Proteins of the CLCA gene family have been proposed to mediate calcium-activated chloride currents. In this study, we used detailed bioinformatics analysis and found that no transmembrane domains are predicted in hCLCA1 or mCLCA3 (Gob-5). Further analysis suggested that they are globular proteins containing domains that are likely to be involved in protein-protein interactions. In support of this, we used bioinformatics analysis, biochemical studies showed that hCLCA1 and mCLCA3, when expressed in HEK293 cells, could be removed from the cell surface and could be detected in the extracellular medium, even after short incubation times. The accumulation in the medium was shown to be brefeldin A-sensitive, demonstrating that hCLCA1 is constitutively secreted. The N-terminal cleavage products of hCLCA1 and mCLCA3 could be detected in bronchoalveolar lavage fluid taken from asthmatic subjects and ovalbumin-challenged mice, demonstrating release from cells in a physiological setting. We conclude that hCLCA1 and mCLCA3 do not contain any transmembrane domains and therefore cannot be chloride channels in their own right.

Many cells are known to possess calcium-dependent chloride channel activities. These include epithelial cells and smooth muscle cells (1, 2). Although we know little about the identity of these channels, members of the CLCA gene family have been suggested to be candidate calcium-sensitive chloride channels (3–5).

Although normally expressed in the gastrointestinal tract, up-regulation of the human calcium-activated chloride channel hCLCA1 has been linked to disease states such as asthma and cystic fibrosis (6–9). This up-regulation was observed in bronchial epithelial cells and goblet cells. In the mouse, the ortholog of hCLCA1 (mCLCA3) exhibits a similar expression profile to hCLCA1 and, in addition, has been localized to mucin granules (10). The expression of mCLCA3 is up-regulated in mouse lung in response to ovalbumin challenge or upon challenge with more complex allergens such as Aspergillus fumigatus, systems that are utilized to model aspects of human asthma (11, 12).

Evidence for the CLCA family as calcium-sensitive chloride channels comes from heterologous expression of a number of CLCA isoforms in a range of cellular systems, which resulted in generation of membrane currents activated with high Ca$$^{2+}$$ concentrations or with ionomycin. These currents were blocked with chloride channel blockers such as niflumic acid and were performed in Cl$$^{-}$$ selective conditions (3, 4).

Even in light of this evidence, questions still remain as to whether members of the CLCA family are themselves responsible for the chloride channel activity, or whether they are regulating the activity of an as yet unidentified ion channel. Calcium-sensitive chloride channels have been measured in cells in which expression of CLCA isoforms could not be detected (13). CLCA family members have also been linked to functions other than that of ion channels. For example, mCLCA3 has been suggested to control mucus production (10). hCLCA2, which is expressed in pulmonary endothelial cells, has been shown to mediate binding of tumor cells via its interaction with $b4$ integrin (14). Furthermore, at least one CLCA isoform (hCLCA3) has been demonstrated to be a secreted protein (15). hCLCA3 is a truncated isoform with no predicted transmembrane domains. CLCA family members are proteolytically cleaved proteins, and there is controversy over which region of hCLCA1 is responsible for the proposed channel activity (16, 17). Hydrophobicity analysis of hCLCA1 has suggested the presence of four transmembrane domains. This observation was supported by studies using epitope-tagged hCLCA1 to identify the intracellular and extracellular domains of the protein (3). To date, no studies have confirmed these observations with untagged hCLCA1 or within a physiological setting.

Contrary to previous suggestions, we report here that hCLCA1 and mCLCA3 do not contain any transmembrane domains and that hCLCA1 is secreted into the extracellular medium when overexpressed. In support of this finding, we were able to identify hCLCA1 and mCLCA3 in the bronchoalveolar lavage (BAL) of asthmatic and of ovalbumin-challenged mice, respectively. These results suggest that the ion channel activity associated with these proteins is likely to be due to a regulatory function and that hCLCA1 could not possess endogenous, calcium-sensitive chloride channel activity.

**EXPERIMENTAL PROCEDURES**

**Bioinformatics Analysis**—Transmembrane analysis was carried out using the programs TMHMM (18), HMMTOP (19), TMAP (20), SOSUI (21), and DAS (22). The von Willebrand factor type A (VWA) domain prediction and analysis was carried out using InterProScan (23), SMART (24), Pfam (25), and PROSITE (26) data bases. The fibronectin type III (FnIII) domain was identified using BLAST (27) and also when searched against the domain data bases Pfam and SMART, using InterProScan. The FnIII domain assignment was confirmed by generating a Hidden Markov Model for this region from a CLCA family protein multiple sequence alignment and searching it against the SCOP Superfamily Hidden Markov Model data base (28) using HHsearch (29). The N-terminal signal sequence was predicted using SignalP (30).
Antibodies and Reagents—A48 anti-hCLCA1 antibody is a rabbit polyclonal antibody raised against the peptide sequence (GYPNAR-RKYIPQQS(C)), corresponding to amino acid residues 681–683 in hCLCA1. The A6637 anti-mCLCA3 rabbit polyclonal antibody was raised against the peptide sequence (GLRTAFTVIKPKYPTDGKS), corresponding to amino acid residues 388–406 in mCLCA3 (produced by Invitrogen). Both antibodies were affinity purified. Horseradish peroxidase (HRP)-conjugated anti-V5 mouse monoclonal antibody (Invitrogen) was used to detect the V5 protein tag. Mouse monoclonal antibody 9E10 was used to detect the Myc protein tag (Calbiochem; Merck Biosciences Ltd., Nottingham, UK). Secondary antibodies used for Western blots were HRP-conjugated goat anti-rabbit IgG (Calbiochem) and HRP-conjugated goat anti-mouse IgG (Sigma-Aldrich). Secondary antibodies used for immunofluorescence were goat anti-rabbit IgG (AlexaFluor 568, goat anti-mouse IgG-AlexaFluor 568, and goat anti-mouse IgG-AlexaFluor 488 (all from Molecular Probes). Secondary antibody used for immunogold labeling was goat anti-rabbit IgG conjugated to 5 nm of colloidal gold (British BioCell International, Cardiff, UK).

