Institute of Veterinary Pathology, Department of Veterinary Medicine,
Freie Universität Berlin

Are CLCA Proteins Metalloproteases?

Comparison of the Cleavage Processes of the Secreted mCLCA3 and the Transmembrane mCLCA6 Proteins

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Inaugural-Dissertation
zur Erlangung des Grades eines
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Für Verena

Contents

List of abbreviations

ADAM	**A D**isintegrin **a**nd **M**etalloprotease
AHR	**A**irway **H**yper**r**esponsiveness
CaCC	**Ca**lcium activated **C**hloride **C**urrents
cAMP	**C**yclic **A**denosine **M**ono**p**hosphate
cDNA	**C**omplementary **D**eoxyribo**n**ucleic **A**cid
CF	**C**ystic **F**ibrosis
CFTR	**C**ystic **F**ibrosis **T**ransmembrane conductance **R**egulator
CLCA	**C**h**l**oride channels, **c**alcium **a**ctivated
COPD	**C**hronic **o**bstructive **p**ulmonary **d**isease
DNA	**D**eoxyribo**n**ucleic **A**cid
ERK	**E**xtracellular-signal **R**egulated **K**inase
et al.	**et al**ii (latin for “and others”)
FAK	**F**ocal **A**dhesion **K**inase
FnIII	**F**ibronectin type **III** domain
HEK293	**H**uman **E**mbryonic **K**idney **293** cells
IL	**I**nter**l**eukin
JAK	**Ja**nus**k**inase
LFA-1	**L**ymphocyte **F**unction-**A**ssociated Antigen **1**
LPS	**L**ipo**p**oly**s**accharide
Lu ECAM-1	**Lu**ng **E**ndothelial **C**ell **A**dhesion **M**olecule 1
Mac-1	**Mac**rophage-**1** antigen
MALDI-TOF-MS	**M**atrix-**a**ssisted **L**aser **D**esorption/**I**onization **T**ime **o**f **F**light **M**ass **S**pectrometry
mCLCA3	**M**urine **c**h**l**oride channel, **c**alcium **a**ctivated protein no. **3**
MMP	**M**atrix **M**etallo**p**rotease
mRNA	**M**essenger **R**ibo**n**ucleic **A**cid
MUC	**Muc**in gene
p53	**P**rotein **53** (due to size of 53 kDa)
RAO	**R**ecurrent **A**irway **O**bstruction
Rce1p	**R**as **c**onverting **e**nzyme **1** **p**rotease
STAT	**S**ignal **T**ransducer and **A**ctivator of **T**ranscription
Th2	**T** **h**elper cell type **2**
TM	**T**rans**m**embrane domain
TNF-α	**T**umor **N**ecrosis **F**actor **α**
VWA	**V**on **W**illebrand factor **A**

1 INTRODUCTION

Members of the CLCA protein family – originally termed **c**h**l**oride channels, **c**alcium **a**ctivated – were discovered in the early 1990ies by two independent research groups in parallel, either as modulators of **c**alcium **a**ctivated **c**hloride **c**onductances (CaCCs) or as cellular adhesion molecules. Intensive research has not yet succeeded in clarifying this discrepancy but rather revealed more and more possible functions and roles in diseases for the CLCA protein family. Amongst those is the potential role of CLCA proteins as modulators of **c**ystic **f**ibrosis (CF), a lethal inherited disease affecting approximately one out of 2,000 newborns. In cystic fibrosis patients, a mutation of the **C**ystic **F**ibrosis **T**ransmembrane Conductance **R**egulator (CFTR) chloride channel leads to a complex exocrinopathy in organs including lung, intestine, pancreas, liver, vas deferens and sweat glands.

Up to 8 CLCA family members in 12 species have been described until today which show a broad tissue expression pattern including the CF relevant tissues lung and intestine. Besides human CLCA proteins, the most relevant family members for CF research to date are murine CLCA proteins with respect to CF mouse models. In recent years, human hCLCA1 and its murine orthologue mCLCA3, both representing secreted proteins of goblet cells, as well as human hCLCA4 and its murine orthologue mCLCA6, both located in the intestine, have so far been the most promising candidates for modulation of the CF phenotype.

Interestingly, all CLCA family members investigated biochemically so far generally undergo post-translational cleavage into two subunits but differ in the post-cleavage processing of these subunits. CLCA proteins of goblet cells, including hCLCA1 and mCLCA3, are fully secreted as heterodimers whereas in CLCA family members of non-goblet cell epithelial cells only the amino-terminal subunit is shed by the cell. In those CLCA proteins a transmembrane domain anchors the carboxy-terminal subunit to the plasma membrane. To date, this transmembrane domain has only been demonstrated for hCLCA2, a human CLCA family member. Little is known about a potential transmembrane domain in other human or murine CLCA proteins.

The cleaving agent of CLCA proteins remains unknown so far as does the functional role of the cleavage event. Preliminary experiments regarding a HEXXH zinc binding amino acid motif in CLCA proteins, typical of metalloproteases, suggest an autoproteolytic cleavage mechanism and thus a potential protease activity of CLCA proteins. In proteases, autoproteolysis is often essential for either their cellular transport or the activation of their enzymatic activity.

This project aimed at characterizing an autoproteolytic cleavage activity of CLCA proteins. Furthermore, the study aimed at examining murine CLCA family members for a potential transmembrane domain and comparing the cleavage processes of two murine CLCA proteins with and without a transmembrane domain. The results will help to determine the *modus operandi* of CLCA proteins and to understand their modulatory role in diseases such as CF and their biomedical relevance in other diseases.

2 LITERATURE

2.1 Tissue expression patterns of CLCA proteins

Four to eight CLCA protein family members (Figure 1) have been described in 12 species (Patel et al. 2009; Plog et al. 2009) with a broad tissue expression pattern. CLCA family members were given a prefix based on their species (h=human, m=murine, p=porcine, b=bovine, r=rat, e=equine) and a number according to the chronological order of their discovery. For example, human hCLCA1 was the first human CLCA family member to be discovered. This led to confusing pairs of orthologues like human hCLCA1 and its murine orthologue mCLCA3, which is the third discovered murine CLCA family member. To date, the numbering of CLCA family members is still inconsistent. For rat CLCA family members, the numbering has already been revised by Plog and colleagues (Plog et al. 2009), but this manuscript refers to the original nomenclature of the respective first publication. Originally, CLCA proteins were classified as putative chloride channels. To date it has been established that CLCA proteins do not represent ion channels and can therefore no longer be named chloride channels, calcium activated. Future renaming of CLCA proteins will include renumbering of the family members. First attempts have proposed the name chloride channel regulators (CLCR; (Gibson et al. 2005)), but final redefinition was postponed until the conclusive establishment of CLCA protein function.

