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Molecules and Cells

The Murine Goblet Cell Protein mCLCA3 Is a Zinc-Dependent Metalloprotease with Autoproteolytic Activity

Melanie K. Bothe, Lars Mundhenk, Matthias Kaup[1], Christoph Weise[2], and Achim D. Gruber*

Several members of the CLCA family of proteins, originally named chloride channels, calcium-activated, have been shown to modulate chloride conductance in various cell types via an unknown mechanism. Moreover, the human (h) hCLCA1 is thought to modulate the severity of disease in asthma and cystic fibrosis (CF) patients. All CLCA proteins are post-translationally cleaved into two subunits, and recently, a conserved HEXXH zinc-binding amino acid motif has been identified, suggesting a role for CLCA proteins as metalloproteases. Here, we have characterized the cleavage and autoproteolytic activity of the murine model protein mCLCA3, which represents the murine orthologue of human hCLCA1. Using crude membrane fractions from transfected HEK293 cells, we demonstrate that mCLCA3 cleavage is zinc-dependent and exclusively inhibited by cation-chelating metalloprotease inhibitors. Cellular transport and secretion were not affected in response to a cleavage defect that was introduced by the insertion of an E157Q mutation within the HEXXH motif of mCLCA3. Interspecies conservation of these key results was further confirmed with the porcine (p) orthologue of hCLCA1 and mCLCA3, pCLCA1. Importantly, the mCLCA3E157Q mutant was cleaved after co-transfection with the wild-type mCLCA3 in HEK293 cells, suggesting that an intermolecular autoproteolytic event takes place. Edman degradation and MALDI-TOF-MS of the protein fragments identified a single cleavage site in mCLCA3 between amino acids 695 and 696. The data strongly suggest that secreted CLCA proteins have zinc-dependent autoproteolytic activity and that they may cleave additional proteins.

INTRODUCTION

CLCA proteins, originally termed chloride channels, calcium-activated, have been implicated in a variety of cellular functions, most consistently with the mediation of transmembrane anion conductance (Cunningham et al., 1995; Gruber et al., 1998; Hamann et al., 2009). However, instead of forming genuine transmembrane channels, CLCAs are fully or partially secreted proteins that may regulate other chloride conductances via a receptor-mediated pathway (Gibson et al., 2005; Mundhenk et al., 2006). They were thus proposed to at least partially compensate for the defective chloride currents in CF patients (Ritzka et al., 2004). The most CF-relevant human CLCA family member, hCLCA1 (Ritzka et al., 2004; van der Doef et al., 2010), and its murine (mCLCA3) (Gibson et al., 2005; Leverkoehne and Gruber, 2002; Mundhenk et al., 2006) and porcine (pCLCA1) (Plog et al., 2009) orthologues are secreted into the respiratory and intestinal mucous layers by goblet cells. As the secretory pathway of all CLCA proteins involves the cleavage of a precursor molecule in the endoplasmic reticulum (Mundhenk et al., 2006; Plog et al., 2009), we reasoned that characterizing the cleavage of key CLCA proteins is crucial for understanding the structure-function relationships of this protein family.

Cleavage of CLCA proteins occurs *in vivo* as well as in heterologous mammalian expression systems, implying that an universal cleavage agent exists that is still unknown (Gruber et al., 2002; Leverkoehne and Gruber, 2002). Recently, a HEXXH zinc-binding amino acid motif, typical of metalloproteases, has been identified in human and invertebrate CLCA proteins, which may confer zinc dependent autoproteolytic cleavage (Pawlowski et al., 2006). However, the zinc dependency of the cleavage process and the autoproteolytic activity of CLCA proteins have not yet been experimentally addressed.

Here we show the zinc dependency of the cleavage process, identify and characterize the exact cleavage site as well as the cellular transport of a cleavage-defective mutant, and experimentally address the autocatalytic activity of CLCA proteins. This study focuses on mCLCA3, an orthologue of human hCLCA1, as a model that represents the best characterized member of the CLCA protein family (Gibson et al., 2005; Leverkoehne and Gruber, 2002; Mundhenk et al., 2006).