Plasmid Constructs—Myc-tagged hCLCA1 (Myc-hCLCA1) was generated as described by Gruber et al. (3) and was a gift from Dr. Bendicht Pauli. The construct used for these studies had the Myc tag placed between amino acids 366 and 367. hCLCA1-V5-His construct (pcDNA3.1-V5-His-TOPO-hCLCA1) was obtained from Invitrogen. The pcDNA3.1-V5-His-TOPO-mCLCA3 (pcDNA3.1-V5-His-TOPO-mCLCA3) was generated using full-length hCLCA1 (PubMed accession number AF270306) with a short Kozak sequence inserted prior to the initiation methionine. The pcMCLCA construct (pcDNA3.1-V5-His-TOPO-mCLCA3) was generated using full-length mCLCA3 (PubMed accession number AB017156). Full-length mCLCA3 was amplified from in-house mouse lung cDNA templates with F3/F4 turbo hot start and cloned into pcDNA3.1 using TOPO technology (Invitrogen). For generation of the HEK293 clone stably expressing hCLCA1, pCIN5-hCLCA1 was transfected into pCIN5_p1 vectors were from Invitrogen. For generation of the HEK293 clone of hCLCA1-V5-His, Myc-tagged hCLCA1, mCLCA3, or GFP transiently transfected with hCLCA1-V5-His, Myc-tagged hCLCA1, mCLCA3, or GFP in the presence or absence of 0.8 mg/ml Geneticin. HEK293 cells were transiently transfected in 1% non-essential amino acids (all from Invitrogen). HEK293 inactivated fetal bovine serum; 2% penicillin, streptomycin, and glutamine (all from Invitrogen). PNS (5 mM MgCl2, and 1 mM ATP) (Sigma) containing HALT protease inhibitor mixture for Western blot analysis. Medium samples were spun at 1500 rpm for 5 min to remove any cells, supernatants were trichloroacetic acid-precipitated, and pellets were resuspended in 100 l of radioimmunoprecipitation assay buffer (Upstate, Dundee, UK) containing HALT protease inhibitor mixture for Western blot analysis. Medium. Samples were spun at 1500 rpm for 5 min to remove any cells, supernatants were trichloroacetic acid-precipitated, and pellets were resuspended in 100 l of PBS to enable equivalent loading of lanes on the gel. For the electron microscopic immunogold labeling, cells grown on poly-L-lysine-coated, 6-well plates were treated as described above. For both immunochemistry procedures, cells were then washed five times with 1 ml of PBS containing 1% bovine serum albumin and then immunolabeled as described below.

Time Course of hCLCA1 Release and Brefeldin A Treatment—HEK-hCLCA1 cells, grown on 6-well plates, were washed five times with 1 ml of serum-free medium, followed by incubations of 1–4 h in the last 30 min of each time-point with 15 l of PBS, 15 l of protein from the pH 11 wash, and the equivalent volume of the other washes were loaded per lane on the gel. For the corresponding immunofluorescence, cells grown on poly-L-lysine-coated coverslips were washed twice in Hanks’ balanced salt solution, followed by a wash in either PBS or pH 11 alkaline wash for 30 min at 4 ºC. For the electron microscopic immunogold labeling, cells grown on poly-L-lysine-coated, 6-well plates were treated as described above. For both immunochemistry procedures, cells were then washed five times with 1 ml of PBS containing 1% bovine serum albumin and then immunolabeled as described above.

