 may be restricted to specific regions within the gastrointestinal tract. pCLCA1

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message was not detected by RT-PCR of total RNA samples from large intestinal mucosa or stomach. pCLCA1 message was detected in porcine tracheal epithelia, but not in samples taken from epithelia of the large bronchi or from lung parenchyma (Fig. 4).

Investigation of pCLCA1 expression in other porcine exocrine tissues demonstrated the presence of pCLCA1 mRNA in the parotid, sublingual, and submandibular salivary glands. Surprisingly, however, considering the classically exocrine nature of the pancreas, no pCLCA1 message was detected in this tissue (Fig. 4). Investigation of pCLCA1 expression in a number of other tissues known to express chloride channels failed to detect pCLCA1 mRNA in total RNA samples from heart, skeletal muscle, liver, and kidney (data not shown).

In situ hybridization with 35S-labeled antisense cRNA probes confirmed the results of the RT-PCR studies. Tracheal expression of pCLCA1 was localized to the surface epithelium and in the underlying submucosal glands (Fig. 5A). Expression of pCLCA1 message appeared to be highest in the glands themselves, rather than in the epithelium lining the collecting ducts opening onto the tracheal surface. No signal was observed elsewhere within the tracheal tissue sections, or in the sense control sections (not shown).

Epithelial cells in both the ileal crypts and villi (Fig. 5, B and C) exhibited a diffuse but moderately strong signal, indicative of pCLCA1 expression throughout this region. No gradient of distribution was apparent along the crypt-villus axis. Extremely strong signal was, however, associated with isolated epithelial cells scattered throughout the villus. The lamina propria, muscularis mucosae, and submucosal regions were all free of specific signal, as were the sense control sections (not shown). Consistent with the results of the RT-PCR studies, specific signal was not detected following hybridization in pulmonary, colonic, pancreatic, or cardiac muscle sections (not shown).

Relative abundance of pCLCA1 mRNA in ileal mucosa. Investigations of the relative abundance of pCLCA1 mRNA in ileal RNA samples determined by semi-quantitative RT-PCR, and based on the amplification of an internal standard in the PCR indicated that the relative ileal mucosal abundance of pCLCA1 mRNA was between 1 and 5 amol/μg total RNA. This abundance is approximately ten times the level of CFTR mRNA reported in nasal biopsy samples by Beck et al. (1), or 10 to 50 molecules of pCLCA1 mRNA per cell.

Effect of pCLCA1 expression on chloride efflux from mouse 3T3 fibroblast cells. Stable pCLCA1 transfectants were produced in 3T3 fibroblasts by electroporation and selection with geneticin. Stably transfected 3T3 cells, containing pCLCA1 under the control of the CMV promoter expressed significant levels of pCLCA1 mRNA as detected by RT-PCR. A high expressing transfected lines maintained with geneticin selection was used to measure rates of release of 36Cl. Confluent monolayers of 3T3 cells contained ~500 nmol of Cl−/mg cell protein after equilibration in a loading

Fig. 4. Tissue expression of pCLCA1 as determined by RT-PCR. Five micrograms total RNA were reverse transcribed with MMLV reverse transcriptase and gene-specific primer (see MATERIALS AND METHODS), PCR-amplified for 30 cycles using internal primers, and then visualized in a 0.8% agarose gel containing 0.25 μg/ml ethidium bromide. a: Intestinal and airway expression of pCLCA1. Lane 1, pBR322-BstN I standards (1,857, 1,058, 929, 383, and 121 bp); lane 2, ileum; lane 3, colon; lane 4, trachea; lane 5, bronchi; lane 6, lung; lane 7, pCLCA1-positive control; lane 8, no template control. b: Pancreatic and salivary gland expression of pCLCA1. Lane 1, pBR322-BstN I standards (1,857, 1,058, 929, 383, and 121 bp); lane 2, pancreas; lane 3, parotid gland; lane 4, submandibular gland; lane 5, sublingual gland; lane 6, pCLCA1-positive control; and lane 7, no template control.
Fig. 5. Bright-field photomicrographs demonstrating in situ hybridization of a $^{35}$S-labeled pCLCA1 antisense cRNA probe in porcine tissue sections. Tracheal epithelium and submucosa with arrows identifying submucosal glands (A), ileal villi with arrows indicating location of a crypt (B), and ileal villus tip (C).
solution containing 130 mM Cl\(^{-}\). The average initial rate of chloride efflux/exchange from cells loaded with \(^{36}\)Cl was about 20 nmol·min\(^{-1}·mg\) protein\(^{-1}\). This basal efflux/exchange rate was similar in untransfected 3T3 cells (not shown) and in transfected 3T3 cells expressing pCLCA1 (Fig. 6). Hence there was no evidence for a change in basal chloride conductance activity in 3T3 cells expressing the pCLCA1 protein. Addition of 10 \(\mu\)M ionomycin to the exchange media increased initial chloride efflux/exchange rates to \(\sim 55\) nmol·min\(^{-1}·mg\) cell protein\(^{-1}\) in transfected 3T3 cells expressing pCLCA1 (Fig. 6). Increased chloride efflux rates in cells expressing pCLCA1 and treated with ionomycin correlated with a reduced content of \(^{36}\)Cl remaining in these cells at the end of a 14-min efflux study. This increased chloride efflux rate appears to reflect the activation of a calcium-dependent chloride conductance activity in the cytoplasmic membrane of 3T3 cells expressing pCLCA1. At 2, 4, and 6 min after treatment with 10 \(\mu\)M ionomycin the \(^{36}\)Cl efflux rate from untransfected 3T3 cells was indistinguishable from the efflux rate observed in transfected cells that did not receive ionomycin.