MATERIALS AND METHODS

Plasmid constructs and antibodies

Plasmids mCLCA3, mCLCA3-YFP and pCLCA1 have been described previously (Leverkoehne and Gruber, 2002; Mundhenk et al., 2006; Plog et al., 2009). The E157Q mutation was

Department of Veterinary Pathology, Freie Universitaet Berlin, Germany, [1]Department of Laboratory Medicine and Pathobiochemistry, Charité Berlin, Germany, [2]Institute of Chemistry and Biochemistry, Freie Universitaet Berlin, Germany
*Correspondence: gruber.achim@vetmed.fu-berlin.de

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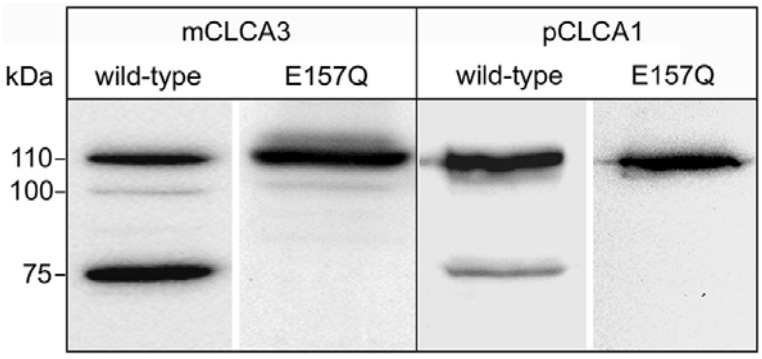

Fig. 1. Inhibition of precursor cleavage in E157Q mutants. Expression of wild-type proteins and E157Q mutants of both mCLCA3 and pCLCA1 in cell lysates of transiently transfected HEK293 cells was examined by immunoblot. The precursor molecules had a size of 110 kDa while the subunits were detected at 75 kDa.

introduced into the wild-type mCLCA3 and pCLCA1 plasmids (Primers: mCLCA3E157Q forward catcagtgggcccacttccgatg, mCLCA3E157Q reverse gacaaaggtcctgtcttgtggccc, pCLCA1 E157Q forward caccaatgggcccacctgcgatg, pCLCA1E157Q reverse aacaaatacccttccttgtggtcc) according to the Phusion® site-directed mutagenesis protocol (Finnzymes) and verified by sequencing. For immunoblot detection of the mCLCA3 and pCLCA1 proteins, antibodies α-p3b2 and p1-N-1ab-p, respectively, were used (Leverkoehne and Gruber, 2002; Plog et al., 2009). Rabbit antibody α-m3-C-1p was raised against a synthetic oligopeptide corresponding to amino acids 866 to 879 in the carboxy-terminal subunit of the mCLCA3 protein and immunopurified.

Cell culture experiments

Following transient transfection (24 h) using Turbofect™ (Fermentas), HEK293 cells were washed with PBS and incubated in adenovirus expression medium (AEM, Invitrogen) for 4.5 h. The cell supernatants were subjected to standard ethanol precipitation, the cells were lysed in standard lysis buffer, and both were analyzed by immunoblotting. Incubation of the samples with Endo H or PNGase F (New England Biolabs) prior to immunoblot analysis was performed as described previously (Bothe et al., 2008). For treatment of mCLCA3-overexpressing HEK293 cells with TPEN or marimastat, cells were incubated in AEM supplemented with or without 20 μM TPEN or 20 μM marimastat for 4 h.

Membrane preparation and activity assays

The zinc dependency of mCLCA3 cleavage was tested in crude membrane extractions as described previously (Kaup et al., 2002) with minor modifications. All of the steps were carried out at 4°C. Cell pellets of mCLCA3-overexpressing HEK293 cells were resuspended in 0.1× PBS, incubated for 10 min, homogenized in a Dounce homogenizer, and centrifuged at 500 × *g* for 15 min, followed by 2,600 × *g* for 15 min. To test the effects of various metal cations, the membrane pellet was resuspended in PBS, divided into 20 μl aliquots and supplemented with 1 mM of $MgCl_2$ (Mg^{2+}), $CaCl_2$ (Ca^{2+}), $ZnCl_2$ (Zn^{2+}), a combination of these cations or left untreated.

To test the effects of protease inhibitors, resuspended membrane pellets supplemented with 1 mM Mg^{2+}, Ca^{2+} and Zn^{2+} were incubated with either 1 mM EDTA (Applichem), 1 mM EGTA (Applichem), 1 mM 1,10-phenanthroline (Applichem), 100 nM tetrakis (2-pyridylmethyl) ethylenediamine (TPEN; Sigma-Aldrich), 1× proteoblock™ [100 mM 4-(2-Aminoethyl)-benzensulfonylfluorid (AEBSF), 80 μM aprotinin, 5 mM bestatin, 1.5 mM E-64, 2 mM leupeptin, 1 mM pepstatin A; Fermentas], 1 mM marimastat (Merck) or left untreated. Each sample was adjusted to 24 μl with PBS and incubated for 30 min at 37°C, followed by subsequent immunoblot analysis. Time course experiments determined the optimal incubation time of 30 min.