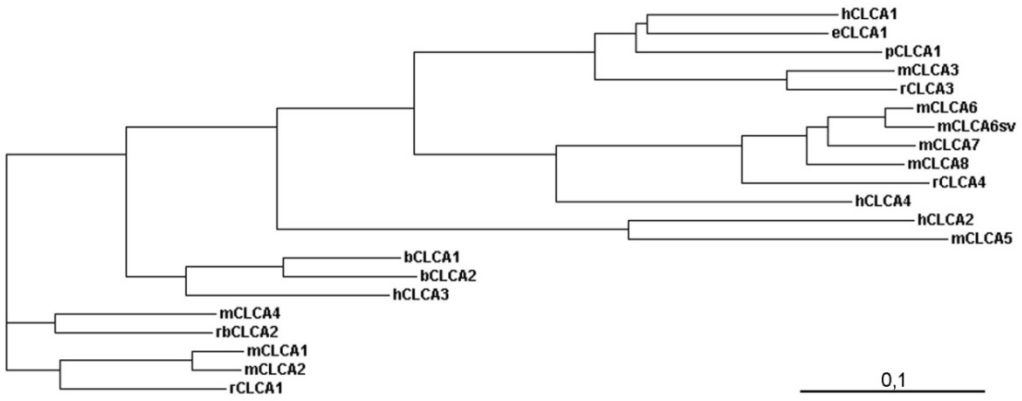

Figure 1: Schematic phylogenetic tree of the most relevant CLCA proteins according to their amino acid sequence identities. Sequence alignment and calculation of the phylogenetic tree were carried out with the ClustalW2 algorithm (Larkin et al. 2007). h = human, m = murine, e = equine, p = porcine, b = bovine, r = rat

Based on their amino acid similarities, the family members can be grouped into 4 clusters (Plog et al. 2009). The expression pattern of CLCA proteins according to their clusters will be described in the following section.

2.1.1 CLCA cluster 1

Members of the CLCA cluster 1 are expressed in goblet cells of mucous membranes, predominantly of the respiratory and the intestinal tracts (Anton et al. 2005; Gruber et al. 1998a; Leverkoehne and Gruber 2002; Plog et al. 2009). CLCA mRNA and/or protein of the following CLCA family members have been detected in the following cells and tissues:

species	CLCA	tissue and cellular expression patterns	mRNA/ protein
human	hCLCA1	intestinal goblet cells and epithelial cells (Gruber et al. 1998a)	mRNA
		intestine, uterus, stomach, testis, kidney, fetal spleen (Agnel et al. 1999)	mRNA
		colon (Bustin et al. 2001)	mRNA
		airways (Toda et al. 2002)	mRNA
		airway goblet cells (Hoshino et al. 2002)	protein
		brain (Zhang et al. 2007)	mRNA
		conjunctival epithelium (Seo et al.)	protein
murine	mCLCA3	uterus, stomach, goblet cells of small intestine and colon, trachea (Komiya et al. 1999)	mRNA
		uterus, stomach, small intestine, colon, ovary; inducible in airway goblet cells (Zhou et al. 2001)	mRNA protein
		uterus, stomach, goblet cells of lung, small intestine, colon and trachea (Leverkoehne and Gruber 2002)	protein
porcine	pCLCA1	enterocytes of ileal crypts and villi (Racette et al. 1996)	protein
		crypt & villus epithelia, trachea: surface epithelium and submucosal glands (Gaspar et al. 2000)	mRNA

		intestine, goblet cells of the respiratory tract, conjunctival mucous membranes, gall bladder, parotid glands, pancreas, bile duct (Plog et al. 2009)	mRNA protein
rat	rCLCA3	brain (Wahl et al. 2009)	mRNA
equine	eCLCA1	mucin-producing cells of the respiratory and intestinal tract, cutaneous sweat glands, renal mucous glands (Anton et al. 2005)	mRNA protein

Table 1: Tissue expression patterns of cluster 1 CLCA proteins.

Besides their modulatory role on calcium activated chloride conductances, family members of this cluster have predominantly been linked to mucus overproduction in respiratory diseases such as CF, asthma and chronic obstructive pulmonary disease in humans and other species .

2.1.2 CLCA cluster 2

The CLCA cluster 2 has been the subject of recent debate, especially in case of the human hCLCA2. This family member was originally identified on the mRNA level in trachea, uterus, prostate, testis and kidney (Agnel et al. 1999), lung and mammary gland (Gruber et al. 1999) but later on turned out to be rather or additionally expressed in conjunctival and corneal epithelium (Itoh et al. 2000), nasal epithelium (Mall et al. 2003) and additionally skin, vagina, esophagus and larynx (Connon et al. 2005; Connon et al. 2004). Previous data on the expression pattern of CLCA of cluster 2 mRNA and/or protein are summarized in table 2:

species	CLCA	tissue and cellular expression patterns	mRNA/ protein
human	hCLCA2	trachea, lung, mammary gland (Gruber et al. 1999)	mRNA
		trachea, uterus, prostate, testis, bladder, stomach, kidney (Agnel et al. 1999)	mRNA
		cornea (Itoh et al. 2000)	mRNA
		colon (Bustin et al. 2001)	mRNA
		endothelial cells of pulmonary arteries, arterioles and	mRNA

		venules (Abdel-Ghany et al. 2001)	protein
		native nasal tissue (Mall et al. 2003)	mRNA
		cells of stratified squamous epithelium adjacent to the basement membrane of the skin, corneal, vaginal, esophageal, laryngeal epithelia (Connon et al. 2004)	protein
murine	mCLCA5	intestine, lung, heart, stomach, spleen, eye, skeletal muscle, testes (Evans et al. 2004)	mRNA
		intestine, lung, heart, stomach, spleen, pancreas, aorta, kidney, esophagus, uterus, lactating mammary gland (Beckley et al. 2004)	mRNA

Table 2: Tissue expression patterns of cluster 2 CLCA proteins.
Members of this cluster have been predominantly described in cell-cell adhesion (Connon et al. 2005) and mediation of tumor growth (Abdel-Ghany et al. 2001; Beckley et al. 2004; Gruber and Pauli 1999b; Walia et al. 2009).

2.1.3 CLCA cluster 3

The human family member hCLCA3 represents a truncated CLCA protein, possibly a pseudogene with a premature stop codon (Gruber and Pauli 1999a). The truncated hCLCA3 might therefore possess a distinct function compared to other CLCA proteins (Gruber and Pauli 1999a). The expression patterns of CLCA proteins of cluster 3 are summarized in table 3. Murine mCLCA1 and mCLCA2 are highly homologous and share approximately 98% amino acid identity and are therefore described as mCLCA1/2 (Leverkoehne et al. 2002).

species	CLCA	tissue and cellular expression patterns	mRNA/ protein
human	hCLCA3	trachea, uterus, prostate, testis, kidney (Agnel et al. 1999)	mRNA
		lung, trachea, spleen, thymus, mammary gland (Gruber and Pauli 1999a)	mRNA
murine	mCLCA1/2	respiratory epithelia of lung and trachea (Gandhi et al. 1998)	mRNA