Membrane Stripping—HEK-hCLCA1 cells were detached from a T75 flask (~1 × 107 cells) with Versene, washed twice with Hanks’ balanced salt solution (Invitrogen), and split into three equal volumes. Cells were pelleted and resuspended in 200 l of either PBS, pH 7.4; pH 2.5 and 0.9% NaCl, adjusted to pH 2.5 with acetic acid); or pH 11 alkaline wash (0.1 mM sodium carbonate, pH 11) (33) for 20 min at 4 ºC. Cells were removed by spinning at 1500 rpm for 5 min, and the supernatant was then spun at 40,000 × g for 30 min at 4 ºC in a Beckman TL-100 ultracentrifuge to ensure removal of all cellular membranes. 150 l of supernatants were trichloroacetic acid-precipitated and resuspended in 150 l of PBS. 15 l of protein from the pH 11 wash, and the equivalent volume of the other washes were loaded per lane on the gel. For the corresponding immunofluorescence, cells grown on poly-L-lysine-coated coverslips were washed twice in Hanks’ balanced salt solution, followed by a wash in either PBS or pH 11 alkaline wash for 20 min at 4 ºC. For the electron microscopic immunogold labeling, cells grown on poly-L-lysine-coated, 6-well plates were treated as described above. For both immunochemistry procedures, cells were then washed five times with 1 ml of PBS containing 1% bovine serum albumin and then immunolabeled as described below.

Time Course of hCLCA1 Release and Brefeldin A Treatment—HEK-hCLCA1 cells, grown on 6-well plates, were washed five times with 1 ml of serum-free medium, followed by incubations of 1–4 h in the last 30 min of each time-point with 15 l of PBS, 15 l of protein from the pH 11 wash, and the equivalent volume of the other washes were loaded per lane on the gel. For the corresponding immunofluorescence, cells grown on poly-L-lysine-coated coverslips were washed twice in Hanks’ balanced salt solution, followed by a wash in either PBS or pH 11 alkaline wash for 20 min at 4 ºC. For the electron microscopic immunogold labeling, cells grown on poly-L-lysine-coated, 6-well plates were treated as described above. For both immunochemistry procedures, cells were then washed five times with 1 ml of PBS containing 1% bovine serum albumin and then immunolabeled as described below.
\textbf{RESULTS}

\textit{Bioinformatics Analysis Predicts No Transmembrane Regions for hCLCA1 and mCLCA3}—Proteins of the CLCA family have been described as integral membrane proteins with either four or five transmembrane domains (3, 15, 36, 37). Our own analysis, using five different programs, did not predict either hCLCA1 or mCLCA3 to possess any \(\alpha\)-helical transmembrane domains. The CLCA family was further analyzed to determine the potential domain structure. A VWA domain is predicted in the central region of the protein. The majority of well-characterized VWA domains are found in cell adhesion and extracellular matrix proteins (38) and thought to be involved in protein-protein interactions, frequently involving divalent cations. However, more distantly related VWA domains have also been found in intracellular proteins, many being components of multi-protein complexes. Approximately half of all VWA domains contain a MIDAS (metal ion-dependent adhesion site) motif (39), which contains key residues required for metal-ion binding. These key residues are all conserved in the VWA domains of hCLCA1 and mCLCA3, suggesting metal-ion binding of this motif in these proteins. The second transmembrane domain previously described (3, 15, 36, 37) for CLCA is located within the globular VWA domain, which thus supports the results of the transmembrane prediction algorithms that this is in fact not a transmembrane domain. An \(\sim 100\)-residue region toward the C terminus of the CLCA family was predicted to be an FnIII domain. FnIII domains are found in a variety of proteins, the majority of which are involved in cell surface binding in some manner or are receptor protein tyrosine kinases or cytokine receptors (40). The remaining regions of the CLCA family do not appear to show obvious similarities to any functionally annotated domains, although the region located between the VWA and FnIII domains, which is predicted to have an all-\(\beta\) secondary structure composition, does show homology to a protein (UniProt-Q8P8U63) in an archaeabacterium, \textit{Methanosarcina mazei}. This protein also possesses the VWA and FnIII domains, but N-terminal of the VWA domain is a CHAP (cysteine, histidine-dependent amidohydrolases/peptidases) domain, which appears to be absent in the CLCA family. This region, situated between the N-terminal signal sequence and the VWA domain, instead contains eight cysteine residues conserved across the CLCA family and is predicted to have an \(\alpha/\beta\) composition (Cys-containing domain). The very C-terminal region of the CLCA family does not appear to be conserved in sequence between family members. This extensive analysis predicts that the CLCA proteins have a globular domain structure, a summary of which is shown in Fig. 1, for hCLCA1 and mCLCA3.

\textit{hCLCA1 Is Strongly Associated with Cell Membranes but Can Be Removed from the Cell Surface}—We examined the nature of the association of hCLCA1 with cell membranes, using biochemical methods and Western blot analysis (Fig. 2A). Following the disruption of HEK-hCLCA1 cells, the nuclei were pelleted. The PNS was found to contain both full-length protein (125 kDa) and the N-terminal cleavage product of hCLCA1. In our studies, the N-terminal cleavage product, previously reported to be 90 kDa (3), appears to migrate at 83 kDa. The PNS was centrifuged to pellet cell membranes, including intact organelles. Full-length and processed hCLCA1 were present in the membrane pellet, whereas no hCLCA1 was detected in the supernatant, representative of the cytosol. The membrane/organelle pellet was subjected to phase separation with Triton X-14 detergent (32). Full-length hCLCA1 partitioned into the aqueous phase, as did a significant proportion of the N-terminal cleavage product. This observation suggested that hCLCA1 is not an integral membrane protein.