Immunoblot analysis

Samples were analyzed by immunoblot as described previously (Plog et al., 2009). For membrane activity assays, the band intensities were measured in three independent experiments. Data were calculated as the percent intensity relative to each sample prior to incubation (set to 100%) and listed with the standard deviations. Statistical significance was determined using an unpaired, two-sided Welch's *t*-test.

Immunoprecipitation, Edman degradation and MALDI-TOF-MS

Lysates from transiently mCLCA3-YFP-overexpressing HEK293 cells were first incubated with anti-YFP antibody (Applied Biological Materials Inc.) followed by protein A/G Plus agarose beads (Thermo Scientific). Mock-transfected cells served as negative controls. The bisected immunoprecipitates were transferred onto PVDF membranes following SDS-PAGE. Bands were either detected by immunoblot with a-m3-C-1p or visualized by Coomassie staining. Edman degradation of Coomassie-stained bands was performed by the Proteomefactory, Berlin, Germany. For MALDI-TOF-MS analysis, excised Coomassie-stained bands representing the mCLCA3-YFP precursor, as well as the amino- and carboxy-terminal subunits, were digested by trypsin. The precursor, as well as the amino-terminal subunit, was also digested by AspN protease prior to MALDI-TOF-MS.

RESULTS

E157Q mutations affect cleavage but not cellular transport of CLCA proteins

The cleavage of a precursor protein into two subunits, a common feature of CLCA proteins (Pauli et al., 2000), has been suggested to depend on a HEXXH motif (Pawlowski et al., 2006). Introduction of an E157Q mutation into the HEXXH motif in the mCLCA3 protein and the porcine orthologue pCLCA1 abrogated the cleavage event (Fig. 1).

In the following experiments, we focused on mCLCA3 and characterized the cellular transport of the mutant version of this protein. Consistent with previously published data (Mundhenk et al., 2006), the precursor of the wild-type mCLCA3 protein was exclusively detected in the cell lysate. Notably, mCLCA3 is sensitive to Endo H and PNGase F, which is indicative of a high mannose-containing glycoprotein (Fig. 2). In contrast, the mutant protein in the cell lysate includes a small fraction of an Endo

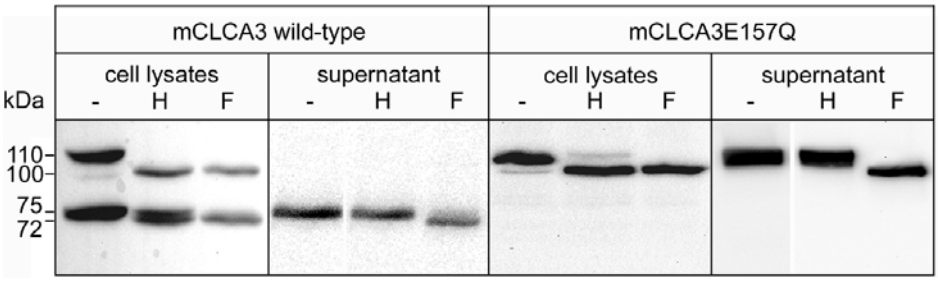

Fig. 2. Uncleaved mCLCA3E157Q precursor passes through the Golgi and is released into the cell supernatant. Immunoblot analyses of cell lysates and supernatants of HEK293 cells transfected with wild-type mCLCA3-pcDNA3.1 or the mCLCA3E157Q mutant. Samples were treated with Endo H (H) or PNGase F (F) or remained untreated (-). The precursor molecule of 110 kDa shifted to 100 kDa in size while the amino-terminal subunit shifted from 75 to 72 kDa.