		mammary gland, respiratory and intestinal epithelia, gall bladder, pancreas, kidney, uterus, epididymis, keratinocytes of the skin, esophagus and cornea (Gruber et al. 1998b)	mRNA
		kidney, liver, spleen (Romio et al. 1999)	mRNA
		mammary gland (Lee et al. 1999)	mRNA
		skin, adrenal gland, heart, kidney, thymus, stomach, small intestine, muscle (Elble and Pauli 2001)	mRNA
		colon, stomach, jejunum, atrium, ventricle, portal vein, pulmonary artery (Britton et al. 2002)	mRNA
		mCLCA1 only: liver, aorta, spleen, lymph nodes, bone marrow mCLCA2 only: mammary glands and fetus day 8.5 (Leverkoehne et al. 2002)	mRNA
		pancreatic zymogen granules, submandibular granular duct cells, luminal membranes of parotid and submandibular ducts, gastric parietal and small intestinal crypts, renal distal and proximal tubule cells (Roussa et al. 2010)	protein
	mCLCA4	smooth muscle cells of intestine, stomach, esophagus, uterus, bladder, aorta, lung; skeletal muscle, heart (Elble et al. 2002)	mRNA
bovine	bCLCA1	trachea (Cunningham et al. 1995; Ran and Benos 1991)	mRNA protein
		corneal epithelium (Zhang et al. 2002)	mRNA
	bCLCA2 alias Lu-ECAM-1	endothelium of venules in lung (Zhu et al. 1991) bronchi and trachea (Elble et al. 1997)	mRNA protein
rat	rCLCA1	striated ducts of submandibular glands, ileum, lung (Yamazaki et al. 2005)	mRNA protein
	rbCLCA1	neurons and glial cells of cerebrum and cerebellum, kidney, small intestine, stomach (Jeong et al. 2005)	mRNA

	rbCLCA2	cerebrum, cerebellum, kidney, stomach, spinal cord, lung, small intestine (Yoon et al. 2006)	mRNA
	ratCaCC1	zymogen granules of the pancreatic ducts (Thevenod et al. 2003)	mRNA protein

Table 3: Tissue expression patterns of cluster 3 CLCA proteins. Alias = bCLCA2 was first discovered as **Lu**ng **E**ndothelial **C**ell **A**dhesion **M**olecule 1 (Lu ECAM-1)

The functional role of cluster 3 has not yet been resolved. Members of the cluster were previously described to play a role in cell adhesion (Furuya et al. 2010) or metastasis and metastatic growth of tumor cells (Abdel-Ghany et al. 2003; Abdel-Ghany et al. 2002; Goetz et al. 1996; Zhu et al. 1992; Zhu et al. 1991). However, expression of mCLCA1/2 in the gastro-intestinal tract (Roussa et al. 2010) as well as rbCLCA1 and rbCLCA2 in the brain (Jeong et al. 2005; Yoon et al. 2006) might also allow for another, yet unidentified biomedical significance.

2.1.4 CLCA cluster 4

Human hCLCA4 was the first CLCA family member to be detected in the brain (Agnel et al. 1999). Its murine orthologue mCLCA6, however, could not be detected in the brain but in intestine, stomach, eye and spleen (Evans et al. 2004).

species	CLCA	tissue and cellular expression patterns	mRNA/ protein
human	hCLCA4	brain, intestine, bladder, uterus, prostate, stomach, testis, salivary gland, mammary gland, trachea (Agnel et al. 1999)	mRNA
		native nasal tissue (Mall et al. 2003)	mRNA
		oral tongue squamous epithelium (Ye et al. 2008)	mRNA
murine	mCLCA6	stomach, intestine, liver (Evans et al. 2004)	mRNA
rat	rCLCA4	uterus, muscle, liver, lung, heart (Song et al. 2009)	mRNA protein

Table 4: Tissue expression patterns of cluster 4 CLCA proteins.

Apart from the hCLCA4 protein which has been discussed to either play a role in modulation of the CF phenotype (Ritzka et al. 2004) and Crohn's disease (Comelli et al. 2009) or in oral tongue squamous cell carcinomas (Ye et al. 2008), the biomedical role of none of the CLCA family members of cluster 4 has yet been identified.

2.2 Biomedical relevance of CLCA proteins

2.2.1 Diseases with secretory dysfunction

CLCA proteins have been linked to diseases with secretory dysfunctions like **c**ystic **f**ibrosis (CF), asthma or **c**hronic **o**bstructive **p**ulmonary **d**isease (COPD). Their explicit role in these diseases is not yet fully understood; both a modulatory role of **ca**lcium activated **c**hloride **c**onductances (CaCC) and a regulatory role on mucus hypersecretion have been proposed (Gruber et al. 2000; Patel et al. 2009).

2.2.1.1 Modulation of calcium activated chloride channels in cystic fibrosis

Chloride channels play a role in different cellular processes including fluid secretion of various epithelia including lung or intestine and excitation of neurons or muscle cells (Eggermont 2004). Defects in chloride conductances, also called channelopathies, lead to several diseases including CF (Planells-Cases and Jentsch 2009). CF is a lethal, autosomal recessive disease with an incidence of 1/2,000 – 3,000 in nations of European origin (Jonsdottir et al. 2008). The pathogenesis of the disease is based on one of several mutations of the **C**ystic **F**ibrosis **T**ransmembrane **C**onductance **R**egulator (CFTR) chloride channel (Anderson et al. 1991; Kerem et al. 1989). Defective chloride secretion and hyperabsorption of sodium leads to an exocrinopathy in organs including lung, intestine, pancreas, liver, vas deferens and sweat glands (Schwiebert et al. 1998). The mortality of the patients is due to the lung phenotype of CF where plugs of sticky, dehydrated mucus promote recurrent lung infections with bacteria such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, resulting in chronic pneumonia (Schwiebert et al. 1998). Other clinical symptoms include pancreatic insufficiency, bile duct obstruction, intestinal obstruction (meconium ileus), infertility in males and high sweat chloride concentration (Schwiebert et al. 1998).

To date, more than 1,000 different mutations in the CFTR causing CF are known (Jonsdottir et al. 2008). Interestingly, the severity of the phenotypes of CF patients differs between individuals carrying same mutation (Kerem et al. 1990). This phenomenon might be due to either environmental or genetic modulators. In CF mouse models, the severity of the intestinal phenotype also varies due to the genetic background of the mice (Rozmahel et al. 1996), pointing towards genetic rather than environmental factors. Among those genetic factors, CaCCs of yet unknown molecular identity may at least in part compensate for the CFTR defect (Figure 2; (Anderson and Welsh 1991; Bronsveld et al. 2001; Rozmahel et al. 1996).

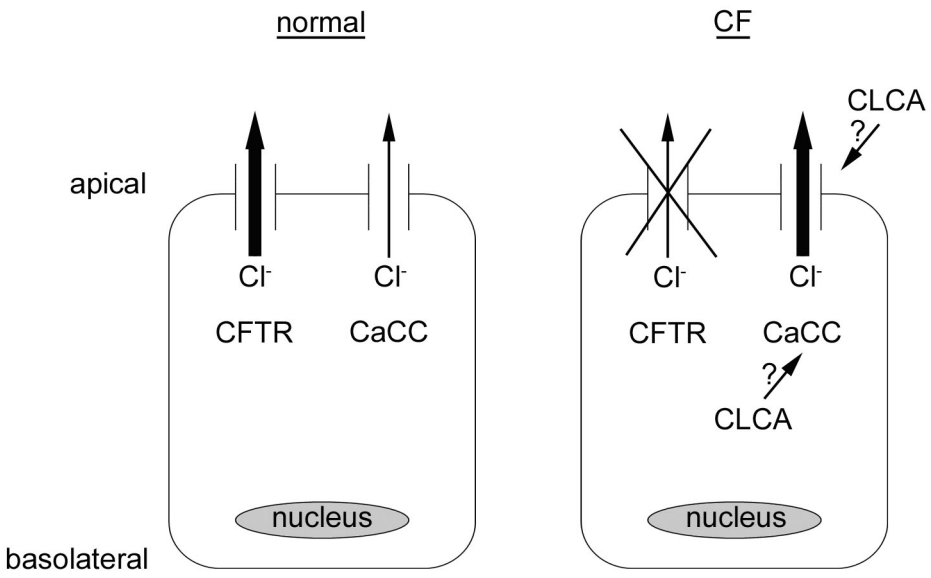

Figure 2: Schematic illustration of chloride conductance in normal versus CF cells. CFTR = **C**ystic **F**ibrosis **T**ransmembrane Conductance **R**egulator chloride channel, CaCC = **c**alcium **a**ctivated **c**hloride **c**onductances, Cl^- = chloride anions, CLCA = **c**h**l**oride channels, **c**alcium **a**ctivated