Expression of pCLCA1 and hCLCA1 mRNA in selected tissues. Differences in nucleic acid sequence of cDNA coding for pCLCA1 and for hCLCA1 could be a result of sequence divergence paralleling human and porcine population evolution. Alternatively, sequence differences could reflect gene duplication and independent evolution of two related genes at a stage in mammalian evolution preceding porcine-human speciation events. The possibility that pCLCA1 and hCLCA1 represent two closely related, but independent genes that are both expressed in some exocrine tissues was investigated by reverse-transcriptase PCR. Total RNA samples from tracheal epithelium, small intestine and submandibular salivary gland were obtained from fresh porcine tissues. Human RNA from the same tissues was obtained from a commercial source. The integrity of mRNA in the pig and human total RNA samples was investigated in reverse transcription reactions primed with oligo-dT and used as a template in a PCR reaction containing primers designed to amplify a fragment of \(\beta\)-actin cDNA. Primer pairs were also chosen to amplify an 884-bp product from pCLCA1, and a 767-bp product from hCLCA1 cDNA. Specificity of amplification of the CLCA1 species was determined by the use of antisense primers specific to unique cDNA sequence present in the 3’ untranslated region of the two cDNAs.

The \(\beta\)-actin and the hCLCA1 cDNA fragments amplified in these PCR reactions are shown in Fig. 7. Integrity of the mRNA in the three total RNA samples from human tissues and the three samples from the corresponding pig sources is indicated by the \(\beta\)-actin band migrating below the 0.5-kb marker. Expression of hCLCA1 mRNA is shown in human small intestinal epithelium, and in porcine tracheal and small intestinal epithelium (Fig. 7a). Results of parallel PCR amplifications carried out using the same reverse transcriptase reactions as template, but employing the primer pair specific for amplification of an 884 base-pair fragment of pCLCA1 are shown in Fig. 7b. pCLCA1 expression was highest in porcine tracheal and small intestinal epithelium, with lower levels detected in submandibular salivary gland. pCLCA1 expression has been observed in some RT-PCR reactions from human tracheal and ileal RNA, but there was no detectable PCR product in the reactions shown in Fig. 7b. These results suggest that porcine trachea and ileum express the highest levels of pCLCA1 and hCLCA1 of the six tissue sources examined in this study. Unique detection of hCLCA1 in human ileal tissue, and pCLCA1 in porcine submandibular salivary gland provides some evidence that the RT-PCR procedure is able to distinguish between species-specific variants of the same gene.

To summarize, mRNA species coding for two related calcium-regulated chloride channels, pCLCA1 and hCLCA1, are co-expressed in porcine trachea and small intestine. However, differences in expression patterns in human ileum and porcine submandibular salivary gland provide evidence for the existence of separate species of mRNA coding for two related chloride channels.

**DISCUSSION**

Attempts to connect the chloride conductance activity in ileal brush-border membranes with a specific protein have led to the identification of the pCLCA1 gene. The pCLCA1 sequence is most highly homologous to that of hCLCA1, with which it shares a 78% amino acid identity, and bears lower but still significant homology to other reported members of the family.
Motifs that are conserved between the predicted protein products of pCLCA1 and the other genes include a number of cysteine residues upstream of the first potential transmembrane domain, as well as four hydrophobic, potentially transmembrane domains. The multiple consensus sites for N-linked glycosylation are also conserved, and are consistent with the findings of immunoprecipitation studies conducted on intestinal brush-border membrane proteins (18).