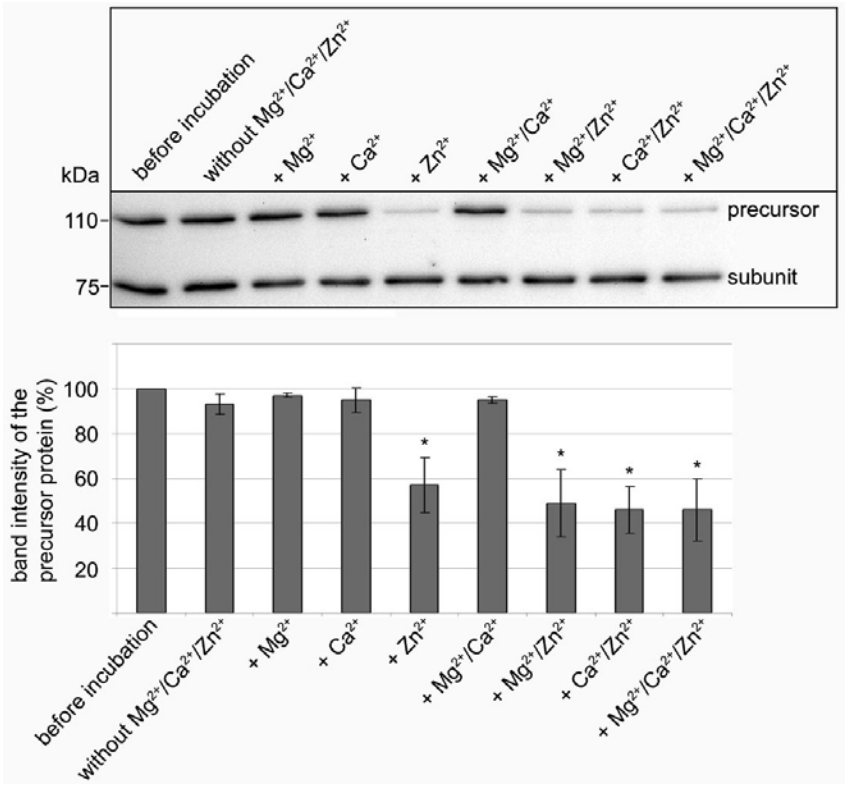

Fig. 3. Cleavage of the mCLCA3 precursor protein is zinc-dependent. Upper panel, samples from membrane preparations of transiently mCLCA3-overexpressing HEK293 cells were incubated with either 1.0 mM magnesium (Mg^{2+}), calcium (Ca^{2+}), zinc (Zn^{2+}) or a combination of these ions, separated by SDS-PAGE and examined by immunoblot using the anti-mCLCA3-amino-terminal antibody α-p3b2. Lower panel, cleavage of the mCLCA3 precursor was quantified by chemiluminescence using Quantity One software for digital quantification of relative band intensities. Data represent the mean values and standard deviations from three independent experiments. *= $p < 0.01$.

H-resistant form, and the mutant could also be detected in the supernatant. In the case of the supernatant fraction, only an Endo H-resistant and PNGase F-sensitive precursor was present, indicating that the uncleaved but complex glycosylated mCLCA3E157Q protein passes through the Golgi apparatus and is released into the supernatant despite the block in cleavage.

Zn^{2+} triggers the cleavage of mCLCA3

The HEXXH amino acid motif is responsible for zinc binding in metalloproteases and other proteins (Hooper, 1994). The impaired cleavage of the E157Q mutant HEXXH motif in hCLCA1 has led to the hypothesis that Zn^{2+}-dependent cleavage occurs in secreted CLCA proteins (Pawlowski et al., 2006). In our membrane activity assay, the individual addition of Mg^{2+} and Ca^{2+} did not reduce the band intensities of the mCLCA3 precursor protein, indicating that these ions failed to trigger mCLCA3 cleavage (Fig. 3). However, upon addition of Zn^{2+}, alone or in combination with any of the other cations, the precursor was cleaved, as observed by a marked reduction in the intensity of the precursor band, indicating that Zn^{2+} is required for the cleavage of mCLCA3.

Chelating agents inhibit the cleavage of mCLCA3

To test whether chelating agents like EDTA, EGTA and 1,10-phenanthroline (Kaup et al., 2002) or TPEN (Howes et al., 2007) inhibit the cleavage of mCLCA3, we incubated the crude membrane extracts with different chelating agents (Fig. 4A). After incubation, the intensity of the protein band representing the precursor was significantly reduced in intensity when compared to the band intensity before incubation. This reduction was inhibited by EDTA, EGTA, 1,10-phenanthroline and TPEN, but not by proteoblock™, a combination of broad-spectrum protease inhibitors, indicating that a metalloprotease cleaves the precursor. Inhibition of cleavage by the chelating agents was not limited to the membrane assay, as incubation of mCLCA3-overexpressing HEK293 cells with TPEN resulted in the appearance of an additional band in the supernatant of TPEN-treated cells that migrated the same distance on the gel as the uncleaved precursor molecule (corresponding to 110