Members of the CLCA family of proteins have been shown to modulate CaCCs (Figure 2) in a yet unknown mechanism (Gruber et al. 2000; Hamann et al. 2009) and several CLCA family members have been proposed to alter the severity of the phenotype of CF patients and CF mouse models (Hauber et al. 2004; Ritzka et al. 2004; van der Doef et al. 2010; Young et al. 2007).

The most CF relevant human CLCA family members are hCLCA1 and hCLCA4. hCLCA1 is located in goblet cells of the respiratory and intestinal tract (Gruber et al. 1998a) while hCLCA4 has been detected in the intestinal tract and trachea, but not in the lung (Agnel et al.

1999). In the gastrointestinal tract, a significant association has been observed for the electrophysiological phenotype of CF patients with allele distributions of hCLCA1 and hCLCA4 (Ritzka et al. 2004). Allelic variants of hCLCA1 have also been shown to alter the severity of the meconium ileus of CF patients (van der Doef et al. 2010). In the respiratory tract of CF patients, hCLCA1 has been shown to be upregulated on the mRNA level (Hauber et al. 2003; Hauber et al. 2004), implying a modulatory role of human CLCA proteins in the disease.

In analogy to human CLCA proteins in CF patients, murine CLCA family members are discussed to modulate the phenotype of CF mouse models. Such models develop severe meconium ileus like in human disease, but CF mice do not reproduce the spontaneous lung disease typical of CF patients (for review on CF mouse models, see (Wilke et al. 2011)). The murine orthologue of hCLCA1, named mCLCA3, is located in goblet cells of the respiratory and intestinal tract (Leverkoehne and Gruber 2002). In the small intestine of CF mouse models ($cftr^{tm1Cam}$, $cftr^{TgH(neoim)1Hgu}$), mCLCA3 was upregulated on the mRNA level (Leverkoehne et al. 2006). In contrast, a study using other CF mouse models ($cftr^{tm1Unc}$) reported reduced expression of mCLCA3 on the protein level (Brouillard et al. 2005). This indicates either a post-translational regulation of mCLCA3 or the expression of mCLCA3 is specific for each CF mouse model. Restoration of mCLCA3 mRNA and protein in CF mouse models led to amelioration of the intestinal phenotype (Young et al. 2007), implying a modulatory key role for mCLCA3 in CF mouse models. The murine orthologue of hCLCA4, named mCLCA6, has also been detected in the intestine on the mRNA level (Evans et al. 2004), but neither the cellular expression pattern of this family member nor its role in CF mouse models have yet been determined.

The *modus operandi* in which CLCA proteins modulate CaCC has been the subject of recent debate. Both modulatory roles as signalling molecules or chloride channel activating roles as proteases have been proposed, but none has been substantially corroborated yet (Gibson et al. 2005; Hamann et al. 2009; Mundhenk et al. 2006; Pawlowski et al. 2006). Of note, Young and colleagues reported amelioration of the phenotype of CFTR -/- mice after restoration of mCLCA3, but the restoration of mCLCA3 did not change the electrophysiological properties of CFTR -/- mice (Young et al. 2007). Thus, modulation of the CF phenotype might be based on a CLCA function distinct from chloride channel regulation.

2.2.1.2 Mucus hypersecretion in cystic fibrosis, asthma and chronic obstructive pulmonary disease

In addition to modulation of CaCCs, secreted CLCA family members of goblet cells, namely hCLCA1 and mCLCA3, have also been shown to play a key role in mucus hypersecretion. Goblet cell hyperplasia and –metaplasia as well as mucus hypersecretion are a shared key feature of diseases like CF, COPD or asthma (Rogers 2007), suggesting a modulatory role of CLCA proteins not only in CF.

Airway epithelia of both CF and asthmatic patients show upregulation of hCLCA1 on the mRNA level (Hauber et al. 2004; Hoshino et al. 2002; Nakanishi et al. 2001; Toda et al. 2002). The murine orthologue of hCLCA1, mCLCA3, was also upregulated on the mRNA level in ovalbumin-challenged mouse models that express the asthma phenotype including airway hyperresponsiveness (AHR), goblet cell hyperplasia and –metaplasia (Nakanishi et al. 2001). Conversely, downregulation of mCLCA3 mRNA suppressed mucus overproduction and AHR in those mouse models (Nakanishi et al. 2001). Unfortunately, the mouse models used did not allow distinction of the role of mCLCA3 in either mucus overproduction or AHR alone. *In vitro*, overexpression of hCLCA1 or mCLCA3 mRNA in the mucoepidermoid cell line NCI-H292 increases mucus production (Hoshino et al. 2002; Nakanishi et al. 2001; Zhou et al. 2002), corroborating the role in goblet cell metaplasia. To separate the role of mCLCA3 in goblet cell hyperplasia and –metaplasia from its role in AHR *in vivo*, Patel and colleagues have investigated mouse models that distinctively expressed either goblet cell hyperplasia or AHR (Patel et al. 2006). Goblet cell hyperplasia induced mCLCA3 in a mouse model *in vivo* but interestingly mCLCA3 was not inducible with AHR (Patel et al. 2006). Conversely, mClca3 gene transfer to mouse airway epithelium induced goblet cell metaplasia but not AHR (Patel et al. 2006). This indicates that the role of hCLCA1 and mCLCA3 in asthma is rather goblet cell hyper- and metaplasia and mucus overproduction than airway hyperresponsiveness.

To further corroborate the role of mCLCA3 in goblet cell hyperplasia, mCLCA3 -/- mice were generated. In an initial study, mCLCA3 deficient mice bred on the C57BL/6 background have shown severe goblet cell hyperplasia after ovalbumin challenge similar to wild-type mice (Robichaud et al. 2005). This might be due to a compensatory effect of another CLCA family member, because Patel and colleagues have reported upregulation of mCLCA5 mRNA after virus induction of mucus cell metaplasia in the lungs of mCLCA3 -/- mice (Patel et al. 2006). Nothing is known about a similar compensatory mechanism in humans. Of note, in mCLCA3 -/- mice on 129SvJ background, differences compared to the wild-type mice including reduced goblet cell hyperplasia and mucus overproduction have been observed (Long et al.

2006; Robichaud et al. 2005). Those strain dependent differences in the phenotype suggest that the regulatory role of CLCA protein in mucus overproduction might be more complex than previously thought.