The A48 antibody, raised against an epitope corresponding to amino acid residues 681–693, recognizes the N-terminal cleavage product. Several possible cleavage sites have been proposed for hCLCA1 (3). One of these sites, situated between amino acids 660 and 661, can now be ruled out because the A48 epitope is downstream of that position.

Immunofluorescent labeling of hCLCA1 on non-permeabilized HEK-hCLCA1 cells revealed that hCLCA1 was expressed at the cell surface and could be detected on the external surface with A48 antibody (Fig. 2C). Utilizing high or low pH solutions as stripping agents has been shown to remove membrane-associated proteins from cell membranes, whereas integral membrane proteins remain in the lipid bilayer (33). This technique was used to determine whether the hCLCA1 plasma membrane association could be disrupted by pH stripping of the HEK-hCLCA1 cells.

Western blot analysis of cells subjected to a range of washing conditions (Fig. 2B) showed that PBS removed some hCLCA1 from the cell surface. More was removed with pH 2.5 wash, whereas pH 11 wash released significant amounts of both full-length and N-terminal processed protein. The ability to remove these two forms of hCLCA1 from the plasma membrane suggests that both the full-length protein and N-terminal cleavage product have no transmembrane domain.

Immunofluorescence detection of hCLCA1 on cells treated with PBS (Fig. 2C) or pH 11 wash (Fig. 2D) revealed that surface expression on the pH 11-washed cells was still evident, despite the removal of a considerable amount of hCLCA1. The cells were seen to remain intact following the pH 11 wash (Fig. 2, D and F), demonstrating that they were not damaged by this treatment and therefore that the hCLCA1 detected in the wash came only from the cell surface. These data suggest that stringent washing removed less tightly bound hCLCA1, whereas that remaining could be bound to an interacting protein anchored in the membrane. Electron microscopic examination of...
surface labeled cells demonstrated immunogold labeling of hCLCA1, which appeared to be loosely attached to the plasma membrane and had a random distribution pattern (Fig. 2E).

Following pH 11 washing, the hCLCA1 that remained attached to the plasma membrane appeared to be predominantly localized to microvilli (Fig. 2F).

**Full-length hCLCA1 and Its N- and C-terminal Cleavage Products Can Be Detected in the Culture Medium from HEK293 Cells Transfected with hCLCA1—HEK293 cells were transiently transfected with an hCLCA1 construct containing a C-terminal V5 tag or with a GFP construct as a control. Surface immunolabeling of non-permeabilized hCLCA1-V5-His-transfected cells produced a punctate staining pattern on the external surface, using both A48 and anti-V5 antibodies (Fig. 3A). Western blot analysis of cell lysates of transfected cells detected both full-length protein and N-terminal cleavage product in the hCLCA1-V5-His-transfected cell lysates, but not in the GFP-transfected cell lysates, using A48 antibody (Fig. 3B). However, anti-V5 antibody only detected the full-length protein in the hCLCA1-V5-His cell lysate, even though the C-terminal processed form would be expected to be more abundant. Culture medium taken from these cells, when examined by Western blotting, was shown to contain full-length protein and the N-terminal processed hCLCA1 by A48 labeling, whereas anti-V5 antibody detected the full-length protein and a faint band around 50 kDa, thought to be the smaller C-terminal cleavage product. The 50-kDa band had a diffuse appearance, indicative of glycosylation. When hCLCA1 was expressed in HEK293 cells, the absence of the C-terminal cleavage product from cell lysate and the low levels of this product detected in the culture medium suggest that the C-terminal fragment is unstable once cleavage has taken place.

**Lack of Detection of the Myc Tag at the Plasma Membrane of HEK293 Cells Expressing Myc-hCLCA1 Indicates that the Myc Epitope Is Masked at This Location**—Gruber et al. (3) previously investigated the transmembrane topology of hCLCA1 using transient transfection of HEK293 cells with Myc-tagged constructs, which had the tag placed at different sites within the hCLCA1 sequence. One of these constructs (m2, which had the Myc tag placed between amino acids 366 and 367) was suggested to be located on the cytoplasmic side of the plasma membrane. This construct was used here for further investigations. Transient transfection of the construct in HEK293 cells, followed by Western blot analysis, revealed the presence of both the full-length protein and N-terminal cleavage product of hCLCA1 in the cell lysate and the N-terminal cleavage product in the culture medium (Fig. 4A). The detection of hCLCA1 in the culture medium, using both A48 and anti-Myc antibody, supports the hypothesis that hCLCA1 can be released from cell membranes.
hCLCA1 and mCLCA3 are Non-integral Membrane Proteins

ure to detect the Myc epitope at the cell surface and detection of the protein in the culture medium suggested that rather than being internal, the Myc epitope was masked at the plasma membrane.