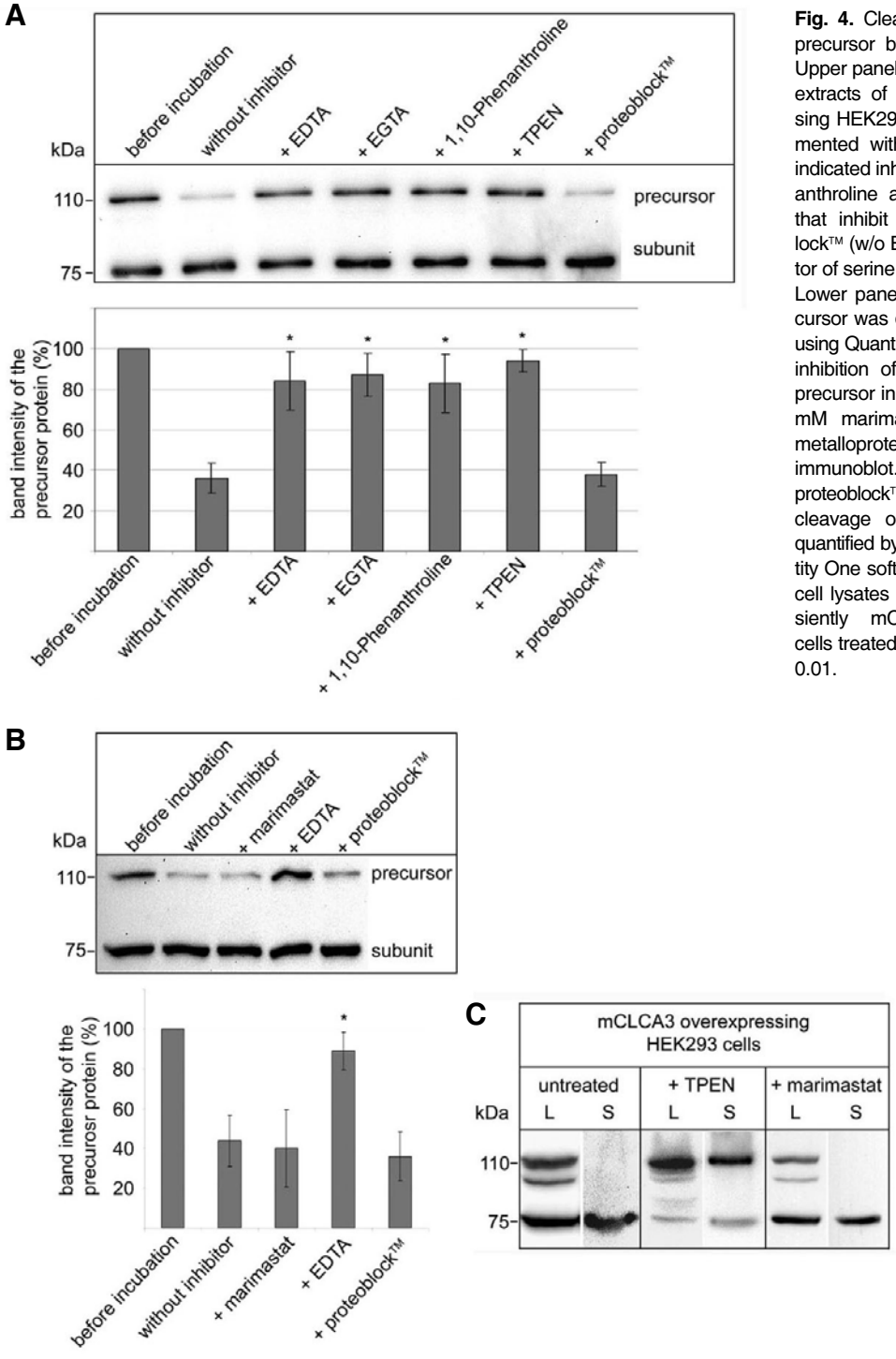

Fig. 4. Cleavage inhibition of the mCLCA3 precursor by metalloprotease inhibitors. (A) Upper panel, immunoblot of crude membrane extracts of transiently mCLCA3-overexpressing HEK293 cells incubated in PBS supplemented with Mg^{2+}, Ca^{2+} and Zn^{2+} and the indicated inhibitors. EDTA, EGTA, 1,10-phenanthroline and TPEN are chelating agents that inhibit metalloproteases, and proteoblock™ (w/o EDTA) is a broad-spectrum inhibitor of serine, cysteine and aspartic proteases. Lower panel, cleavage of the mCLCA3 precursor was quantified by chemiluminescence using Quantity One software. (B) Upper panel, inhibition of the cleavage of the mCLCA3 precursor in crude membrane extracts by 1.0 mM marimastat, a broad-spectrum matrix metalloprotease inhibitor, was analyzed by immunoblot. Samples treated with EDTA and proteoblock™ served as controls. Lower panel, cleavage of the mCLCA3 precursor was quantified by chemiluminescence using Quantity One software. (C) Immunoblot analysis of cell lysates (L) and supernatants (S) of transiently mCLCA3-overexpressing HEK293 cells treated with TPEN or marimastat. *= $p < 0.01$.