In addition to CF and asthma, mucous hypersecretion is also a key feature of COPD in humans and the corresponding disease **r**ecurrent **a**irway **o**bstruction (RAO) in horses. In humans, hCLCA1 mRNA turned out to be upregulated in airways of COPD patients (Hauber et al. 2005a; Wang et al. 2007) and a hCLCA1 gene polymorphism was discussed as a tool for predicting COPD susceptibility (Hegab et al. 2004). In RAO affected horses, the equine orthologue eCLCA1 mRNA was originally reported to be overexpressed (Anton et al. 2005). But as the number of eCLCA1 mRNA copies per goblet cell is the same between horses affected by RAO and healthy horses, this overexpression is due to goblet cell hyperplasia rather than transcriptional upregulation in individual cells (Range et al. 2007). A recent study suggested an alternative, eCLCA1 independent, regulatory pathway leading to mucous overproduction in RAO (Ryhner et al. 2008) and thus the role of CLCA in RAO remains to be determined.

The putative signalling pathway of CLCA proteins leading to mucous overproduction is still under investigation. Expression of Th2 cytokines (IL-9, IL-13, IL-4), LPS, histamine and TNF-α in both cell cultures and mouse models of airway diseases lead to increased hCLCA1 or mCLCA3 mRNA expression (Busse et al. 2005; Endo et al. 2007; Hauber et al. 2007a; Hauber et al. 2005b; Hauber et al. 2007b; Hauber et al. ; Hauber et al. 2004; Kim et al. 2007; Toda et al. 2002; Yasuo et al. 2006; Zhou et al. 2001; Zhou et al. 2002). In general, signals of IL-4 and IL-13 are transmitted by STAT6 and several studies support the hypothesis that an IL-13 induction of hCLCA1 or mCLCA3 expression may take place via the JAK/STAT6 pathway (Lee et al. 2002; Matsunaga et al. 2011; Patel et al. 2009; Thai et al. 2005). Interestingly, cytokine levels of the mouse strain play a crucial role in phenotype development of mCLCA3 -/- mouse models. As mentioned, differences compared to the wild-type mice like reduced goblet cell hyperplasia and mucus overproduction could only be observed in mCLCA3 -/- mice on the 129SvJ background, but not on the C57BL/6 background (Long et al. 2006; Robichaud et al. 2005). 129SvJ mice physiologically express higher levels of Th2 cytokines than C57BL/6 mice (Long et al. 2006; Robichaud et al. 2005). This further corroborates the hypothesis that Th2 cytokines are involved in the CLCA signalling pathway.

The target genes or proteins of CLCA proteins in mucus overproduction are still under investigation. Among the candidate target molecules are mucins, glycoproteins representing the gel forming components of mucus. Mucins are encoded by **muc**us (MUC) genes. The

downstream influence of CLCA proteins on MUC5AC, the most relevant mucus gene, has been intensely studied in the past. Initial studies have suggested that overexpression of hCLCA1 leads to induction of MUC5AC *in vitro* in NCI-H292 cells (Hauber et al. 2005b; Hoshino et al. 2002; Kim et al. 2007; Nakanishi et al. 2001; Seo et al. 2011). In contrast, one study reported the MUC5AC gene not to be induced by hCLCA1 in primary tracheobronchial epithelial cells (Thai et al. 2005). But when measured *in vivo*, upregulation of hCLCA1 mRNA was always associated with upregulation of MUC5AC mRNA (Busse et al. 2005; Endo et al. 2007; Hauber et al. 2007a; Hauber et al. 2005b; Hauber et al. 2007b; Hauber et al. ; Hauber et al. 2004; Kim et al. 2007; Toda et al. 2002; Yasuo et al. 2006; Zhou et al. 2001; Zhou et al. 2002), further corroborating a putative induction of MUC5AC by hCLCA1. In addition to the results on MUC genes, a recent study has reported the association of hCLCA1 with other proteins in co-immunoprecipitation experiments of primary cultures of well differentiated human bronchial epithelial cells (Raiford et al. 2011). **M**yristoylated **A**lanine-**R**ich **C** **K**inase **S**ubstrate (MARCKS), **H**eat **S**hock **P**rotein **70** (HSP70) and **C**ysteine **S**tring **P**rotein (CSP) were detected in immunoblots after precipitation with anti-hCLCA1/mCLAC3 antibody (Raiford et al. 2011). The putative functional relationship between these proteins and hCLCA1 is unclear at present and the precise role of CLCA in the molecular pathway of mucus production still needs to be elucidated.

2.2.2 Cell adhesion under physiologic conditions and in metastasizing tumor cells

Since the discovery of CLCA proteins, the role of CLCA in secretory diseases and their role in tumor metastasis and the development of cancer have been studied in parallel. The first CLCA family member bCLCA2 was initially described as Lu ECAM-1, a lung endothelial cell adhesion molecule (Zhu et al. 1991). Cell adhesion molecules play a key role in cell-to-cell or cell-to-extracellular-matrix interactions of metastasizing tumor cells (Makrilia et al. 2009) as well as lymphocytes (Stoolman 1989). *In vitro*, bCLCA2 was able to bind the lung-colonizing melanoma derived cells B16-F10 (Goetz et al. 1996; Zhu et al. 1991) and an antibody directed against bCLCA2 alias Lu ECAM-1 prevented lung metastasis of tumor cells in a mouse model (Zhu et al. 1992; Zhu et al. 1991).

The role of CLCA in cell adhesion is not restricted to tumor cells. Human hCLCA2 is co-localized with beta4 integrin in basal cells of stratified epithelia, suggesting a general role in cell adhesion for this CLCA homologue (Connon et al. 2005). Furthermore, murine mCLCA1 was shown in lymphatic endothelium to interact with **l**ymphocyte **f**unction-**a**ssociated antigen **1** (LFA-1) and **mac**rophage-**1** antigen (Mac-1), both representing leukocyte-integrins that mediate leukocyte adhesion to endothelium (Furuya et al. 2010). The role of CLCA proteins

in endothelial cell adhesion might therefore not only be restricted to tumor cells, but they may also play a key role in cell adhesion or endothelial adhesion of leukocytes.

Only preliminary data exist on the pathway induced after adhesion of tumor cells to CLCA. Adhesion of human breast cancer cells to lung endothelial hCLCA2 has been shown to be mediated via beta4 integrin expressed on the surface of tumor cells (Abdel-Ghany et al. 2001). After ligation of beta4 integrin to CLCA, FAK/ERK signalling was initiated in tumor cells to promote tumor cell proliferation as shown for murine mCLCA1 (Abdel-Ghany et al. 2002). Therefore, CLCA proteins might bind metastasizing tumor cells and then induce tumor growth at the site of metastasis.

Interestingly, the integrin binding motif identified in several CLCA family members is not shared exactly among all CLCA proteins. Most prominently, it is disrupted in hCLCA1 (Abdel-Ghany et al. 2003). This raises the question of whether all clusters of CLCA proteins may have the same properties and functions.

2.2.3 Role in apoptosis and tumor growth

CLCA proteins have also been described as growth inhibitors of tumor cells. Expression of hCLCA2 mRNA is lost in breast cancer and tumorigenic breast cancer cell lines, suggesting a physiological role of CLCA proteins in tumor suppression or growth inhibition (Gruber and Pauli 1999b). Recently, hCLCA2 was shown to inhibit growth of breast cancer cells and other tumor cells after induction by p53 or DNA damaging agents, indicating that in return downregulation of hCLCA2 in cancer cells might lead to a survival advantage of those cells (Walia et al. 2009). Induction of detachment-sensitive inhibition of tumor cell growth was also reported for the murine hCLCA2-orthologue mCLCA5 and for mCLCA1/2 (Beckley et al. 2004; Elble and Pauli 2001).