The N-terminal Cleavage Product of hCLCA1 Rapidly Accumulates in Culture Medium from HEK-hCLCA1 Cells via a Brefeldin A-sensitive Pathway and Is Detected in BAL Fluid from Asthmatics—The observation that hCLCA1 could be detected in culture medium from HEK-hCLCA1 cells after 24 h led us to investigate how quickly the protein appeared in the medium. Medium samples taken at 1-, 2-, 3-, and 4-h intervals after stringent washing were subjected to Western blot analysis with A48 antibody. The N-terminal cleavage product was shown to rapidly accumulate in the medium, and by 4 h, a significant amount of hCLCA1 could be detected (Fig. 5A). Because the incubation times were short, any contribution from cell debris would be minimal.

The fungal metabolite BFA has been demonstrated to inhibit the constitutive secretory pathway (41, 42). BFA treatment was utilized here to investigate whether the appearance of hCLCA1 in the medium was a result of direct vesicular trafficking from the Golgi. Incubation of HEK-hCLCA1 cells with BFA for 4 h blocked the release of hCLCA1 into the medium (Fig. 5B). Removal of BFA, followed by a 4-h incubation, resulted in recovery of the Golgi apparatus and release of hCLCA1 into the medium. These data provide further support that hCLCA1 is a secreted protein.

Immunolabeling of hCLCA1 on non-permeabilized HEK-hCLCA1 cells incubated in the presence or absence of BFA for 4 h demonstrated that the surface expression of hCLCA1 remained unchanged in both pattern and intensity (Fig. 5C). This indicated that plasma membrane-associated hCLCA1 does not undergo rapid turnover via internalization or dissociation.

Because hCLCA1 is highly expressed in asthmatic lung, we examined samples from asthmatic subjects to rule out the possibility that our observations were a consequence of heterologous overexpression. BAL fluid samples were examined to ascertain whether hCLCA1 could be detected in an extracellular environment. BAL fluid taken from 11 non-asthmatic and 7 asthmatic subjects was analyzed by Western blotting (Fig. 5D). The N-terminal cleavage product of hCLCA1 was detected in BAL fluid from all seven asthmatic subjects (Fig. 5D, lanes 12–18). Only one of the samples from non-asthmatics appeared positive for hCLCA1 (Fig. 5D, lane 2). This patient, although not asthmatic, did suffer from perennial rhinitis, which may explain the presence of hCLCA1 in that sample. The ability to detect hCLCA1 in BAL fluid from asthmatics reinforces the evidence that hCLCA1 is released into the extracellular environment.

Studies of the Murine Ortholog (mCLCA3) Demonstrated That It Is Released into Culture Medium from Transfected HEK293 Cells and Is Present in BAL Fluid of OVA-challenged Mice—To demonstrate that the murine ortholog (mCLCA3) was also secreted, studies were carried out on transiently transfected HEK293 cells. By Western blot analysis, both the full-length and N-terminal processed forms of mCLCA3 were detected in the cell lysate (Fig. 6A). The mCLCA3 proteins appeared to be slightly smaller than the hCLCA1 ortholog, with the full-length band migrating at 110 kDa and the N-terminal cleavage product migrating at 80 kDa. Medium taken 4 h after washing the cells contained the N-terminal cleavage product, demonstrating rapid accumulation in culture medium as seen with hCLCA1. Western blot analysis of lysates of lung tissue taken from naïve and OVA-challenged mice showed that the N-terminal cleavage product was expressed in lung from challenged mice but absent from naïve mouse lung (Fig. 6B), in

---

**Fig. 4.** Myc tag is undetectable at the cell surface, using immunocytochemistry. Myc-tagged hCLCA1 and GFP control constructs were used to transiently transfet HEK293 cells. A, Western blots of cell lysates (25 μg) and culture medium (equivalent volume) were probed with A48 and anti-Myc antibodies. B, immunofluorescence was carried out on Myc-hCLCA1 transfected cells labeled with A48 and anti-Myc antibodies. Permeabilized cells were labeled with A48 (i) and anti-Myc (ii) for internal localization. Non-permeabilized cells were labeled with A48 (iii) and anti-Myc (iv) for surface localization. Finally, cells were double-labeled with both antibodies on permeabilized cells (v) or surface-labeled with A48 followed by permeabilization and internal labeling with anti-Myc (vi). A48, red; anti-Myc, green. Bar = 10 μm.
agreement with previous studies (11, 12, 10). BAL fluid samples taken from five naive and five OVA-challenged mice were examined by Western blot analysis (Fig. 6C). mCLCA3 protein was not detected in the naive samples, whereas the N-terminal cleavage product was present in the challenged mouse samples. These results show that mCLCA3 shares similar molecular characteristics to hCLCA1 and that both proteins are released into the extracellular milieu.