kDa) (Fig. 4C). EDTA, EGTA and 1,10-phenanthroline are chelating agents that specifically chelate Ca^{2+}, Mg^{2+} and Zn^{2+}, whereas TPEN specifically chelates Zn^{2+}, Fe^{2+} and Mn^{2+}. As Zn^{2+} is the only common cation that is chelated by all four reagents, the metalloprotease that is responsible for cleavage must be a zinc-dependent metalloprotease. The zinc-dependent matrix metalloproteases (MMPs) have been intensely studied in the past, and hCLCA1 was suggested to possess properties similar to MMP-11 (Pawlowski et al., 2006). The involvement of a transmembrane MMP or a disintegrin and metalloprotease (ADAM) was tested using the synthetic inhibitor marimastat (Tortorella et al., 2009) in crude membrane extractions, while the role of secreted MMPs (which are unlikely to be present in the crude membrane extractions) was tested by marimastat incubation with mCLCA3-overexpressing HEK293 cells. In contrast to the metal ion chelators, marimastat failed to prevent the cleavage of the precursor of mCLCA3 in both crude membrane extractions and upon incubation of marimastat with

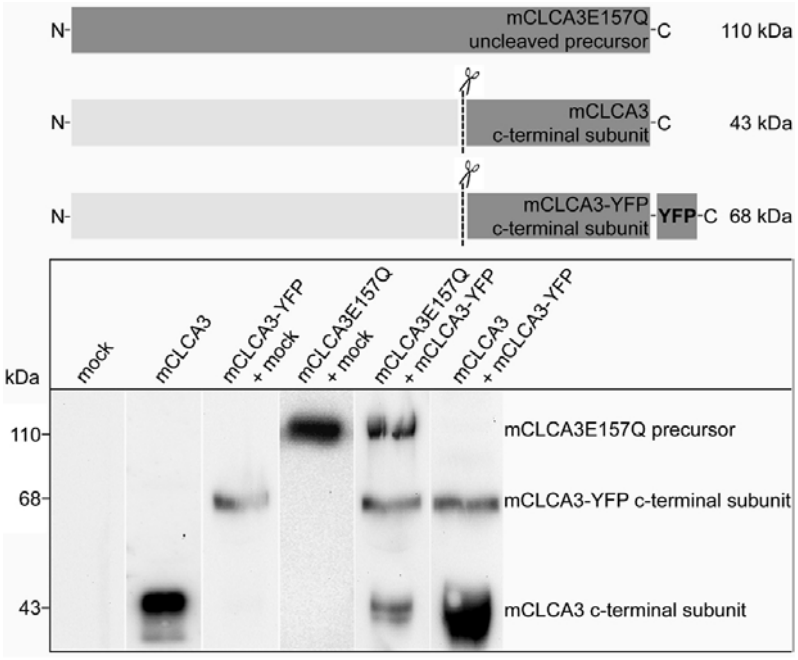

Fig. 5. mCLCA3-YFP is capable of cleaving mCLCA3E157Q *via* intermolecular auto-proteolysis. Immunoblot analysis of supernatants of HEK293 cells co-transfected with different combinations of plasmids encoding mutant mCLCA3 E157Q, mCLCA3-YFP, wild-type mCLCA3 or vector alone (pcDNA3.1; mock). The predicted sizes of the uncleaved precursor of the mutant mCLCA3E157Q and the cleaved carboxy-terminal subunits of mCLCA3 and mCLCA3-YFP protein are depicted in the upper panel. The results are representative of three independent experiments.