Though investigations have first concentrated on the role of CLCA proteins of cluster 2, other CLCA proteins have also been reported to be downregulated in some forms of cancer although they have been originally described in other tissues or with other functions. For example, hCLCA1 was shown to be downregulated in colorectal cancer (Bustin et al. 2001) as was hCLCA4 in tongue squamous cell carcinoma (Ye et al. 2008). Although these family members have originally been associated with other functions, their downregulation in cancer might indicate a role in growth inhibition or suppression of tumor cells.

In addition to its role in secretory disorders or tumorsuppression, a distinct expression site and third function has recently been added to the complex picture of the family member hCLCA1. On the mRNA level, hCLCA1 has been shown to be upregulated in areas of the brain that undergo cell death and additionally specific induction of hCLCA1 in neurons led to neuronal death (Wahl et al. 2009; Zhang et al. 2007), extending the role of CLCA from growth inhibition to cell death pathways.

2.3 Structure and biochemical characteristics of CLCA proteins

CLCA family members were initially described to possess up to five transmembrane domains (Gruber et al. 1998a; Gruber et al. 1999). To date it is known that based on the differential processing of the subunits after post-translational cleavage, two subgroups of CLCA proteins can be distinguished. In one subgroup, both subunits are secreted (Gibson et al. 2005; Mundhenk et al. 2006), and in the other group, the carboxy-terminal subunit possesses a transmembrane segment, resulting in shedding of only the amino-terminal subunit (Elble et al. 2006). These transmembrane CLCA proteins represent type I single-pass transmembrane proteins with the amino-terminal subunit and part of the carboxy-terminal subunit located extracellularly and the carboxy-terminus located intracellularly.
According to the revised structural model of CLCA proteins, the amino-terminal subunit of CLCA proteins consists of an amino-terminal domain (n-CLCA) and a von Willebrand factor type A (vWA) domain (Patel et al. 2009). The exact cleavage site has not yet been determined. The carboxy-terminal subunit contains a fibronectin type III (FnIII) domain and some CLCA proteins possess a transmembrane domain in this subunit (Patel et al. 2009).

2.3.1 n-CLCA domain

The n-CLCA domain of CLCA proteins bears a HEXXH zinc-binding amino acid motif (Pawlowski et al. 2006), which is typical of metalloproteases but can sometimes also be found in other proteins like the bradykinin B1 receptor (Ignjatovic et al. 2002). Experimental mutation E157Q of the HEXXH motif in the human hCLCA1 resulted in abrogated cleavage of the hCLCA1 protein (Pawlowski et al. 2006), indicating that this motif is crucial for the cleavage process. Impeded cleavage in that study could either be due to self-cleavage of the protein or to improper folding, resulting in failure of protease targeting (Patel et al. 2009). Thus, the exact mechanism of the cleavage as a crucial feature of CLCA proteins as well as the cleaving agent remains to be identified (see chapter 2.4.3). Carboxy-terminally to the

HEXXH motif, a cystein-rich motif of five cystein residues (Pauli et al. 2000) might be responsible for disulfide bonds stabilizing the protein.

2.3.2 Von Willebrand factor A domain

The n-CLCA domain of CLCA proteins is carboxy-terminally followed by a **v**on **W**illebrand factor **A** (VWA) domain (Loewen and Forsyth 2005; Patel et al. 2009). This protein-protein-interaction domain is widely distributed in extracellular matrix proteins, integrins, ion channel subunits and protease inhibitors, implicating not only the well-known role of the domain in cell adhesion, but also a possible role in protease regulation (Whittaker and Hynes 2002). Most of the CLCA proteins also possess a **m**etal-**i**on **d**ependent **a**dhesion **s**ite (MIDAS) in the VWA (Loewen and Forsyth 2005; Patel et al. 2009). This MIDAS binds bivalent cations to increase the stability of the protein-protein-interactions (Loewen and Forsyth 2005). However, without structural data of CLCA proteins, the role of the MIDAS in CLCA protein interactions can only be vaguely assumed.

2.3.3 Fibronectin type III domain

The carboxy-terminal subunits of human and murine CLCA proteins possess an extracellular **f**ibro**n**ectin type **III** (FnIII) domain, except hCLCA3 and mCLCA8 which represent CLCA proteins only bearing a shortened amino-terminal subunit (Patel et al. 2009). Apart from fibronectin, FnIII domains are supposed to exist in approximately 2% of all proteins (Bloom and Calabro 2009), including cell-adhesion molecules (Togashi et al. 2009) and enzymes (Matozaki et al. 2010). The FnIII domain is predominantly responsible for protein-protein interactions (Ohashi et al. 2009; Yang et al. 2008). As the FnIII domain of CLCA proteins is located in the extracellular part of the carboxy-terminal subunit in both partially and fully secreted CLCA proteins (Patel et al. 2009), a putative role in protein-protein-interactions with extracellular proteins remains to be determined.

2.4 Cellular processing of CLCA proteins

2.4.1 Signal sequence

Computational sequence analyses predict a putative signal sequence in CLCA proteins (Loewen and Forsyth 2005). Experimental data revealed transport of CLCA family members via the endoplasmic reticulum and the Golgi apparatus either to the plasma membrane or into the extracellular space (Elble et al. 2006; Gibson et al. 2005; Mundhenk et al. 2006; Plog et al. 2009; Range et al. 2007). The signal sequence has been shown to be cleaved off intracellularly in bCLCA2 (Elble et al. 1997). Though the cleavage of the signal sequence has not yet been proven but predicted for other CLCA family members (Gibson et al. 2005; Gruber et al. 1998a) it is likely to assume cleavage of the signal sequence for other CLCA family members.

2.4.2 Glycosylation

CLCA proteins possess multiple potential asparagine-linked glycosylation sites (Loewen and Forsyth 2005), but only preliminary data on the *de facto* glycosylation sites of CLCA proteins are available (Gruber et al. 1999). Glycosylation of the precursor molecule has been performed for hCLCA1, eCLCA1, bCLCA2, mCLCA3, mCLCA5 and mCLCA6 using coupled transcription and translation in the presence of canine microsomal membranes (Elble et al. 1997; Evans et al. 2004; Gruber et al. 1998a; Leverkoehne and Gruber 2002; Range et al. 2007). The precursor molecules of all the proteins mentioned show a shift in size due to glycosylation after addition of canine microsomal membranes. To distinguish between mannose-rich glycosylation in the endoplasmic reticulum and complex glycosylation during the passage through the Golgi apparatus, deglycosylation experiments were performed using CLCA family member overexpressing HEK293 cells. Deglycosylation studies on eCLCA1, pCLCA1 and mCLCA3, CLCA proteins of cluster 1 revealed a mannose-rich glycosylated precursor molecule that reaches the endoplasmic reticulum (Mundhenk et al. 2006; Plog et al. 2009; Range et al. 2007). It is cleaved before complex glycosylation occurs and the resulting subunits both pass the Golgi apparatus and receive a complex glycosylation (Mundhenk et al. 2006; Plog et al. 2009; Range et al. 2007). In contrast, the precursor molecule of hCLCA2, a CLCA family member of cluster 2, passes the Golgi apparatus as an uncleaved, complex glycosylated glycoprotein (Elble et al. 2006). Thus, all CLCA proteins investigated so far pass the endoplasmic reticulum and the Golgi apparatus, but are either cleaved in the endoplasmic reticulum or a post-Golgi compartment.