**DISCUSSION**

Proteins from the CLCA family of have been generally accepted to be integral membrane proteins, based on hydrophobicity studies of bovine bCLCA2, murine mCLCA1, and human hCLCA1 and hCLCA2 (4, 5, 3, 15). However, concerns have been growing as to whether the original topology studies were correct. Firstly, the putative transmembrane topologies are inconsistent between CLCA family members, both in position and number. Secondly, the proposed topology suggests that there are transmembrane regions within the globular VWA domain, an unlikely scenario based on the numerous studies of this well-characterized domain (43). The state of the art bioinformatics programs described here predicted that transmembrane domains are not present in the two family members hCLCA1 and mCLCA3. These observations formed the basis of our biochemical studies.
Transfection of HEK293 cells with hCLCA1 demonstrated that all three forms of the protein (full-length protein and its N- and C-terminal cleavage products) could be detected in the culture medium. The accumulation of the N-terminal cleavage product in the culture medium was shown to be rapid and a result of direct trafficking from the secretory pathway, demonstrated by the inhibition of release following incubation with BFA. These studies signify that hCLCA1 and mCLCA3 are not integral membrane proteins, making it impossible for them to be ion channels. It is also probable that this is the case for other members of the CLCA family because our bioinformatics analysis of several other family members predicted that there are no transmembrane domains, certainly in the N-terminal cleavage product. The structure of the C-terminal smaller cleavage product is not conserved between family members, and it is possible that some of the CLCA proteins may have a transmembrane domain within this region. However, the lack of detection of the C-terminal cleavage product of hCLCA1 in lysates from HEK-hCLCA1 cells suggested that it is likely to be the N-terminal cleavage product that plays a functional role. This is supported by a study on bovine CLCA1 in which a truncated 42-kDa mutant construct generated from the N-terminal cleavage product was found to yield identical chloride currents to the full-length protein when expressed in Xenopus oocytes (16).

It has been proposed that the CLCA proteins may be chloride channel regulators, rather than being ion channels themselves (43–47). Studies with pCLCA1, the porcine ortholog of hCLCA1, revealed its ability to mediate increased chloride currents that were either calcium- or cAMP-dependent, depending on the cell type in which it was expressed. When Caco-2 cells expressing pCLCA1 were differentiated, the calcium-activated chloride current became undetectable, even though pCLCA1 expression was maintained, demonstrating that it lacked inherent chloride current activity. Therefore, pCLCA1 appeared to be acting as a chloride channel regulator, able to enhance cystic fibrosis transmembrane conductance regulator currents as well as increase the amplitude of currents from endogenous calcium-activated chloride channels, whose identity remains unknown (44–46).

We found that full-length hCLCA1 and the N-terminal cleavage product could be removed from the cell surface, with some hCLCA1 remaining attached. Incubation with BFA to inhibit release of newly synthesized proteins demonstrated that the plasma membrane-localized hCLCA1 did not undergo rapid internalization or dissociation from the cell surface. This indicates that the cleavage of hCLCA1 is unlikely to take place via the endocytic pathway. It is possible that the residual hCLCA1 remained on the cell surface through an interaction with an ion channel in the plasma membrane. Electron microscopic examination of surface immunolabeled HEK-hCLCA1 cells showed that this residual hCLCA1 was localized to the microvilli. This is in keeping with an association with ion channels because microvilli are highly specialized regions of the plasma membrane, in which ion channels and transporter proteins are known to reside (48). mCLCA1 has been shown to directly interact with the large conductance potassium channel β2-subunit KCNMB1 when co-expressed in HEK293 cells. This increased the calcium sensitivity and evoked a larger calcium-activated chloride current than when mCLCA1 was expressed alone (49). In this study, a physical association between the two proteins was demonstrated, although the specific domains involved in the interaction have not been identified. These studies add to the mounting evidence that the CLCA proteins are part of a complex of regulatory/auxiliary proteins for ion channels, rather than being ion channels themselves. The localization of hCLCA1 to the external cell surface means that it would be possible for interactions to occur not only on the outside of the plasma membrane but also within the lumen of intracellular organelles.

The N-terminal cleavage products of hCLCA1 and mCLCA3 could be detected in BAL fluid from asthmatic subjects and OVA-challenged mice, respectively, demonstrating release from cells that endogenously express these proteins. mCLCA3 has been shown to be expressed in goblet cells and was immunolocalized to the lumen of the mucin granules (10). mCLCA3 could therefore be released from the cell along with the mucus. As a component of the mucin granules, hCLCA1 and mCLCA3 may be involved in regulation of the airway surface liquid volume and composition via modulation of chloride channel activity in the airway epithelial cells. Detection of hCLCA1 and mCLCA3 in BAL fluid raises the possibility that, as well as having a function within the cells in which they are expressed, as secreted proteins they could also have the ability to interact with proteins on the surface of other cells that are exposed to mucus secretions in the airway lumen.

In addition to the ability of the CLCA proteins to act as chloride channel regulators, other functions have been described for some family members. hCLCA2, hCLCA2, mCLCA1, and mCLCA5 have been shown to play a role in cell adhesion, binding to β4 integrin on the surface of melanoma cells (50, 51). However, this was shown not to be the case for hCLCA1 because the β4 integrin binding motif is disrupted. Down-regulation of some CLCA family members has been linked to breast and colorectal malignancies, suggesting the ability to act as tumor suppressors (52, 53). mCLCA2 is up-regulated under conditions of apoptotic stress (54), with increased expression in mammary tissue during lactation and involution, times when this tissue is undergoing remodeling (10). Asthmatic lung tissue is also undergoing remodeling, and it is tempting to speculate that hCLCA1 and mCLCA3 may be up-regulated here as part of a stress response. The full elucidation of the functions of this family of proteins awaits identification of interacting proteins.