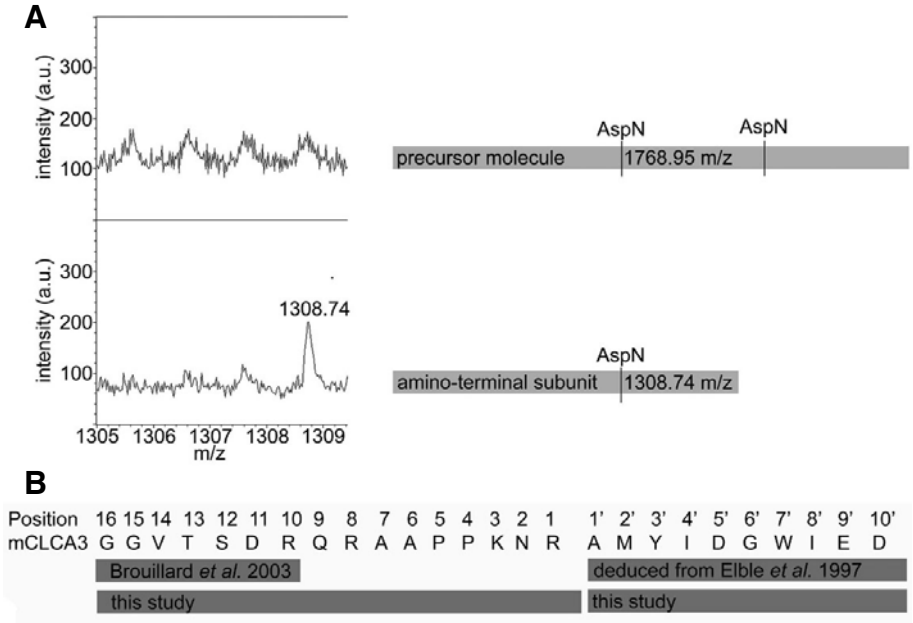

Fig. 6. mCLCA3 is cleaved between R695 and A696. (A) Section of a MALDI-TOF mass spectrum of the precursor and the amino-terminal subunit after digestion with AspN protease. The peptide with 1308.74 m/z was identified by MS/MS sequencing as amino acids 685-695 of mCLCA3. a.u. = arbitrary unit. (B) Edman-degra-dation of the immunoprecipitated mCLCA3-YFP carboxy-terminal subunit identified A696 to lead the carboxy-terminal subunit.

mCLCA3-overexpressing HEK293 cells (Figs. 4B and C).

The mutant mCLCA3E157Q is cleaved in cells that have been co-transfected with mCLCA3-YFP

Because CLCA proteins are too large for chemical synthesis and extensive efforts toward other methods of protein purification proved unsuccessful (data not shown), we took a co-transfection approach to characterize the proteolytic activity of mCLCA3 in this study. Such co-transfection experiments have been described previously for the determination of cleavage or self-cleavage activity of other proteases (Cao et al., 2005; Contin et al., 2003; Gao et al., 2004; Schlomann et al., 2002). In our study, the mCLCA3E157Q mutant protein, serving as a putative substrate, was co-expressed with a regularly processed wild-type mCLCA3-YFP protein. The YFP-tagged protein variant was used to distinguish between the carboxy-terminal subunit of mCLCA3-YFP (68 kDa) and a possible *de novo* cleaved carboxy-terminal subunit of mCLCA3E157Q (43 kDa). Co-transfection of mCLCA3E157Q and mCLCA3-YFP resulted in the secretion of the precursor of mCLCA3E157Q, the carboxy-terminal subunit of mCLCA3-YFP and a *de novo* cleaved carboxy-terminal subunit of mCLCA3E157Q (Fig. 5). The carboxy-terminal subunit of mCLCA3 was exclusively detected after co-transfection of mCLCA3 and mCLCA3-YFP, but not after trans-

fection with mCLCA3-YFP co-transfected with the pcDNA3.1 vector or mCLCA3E157Q that was co-transfected with pcDNA 3.1. Similar cleavage was observed in cell lysates (data not shown), indicating that the cleavage occurs in an intracellular compartment. These results support the hypothesis that the cleavage-competent mCLCA3 may be capable of cleaving mCLCA3E157Q via an intermolecular cleavage event.

mCLCA3 is cleaved at a single cleavage site between R695 and A696

After digestion of the amino-terminal subunit of mCLCA3 with AspN, MALDI-TOF-MS revealed a peak at m/z = 1308 that was undetectable after AspN digestion of the precursor (Fig. 6A). The resulting peptide represents amino acids 685 to 695 of mCLCA3. Interestingly, a second peak was detected at m/z = 1152, representing amino acids 685 to 694. Both assignments were confirmed by MALDI-MS/MS sequencing (data not shown). Edman degradation and MALDI-TOF-MS of the immunoprecipitated carboxy-terminal subunit of mCLCA3-YFP revealed amino acid 696 as the first amino acid of the carboxy-terminal subunit of mCLCA3-YFP (Fig. 6B).