2.4.3 Cleavage of CLCA proteins

As a conserved feature, all CLCA proteins are post-translationally cleaved into an approximately 90 kDa amino-terminal and an approximately 35 kDa carboxy-terminal subunit (Pauli et al. 2000). Although post-translational cleavage is a common feature of all CLCA proteins investigated so far, the sparse data on the cleavage mechanism pose just as many questions as answers.

The cellular compartment where cleavage occurs differs between CLCA family members. All CLCA proteins of cluster 1 investigated to far are cleaved in the endoplasmic reticulum or early Golgi apparatus (Mundhenk et al. 2006; Plog et al. 2009; Range et al. 2007). The same was shown for mCLCA4 as a protein of cluster 3 (Huan et al. 2008) whereas the cluster 2 CLCA protein hCLCA2 is cleaved at the plasma membrane (Elble et al. 2006). It is unclear at present whether cleavage at the plasma membrane is a specific phenomenon of hCLCA2 or a specific phenomenon of human CLCA family members. Knowledge of the cellular compartment could help identifying the cleaving agent, because some proteases show compartment-specific expression, i.e. the **R**as **c**onverting **e**nzyme 1 **p**rotease (Rce1p) was exclusively detected in the endoplasmic reticulum (Schmidt et al. 1998).

Only preliminary data exist on the exact cleavage site of CLCA proteins. For mCLCA3, analysis of the amino-terminal subunit via MALDI-TOF-MS resulted in detection of peptides including amino acid 686, indicating a cleavage site carboxy-terminally to this position (Brouillard et al. 2005). Analysis of the amino-terminal end of the carboxy-terminal subunit of bCLCA2 via Edman degradation showed that the carboxy-terminal subunit of bCLCA2 starts at amino acid 703 (Elble et al. 1997). The carboxy-terminus of the amino-terminal subunit of bCLCA2 was not investigated in that study, thus it is unclear whether CLCA proteins are cleaved at a single or more cleavage sites by one or more cleaving agents.

The cleaving agent of CLCA proteins is also still unknown. CLCA proteins are cleaved both during their cellular transport *in vivo* as well as in heterologous mammalian expression systems, implying a universal cleaving agent (Gruber 2002). Recently, self-cleavage of CLCA proteins has been proposed in accordance with the HEXXH motif mentioned above (Pawlowski et al. 2006). Cleavage was abrogated after mutation of the E of the HEXXH motif in hCLCA1 into Q (Pawlowski et al. 2006). However, autoproteolytic activity of CLCA proteins remains to be proven because abrogation of cleavage might also be due to improper protein folding or accumulation of the protein in the cell. Furthermore, only preliminary data exist on a secreted CLCA family member of cluster 1, leaving the question of whether cleavage of a CLCA protein bearing a transmembrane domain would also be depending on the HEXXH

motif. Of note, autoproteolytic activity would be a further hint towards a possible role of CLCA proteins as metalloproteases.

The functional significance of CLCA cleavage has not yet been identified. The possible functions of protein cleavage include degradation, cellular transport or activation of the protein. Cellular transport of all CLCA protein investigated so far is conducted via the endoplasmic reticulum and the Golgi apparatus. The murine CLCA proteins of cluster 3, namely mCLCA1/2 and mCLCA4, possess a dileucine forward trafficking signal that promotes export from the endoplasmic reticulum (Huan et al. 2008). Mutation of this dileucine signal not only resulted in retention of mCLCA4 in the endoplasmic reticulum but also in abrogated cleavage (Huan et al. 2008). Despite the fact that this motif is exclusively present in mCLCA1/2 and mCLCA4, these data suggest that cellular transport and proteolytic processing of CLCA proteins may be linked to one another. It would thus be interesting to test in return whether an uncleaved CLCA protein bearing the E157Q mutation of the HEXXH motif undergoes physiological cellular transport.

In addition to cellular transport and degradation, cleavage might be essential for activation of a protein (Hughey et al. 2007; Soh et al. 2010). In proteases, for example, proteolytic cleavage might lead to activation of the inactive protease precursor molecule called zymogen as shown for caspases (for review on caspase activation mechanism, see (Boatright and Salvesen 2003). CLCA proteins possess a HEXXH zinc-binding motif, providing a hint towards a potential metalloprotease activity (Pawlowski et al. 2006). Thus, characterization of the cleavage process might lead to a better understanding of a possible role of CLCA proteins as proteases.

2.4.4 Secretion of whole protein versus ectodomain shedding of transmembrane CLCA proteins

Despite initial data that have suggested multiple transmembrane domains in CLCA proteins (Gruber et al. 1999), today it is known that these proteins obviously do not possess more than one transmembrane domain. After passing the Golgi apparatus, CLCA proteins are either secreted by the cell or anchored to the plasma membrane via a transmembrane domain in the carboxy-terminal subunit, resulting in ectodomain shedding of the amino-terminal subunit. Functional and biochemical investigations of CLCA should consider comparison of both subgroups before making general statements about CLCA proteins.

The CLCA proteins of cluster 1, namely hCLCA1, mCLCA3, pCLCA1 and eCLCA1, are fully secreted proteins (Gibson et al. 2005; Mundhenk et al. 2006; Plog et al. 2009; Range et al. 2007). After cleavage in the endoplasmic reticulum, both subunits stay associated with one another, are complex glycosylated in the Golgi apparatus and secreted into the supernatant (Mundhenk et al. 2006). Of note, all fully secreted CLCA proteins are expressed in goblet cells and secreted into the extracellular space. This might indicate an extracellular function, e.g. as signaling molecules.

The precursor molecule of cluster 2 family member hCLCA2 passes the Golgi apparatus and undergoes cleavage at the plasma membrane (Elble et al. 2006). Here, the carboxy-terminal subunit is anchored to the cell via a transmembrane domain and only the amino-terminal subunit is shed by the cell (Elble et al. 2006). Interspecies conservation of this phenomenon has been shown for cluster 2 family member bCLCA2 (Elble et al. 2006) but none of the other clusters of CLCA proteins have been investigated. Two additional murine CLCA proteins, termed mCLCA5 and mCLCA6, have been detected at the plasma membrane (Evans et al. 2004). However, a transmembrane domain has not yet been proven for any of them.

3 AIMS OF THIS PH.D. PROJECT

Several studies have proposed a role of CLCA proteins as modulators of the CF phenotype but their exact modulatory role remained elusive. As a HEXXH zinc-binding amino acid motif in the amino-terminal subunit of CLCA proteins suggested a role for these proteins as metalloproteases (Pawlowski et al. 2006), the cleavage of secreted and transmembrane CLCA proteins themselves was investigated in this study.