Two conserved consensus sites for monobasic proteolytic cleavage are present in both pCLCA1 and hCLCA1, while other CLCA proteins contain only one site. The processing of a single larger protein product into multiple subunits was first documented in Lu-ECAM-1 (7), and a similar pattern of posttranslational processing has been proposed to occur in hCLCA1, hCLCA2 and the murine mCLCA1 (10, 12, 13). Protein protection-labeling studies conducted on porcine ileum had previously identified pCLCA1 candidate proteins in the molecular mass regions of 130 and 23 kDa (9). In porcine enterocytes treated with the glycosylation inhibitor tunicamycin, immunoprecipitation of brush-border membrane proteins with an anti-chloride conductance antibody identified both a 90 kDa protein and two smaller species under 30 kDa (18). The results of these studies are consistent with processing of the pCLCA1 protein product occurring posttranslationally, in a similar fashion to other members of the family.

pCLCA1 also contains multiple consensus sequences for phosphorylation by serine/threonine protein kinases. The predominance of consensus sites for protein kinase C in pCLCA1 is consistent with the calcium-sensitivity exhibited by CLCA proteins that have been shown to be chloride channels. The strong protein kinase A consensus site unique to pCLCA1 at S587 lies in a putative intracellular domain. The physiological relevance of this site is supported by cAMP activation of the conductance pathway associated with pCLCA1 (5). Although attention has focussed on calcium regulation of the CLCA family, other protein members of the family contain potential A-kinase phosphorylation sites. The regulation of channel gating in the CLCA family by multiple and alternative pathways could be worthy of investigation.
The current topology model for hCLCA1 puts the first four conserved C-kinase consensus phosphorylation acceptor sites in the NH₂-terminal extracellular loop, which is inconsistent with a regulatory role in channel gating. However, the same topology model predicts that the three conserved C-kinase sites plus the A-kinase site can influence channel gating from their location in a putative intracellular domain between TM3 and TM4. The model also predicts that all six of the N-linked glycosylation consensus sites conserved between h and pCLCA1 have an extracellular location.

Three of the five Ca/M KII sites in pCLCA1 are conserved with hCLCA1. Overlap of the site at T665 with the first of the two monobasic proteolytic cleavage sites at R662 indicates a potential modulatory role for Ca/M KII kinase in the cleavage of the COOH-terminal fragment. The predicted extracellular location of this T665 site and the other two conserved Ca/M KII sites in the extracellular COOH-terminal region of the protein makes these sites, like the NH₂-terminal C-kinase sites, unlikely candidates for involvement in channel gating.

The relatively homologous murine gob-5 gene, hCLCA1, and pCLCA1 (12, 16) all show some expression in the gastrointestinal tract. Gastrointestinal expression of gob-5 occurs predominantly in small intestinal goblet cells, in colonic mucous neck cells, and in pyloric surface epithelium (12). Gob-5 is not universally expressed in gastrointestinal mucus-secreting cells, however, as message has not been detected in salivary glands or in mucous neck cells elsewhere in the stomach (12). hCLCA1 expression, on the other hand, is restricted to the mucosa of the small and large intestine, although like gob-5, the highest expression is seen in goblet cells (16). Strong pCLCA1 expression was detected in the parotid salivary glands and the mucosal epithelium of the small intestine, with particularly high levels of pCLCA1 message observed in scattered cells throughout the villi, consistent with the location of goblet cells. However, expression was not detected in stomach or colon.

Expression of pCLCA1 in porcine airways was restricted to the trachea and could not be detected in the large bronchi or the lungs, either by in situ hybridization or by the more sensitive RT-PCR technique. Other members of the family that are expressed in the airways include bCLCA1, hCLCA2, hCLCA3, and mCLCA1 (13–15). Because these chloride channels possess distinctive tissue distributions, it seems likely that they are different proteins with functional differences from pCLCA1. Independent detection of pCLCA1 and its closest human homolog, hCLCA1, in tracheal and small intestinal mucosal RNA samples from pig and human argues for the coexistence of related forms of the CLCA1 chloride channel family in the mammalian genome. The presence of multiple CLCA homologs emphasizes the need for careful, gene-specific primer design in investigations of tissue expression patterns using RT-PCR.

The use of DNA standards in “quantitative” RT-PCR would be expected to underestimate the amount of mRNA due to efficiency losses in the reverse transcriptase reaction. Hence, the finding of 10 to 50 molecules of pCLCA1 mRNA per cell may give some indication of a relatively significant role of pCLCA1 in bulk chloride ion passage across the intestinal brush-border membrane. The potential role of proteins that interact with that antibody was indicated by inhibition of nearly 100% of conductive ⁸⁶Cl⁻ uptake by anti-pCLCA1 monoclonal antibody in functional ileal brush-border vesicle assays (18). The brush-border vesicles used in those studies were prepared by homogenizing ileal mucosal scrapings in buffer containing 10 mM Ca²⁺ to promote precipitation of non-brush-border components (18). Those preparation conditions were appropriate for the activation of a Ca²⁺-dependent chloride conductance channel.

The ionomycin-dependent enhancement of chloride efflux from 3T3 cells expressing pCLCA1 indicates that expression of the protein was associated with chloride conductance activity in this tissue culture model. Clarification of an in vivo role of pCLCA1 in intestinal chloride conductance will require additional studies of functional expression and electrophysiological characterization of the pCLCA1 gene product.

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