We have demonstrated that hCLCA1 and mCLCA3 are non-integral membrane proteins that are secreted from cells into the extracellular environment. The ability of the CLCA proteins to mediate calcium-activated chloride currents when expressed in cells led to their nomenclature: chloride current-associated (CLCA). Because it now appears that they cannot themselves be ion channels, this nomenclature seems inappropriate. We propose that the members of the CLCA family are modulators of chloride channel activity and, as such, should perhaps be renamed as CLCR (chloride channel regulator). This would also allow the renumbering of the family members to better reflect their orthologous relationships.

Acknowledgments—We thank Dr. Bendict Pauli for the generous gift of the Myc-tagged hCLCA1 construct and helpful discussion and Dr. Chris Corrigan for the generous gift of the human BAL fluid samples. We also thank Alan White, Keith Brooks, Amanda Jowett, Gael McWalter, Farhana Hussain, and Nicola Waite for expert technical assistance.

REFERENCES
1. Atherton, H., Mesher, J., Poll, C. T., and Danahay, H. (2003) Naunyn-Schmiedeberg’s Arch. Pharmacol. 367, 214–217
2. Large, W. A., and Wang, Q. (1996) Am. J. Physiol. 271, C435–C454
3. Gruber, A. D., Elble, R. C., Ji, H. L., Schreur, K. D., Fuller, C. M., and Pauli, B. U. (1998) Genomics 54, 200–214
4. Cunningham, S. A., Awayda, M. S., Bubien, J. K., Ismailov, I. I., Arrate, M. P., Berdiev, B. K., Benos, D. J., and Fuller, C. M. (1995) J. Biol. Chem. 270, 31016–31026
5. Gandhi, R., Elble, R. C., Gruber, A. D., Schreur, K. D., Ji, H. L., Fuller, C. M., and Pauli, B. U. (1998) J. Biol. Chem. 273, 32006–32101
6. Hoshide, M., Morita, S., Iwashita, H., Nogi, T., Nakaniishi, A., Ashida, Y., Nishimura, O., Fujisawa, Y., and Fujino, M. (2002) Am. J. Respir. Crit. Care Med. 165, 1132–1136
7. Toda, M., Tolle, M. K., Levitt, R. C., and Hamid, Q. (2002) J. Allergy Clin. 27211
hCLCA1 and mCLCA3 are Non-integral Membrane Proteins