DISCUSSION

Although the molecular function of CLCA proteins is far from being resolved, a HEXXH motif in CLCA suggests a new possible function as metalloproteases (Pawlowski et al., 2006). Here, we further characterized the cleavage process to confirm and specify the proteolytic activity of CLCA proteins. Introduction of the E157Q mutation in the HEXXH consensus zinc-binding motif of mCLCA3 and pCLCA1 abrogated the cleavage of both proteins, consistent with the data presented for their human orthologue (Pawlowski et al., 2006). This indicates that inhibition of protein cleavage by the introduction of the E157Q mutation in the HEXXH motif is a universal and evolutionarily conserved event. We focused our study on murine mCLCA3 as the best studied model for CLCA protein processing (Gibson et al., 2005; Leverkoehne and Gruber, 2002; Mundhenk et al., 2006).

The proteolytic processing of proteins may be a critical prerequisite for cellular transport (Creemers et al., 1995). Surprisingly, although cleavage was fully abolished, the mutant mCLCA3E157Q protein exhibited complex glycosylation and was secreted. Nevertheless, proteolytic cleavage may be a critical prerequisite for functionality, and uncleaved CLCA proteins might have reduced or abolished function, which remains to be tested in future work.

Zn^{2+}-dependent cleavage is a key feature of metalloproteases (Hooper, 1994). In this study, the cleavage of mCLCA3 was shown to be Zn^{2+}-dependent and exclusively inhibited by metalloprotease inhibitors, pointing toward a metalloprotease as the cleavage agent. Importantly, marimastat failed to inhibit the cleavage of the mCLCA3 precursor, convincingly arguing that MMPs or ADAM proteases are not involved in the cleavage of mCLCA3.

It has been proposed that the agent cleaving CLCA proteins must be universally expressed (Gruber, 2002) or, alternatively, that cleavage is performed by the protein itself (Pawlowski et al., 2006). Here we show that an active wild-type mCLCA3 protein is capable of processing the cleavage-defective mutant via an intermolecular cleavage event. However, we cannot fully exclude the remote possibility that transfection of cells with wild-type mCLCA3 leads to the activation of a proteolytic cascade that results in the cleavage of mCLCA3E157Q by a distinct protease. Because similar co-transfection experiments have previously proven that autoproteolytic processing of other proteases occurs (Schlomann et al., 2002), we conclude that cleavage of mCLCA3 may also occur autoproteolytically. However, this cleavage appeared to be incomplete, leaving a small fraction of uncleaved precursor protein. One possible explanation is that the physiological cleavage process may be an intra- rather than intermolecular event. Alternatively, close physical interaction of the two proteins may be essential for the cleavage process and might be altered by the YFP tag. In any case, the results clearly suggest that one mCLCA3 protein may be processed by other mCLCA3 molecules by intermolecular cleavage.

The cleavage site of mCLCA3 has only been vaguely localized in previous studies (Brouillard et al., 2005; Elble et al., 1997). Here, we have identified the exact cleavage site between R695 and A696. Our mass spectrometric data revealed a candidate second cleavage site following amino acid 694. This potential second cleavage site, however, could not be corroborated by Edman degradation of the carboxy-terminal subunit. It might, therefore, be an artifact due to side-activity of the AspN protease that occurred at the amine bond of arginine. Alternatively, the protein might be cleaved between amino acids R695 and A696, followed by the degradation of the carboxy-terminus of the amino-terminal subunit by a carboxy-peptidase. A more detailed understanding of the conserved amino acids may serve as a starting point for determining the substrate requirements of CLCA proteases.

The combined results of this study strongly suggest that mCLCA3 and probably its orthologues, including the human hCLCA1, are capable of zinc dependent, intermolecular and/or intramolecular autoproteolysis, establishing the CLCA family as a new class of metalloproteases. In light of the fact that mCLCA3 is secreted by goblet cells (Leverkoehne and Gruber, 2002), it is tempting to speculate that extracellular mucin glycoproteins or other components of the mucous layer might potentially serve as protease substrates. Future studies will address the question of which, if any, association exists between the protease activity and the mediation of transepithelial anion conductance by CLCA proteins (Hamann et al., 2009). Consistent with this notion, other matrix metalloproteases have previously been shown to be capable of modulating epithelial chloride currents (Duszyk et al., 1999). Establishing mCLCA3 as a zinc-dependent metalloprotease may set the stage for a better understanding of the role of CLCA proteins in normal and diseased tissues.

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