8. Hauber, H. P., Manoukian, J. J., Nguyen, L. H. P., Sobol, S. E., Levitt, R. C., Holroyd, K. J., McElvaney, N. G., Griffin, S., and Hamid, Q. (2003) Laryngoscope 113, 1037–1042
9. Hauber, H. P., Tiscopoulous, A., Wallaert, B., Griffin, S., McElvaney, N. G., Daugnault, P., Mueller, Z., Olevstein, E., Holroyd, K. J., Levitt, R. C., and Hamid, Q. (2004) Eur. Respir. J. 23, 846–850
10. Leverkoehne, I., and Gruber, A. D. (2002) J. Histochem. Cytochem. 50, 829–838
11. Nakaniishi, A., Morita, S., Iwashita, H., Sagiya, Y., Ashida, Y., Shirafugi, H., Fujisawa, Y., Nishimura, O., and Fujino, M. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 5175–5180
12. Zhou, Y., Dong, Q., Louahed, J., Dragwa, C., Savio, D., Huang, M., Weiss, C., Tomer, Y., McLane, M. P., Nicolaides, N. C., and Levitt, R. C. (2001) Am. J. Respir. Cell Mol. Biol. 25, 486–491
13. Papassotiriou, J., Eggermont, J., Droogmans, G., and Nilius B. (2001) Pflügers Arch. 442, 273–279
14. Abdel-Ghany, M., Cheng, H.-C., Elble, R. C., and Pauli, B. U. (2001) J. Biol. Chem. 276, 5248–5254
15. Gruber, A. D., Schreur, K. D., Ji, H. L., Fuller, C. M., and Pauli, B. U. (1999) J. Appl. Physiol. Cell Physiol. 45, C1261–C1270
16. Ji, H.-L., DuVall, M. D., Patton, H. K., Satterfield, C. L., Fuller, C. M., and Benos, D. J. (1998) Am. J. Physiol. Cell Physiol. 274, C455–C464
17. Ran, S., Fuller, C. M., Arrate, M. P., Laterre, R., and Benos, D. J. (1992) J. Biol. Chem. 267, 20630–20637
18. Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E. L. L. (2001) J. Mol. Biol. 305, 567–580
19. Tusnady, G. E., and Simon, I. (2001) Bioinformatics 17, 849–850
20. Persson, B., and Argos, P. (1994) J. Mol. Biol. 237, 182–192
21. Hirokawa, T., Boon-Chieng, S., and Mitaku, S. (1998) Bioinformatics 14, 378–379
22. Cesroz, M., Wollin, E., Simon, I., von Heijne, G., and Elofsson, A. (1997) Proteins 29, 873–876
23. Zdobnov, E. M., and Apweiler, R. (2001) Bioinformatics 17, 847–848
24. Letunic, I., Goedeart, L., Dickens, N. J., Doerks, T., Schultz, J., Mott, R., Cicairelli, F., Copley, R. R., Ponting, C. P., and Gough, J. (2001) Nucleic Acids Res. 30, 242–244
25. Bateman, A., Coin, L., Durbin, R., Finn, D. R., Hollich, V., Griffiths-Jones, S., Khanna, A., Marshall, M., Mazon, S., Sonnhammer, E. L. L., Studholme, D. J., Yeats, C., and Eddy, S. R. (2004) Nucleic Acids Res. 32, D138–D141
26. Falquet, L., Pagni, M., Bicher, P., Hulo, N., Sigrist, C. J. A., Hofmann, K., and Bairoch, A. (2002) Nucleic Acids Res. 30, 235–238
27. Aitschui, S. F., Maddo, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Nucleic Acids Res. 25, 3389–3402
28. Gough, J., Karplus, K., Hughey, R., and Chothia, C. (2001) J. Mol. Biol. 313, 903–919
29. Soding, J. (2005) Bioinformatics 21, 951–960
30. Bendtzen, J. D., Nielsen, H., von Heijne, G., and Brunak, S. (2004) J. Mol. Biol. 340, 785–795
31. Balch, W. E., Dunphy, W. G., Braell, W. A., and Rothman, J. E. (1984) Cell 39, 405–416
32. Brusa, S. S., and Rudolf, J. D. (1994) Methods Enzymol. 228, 182–193
33. Fujiki, Y., Hubbard, A. L., Fowler, S., and Lazarow, P. B. (1992) J. Biol. Chem. 275, 97–102
34. Simionescu, N., and Simonescu, M. (1976) J. Cell. Biol. 70, 608–621
35. Hopkins, C. R., and Trowbridge, I. S. (1983) J. Cell Biol. 97, 508–521
36. Gruber, A. D., Fuller, C. M., Elble, R. C., Benos, D. J., and Pauli, B. U. (2000) J. Cell Biol. 93, 97–102
37. Fujiki, Y., Hubbard, A. L., Fowler, S., and Lazarow, P. B. (1992) J. Biol. Chem. 277, 22119–22122
38. Tuckwell, D. (1999) Biochem. Soc. Trans. 27, 835–840
39. Lee, J. O., Rieu, P., Arnaout, M. A., and Liddington, R. (1995) Cell 80, 631–638
40. Bork, P., and Doi, L. F. R. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8990–8994
41. Lippincott-Schwartz, J., Yuan, L. C., Bonifacino, J. S., and Klausner, R. D. (1989) Cell 56, 801–813
42. Dintz, A., and Berger, E. G. (1996) Histochem. Cell Biol. 109, 571–590
43. Whittaker, C. A., and Hynes, R. O. (2002) Mol. Biol. Cell 13, 3369–3387
44. Loewen, M. E., Bekar, L. K., Gabriel, S. E., Walz, W., and Forsyth, G. W. (2002) Biochem. Biophys. Res. Commun. 298, 531–536
45. Loewen, M. E., Smith, N. K., Hamilton, D. L., Grahn, B. H., and Forsyth, G. W. (2003) Am. J. Physiol. Cell Physiol. 285, C1314–C1321
46. Loewen, M. E., Bekar, L. K., Walz, W., Forsyth, G. W., and Gabriel, S. E. (2004) Am. J. Physiol. Gastroutest. Liver Physiol. 287, G33–G41
47. Eggermont, J. (2004) Proc. Am. Thorac. Soc. 1, 22–27
48. Lange, K. (2000) J. Cell. Physiol. 185, 21–35
49. Greenwood, I. A., Miller, L. J., Ohyu, S., and Horwitz, B. (2002) J. Biol. Chem. 277, 22119–22122
50. Abdel-Ghany, M., Cheng, H.-C., Elble, R. C., and Pauli, B. U. (2002) J. Biol. Chem. 277, 34391–34400
51. Abdel-Ghany, M., Cheng, H.-C., Elble, R. C., Lin, H., DiBiase, J., and Pauli, B. U. (2003) J. Biol. Chem. 278, 34946–34949
52. Gruber, A. D., and Pauli, B. U. (1999) Cancer Res. 59, 5488–5491
53. Bustin, S. A., Li, S.-R., and Lorou, S. (2001) DNA Cell Biol. 20, 331–338
54. Elble, R. C., and Pauli, B. U. (2001) J. Biol. Chem. 276, 40510–40517
hCLCA1 and mCLCA3 Are Secreted Non-integral Membrane Proteins and Therefore Are Not Ion Channels
Adele Gibson, Alan P. Lewis, Karen Affleck, Alan J. Aitken, Eric Meldrum and Nicola Thompson

J. Biol. Chem. 2005, 280:27205-27212. doi: 10.1074/jbc.M504654200 originally published online May 25, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M504654200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 52 references, 17 of which can be accessed free at http://www.jbc.org/content/280/29/27205.full.html#ref-list-1