Hypothesis I:

CLCA proteins show characteristics of metalloproteases

- HEXXH amino-acid motif
- zinc-dependent cleavage mechanism
- activation by a protease or autoproteolysis

A protease or proteinase is an enzyme that cleaves another protein. In metalloproteases, the proteolytic mechanism involves a metal. This metal can be bound by specific protein domains, e.g. the group of zinc-dependend metalloproteases possess a HEXXH amino acid motif responsible for zinc-binding (for review, see (Hooper 1994)). Most proteases are expressed as an inactive precursor, the zymogen, and require proteolytic activation. This activation can either be performed by another protein or by the protein itself, in a mechanism called autoproteolysis (Guan et al. 1996; Ra and Parks 2007). The first aim of this study was to characterize the cleavage process of the secreted mCLCA3 protein with regard to the characteristics of metalloproteases.

3.1 Aim no. 1: Characterization of the cleavage process of the secreted mCLCA3 protein

In humans, hCLCA1 and hCLCA4 at this time point appear to be the most relevant CLCA proteins for CF phenotype modulation. Therefore, the cleavage of the biochemically well characterized, secreted mCLCA3, murine orthologue of hCLCA1 representing the most relevant CLCA family member in CF mouse models (see chapter 2.2.1.1) was investigated in this study. The tissue expression pattern and secretory pathway of mCLCA3 have been well characterized in the past. The protein is expressed in goblet cells throughout the body

(Leverkoehne and Gruber 2002) and after post-translational cleavage, both subunits are secreted into the supernatant *in vitro* (Gibson et al. 2005; Mundhenk et al. 2006). Furthermore, the initial experiments concerning the possible protease activity of CLCA proteins have been conducted for hCLCA1, the human orthologue of mCLCA3 (Pawlowski et al. 2006). The major challenges in CLCA research to date are to test the species independency of the result for hCLCA1 and determine the proteolytic activity of secreted CLCA proteins. Therefore the zinc dependency of the cleavage process, the exact cleavage site as well as the cellular transport of a cleavage-defective mutant and the autocatalytic activity of mCLCA3 were experimentally addressed.

Hypothesis 2:

The cleavage processes of transmembrane and secreted CLCA proteins differ in consequence of the transmembrane domain.

CLCA proteins can be divided in two subgroups, namely fully secreted and transmembrane CLCA proteins. The fact that the only transmembrane CLCA protein investigated to far is cleaved at the plasma membrane while secreted CLCA family members are cleaved in the endoplasmic reticulum (see chapter 2.4.3) raised the hypothesis that the cleavage process of secreted CLCA family members differs from the cleavage process of transmembrane CLCA proteins. In order to compare the cleavage mechanism of a secreted and a transmembrane murine CLCA protein, another aim of this study was to identify a murine CLCA protein bearing a transmembrane domain that is expressed in CF relevant tissue.

3.2 Aim no. 2: Identification of a murine transmembrane CLCA protein in tissues relevant for cystic fibrosis

3.2.1 Expression pattern analysis and biochemical characterization of mCLCA5

The human hCLCA2 protein, orthologue to mCLCA5, was the first CLCA protein in which the single carboxy-terminal transmembrane domain was identified (Elble et al. 2006). Its murine orthologue mCLCA5 has been detected at the plasma membrane (Evans et al. 2004) but a transmembrane domain has not yet been established. One study detected mCLCA5 mRNA in the spleen and eye (Evans et al. 2004) but expression in other tissues might be possible. For example, Patel and coworkers reported mCLCA5 to be upregulated in lungs of mCLCA3

-/- mice after virus induced mucus cell hyperplasia (Patel et al. 2006). These knock-out mice displayed no phenotype without challenge, suggesting a compensatory role of mCLCA5 for the loss of mCLCA3 function (Patel et al. 2006). On the other hand, in wild-type mice challenged with IL-13, only mCLCA3 but no other murine CLCA homologue was upregulated (Nakano et al. 2006) but this could be due to the specific challenge used. Thus, despite an additionally reported role in mediation of tumor cell growth (Beckley et al. 2004), mCLCA5 represents a promising candidate for modulation of the CF lung phenotype and was included in this study. In this study, the tissue expression pattern of mCLCA5 on the RNA and protein levels as well as its subcellular distribution was investigated. Furthermore, the potential transmembrane domain of this protein was analyzed.

3.2.2 Expression pattern analysis and biochemical characterization of mCLCA6

The human hCLCA4 protein has been proposed to modulate the gastro-intestinal CF phenotype (Ritzka et al. 2004) and also was reported to be upregulated in the intestine of Crohn's disease patients (Comelli et al. 2009). However, the cellular expression pattern of this homologue in the intestine as well as its orthologues has not yet been determined. The murine mCLCA6 was detected in the intestine on the mRNA level but its expression pattern on the protein level remains elusive (Evans et al. 2004). Nevertheless, similar to mCLCA5, the mCLCA6 protein has also been localized at the plasma membrane (Evans et al. 2004), suggesting but not proving a transmembrane domain. Therefore, the tissue and subcellular expression pattern of mCLCA6 were determined and a putative transmembrane domain was characterized.

3.3 Aim no. 3: Comparison of the cleavage processes of a secreted CLCA protein versus a transmembrane CLCA protein

Finally, the overall aim of the study was to compare the cleavage processes of the secreted family member mCLCA3 with that of a transmembrane murine CLCA protein. Specifically, the cellular transport of a cleavage-defective mutant and the zinc-dependency of the process were analyzed.

4 RESEARCH PUBLICATIONS IN PEER-REVIEWED JOURNALS

4.1 Murine mCLCA6 is an Integral Apical Membrane Protein of Non-goblet Cell Enterocytes and Co-localizes with the Cystic Fibrosis Transmembrane Conductance Regulator

Authors: Bothe MK, Braun J, Mundhenk L, Gruber AD
Year: 2008
Journal: J Histochem Cytochem 56:495-509

DOI: 10.1369/jhc.2008.950592

Declaration of own portion of work in the research publication:

Contributions of MK Bothe: Independent design, preparation, completion and evaluation of all experiments involving protein expression in cell culture, biochemistry, immunohistochemistry and immune electron microscopy

Independent subsequent creation of the entire manuscript with exception of investigations involving laser capture microdissection and quantification of the target gene mRNA.

Contributions of other authors: Design, preparation, completion and evaluation of investigations involving laser capture microdissection and quantification of the target gene mRNA. Subsequent compilation of parts of the manuscript relating to these analyses.

4.2 Murine mCLCA5 is Expressed in Granular Layer Keratinocytes of Stratified Epithelia

Authors: Braun J*, Bothe MK*, Mundhenk L, Beck CL, Gruber AD
*both authors contributed equally
Year: 2010
Journal: Histochem Cell Biol 133(3):285-99

DOI: 10.1007/s00418-009-0667-0

Declaration of own portion of work in the research publication:

Contributions of MK Bothe: Design, preparation, completion and evaluation of investigations involving *in silico* sequence analyses, biochemical analyses and immunohistochemistry

Subsequent creation of parts of the manuscript relating to these analyses including:
In the materials and methods section: *in silico* sequence analyses; immunohistochemistry; biochemical protein analyses
In the results section: *in silico* sequence analyses; tissue expression pattern (by immunoblots, immunohistochemistry and confocal laser scanning microscopy); biochemical data analysis
In the discussion section: the paragraph on the biochemical data analysis; interpretation of the overall results

Contributions of all authors: Independent design, preparation, completion and evaluation of all investigations involving laser capture microdissection and quantification of the target gene mRNA. Independent design, preparation and evaluation of all investigations involving immune electron microscopy. Subsequent compilation of the manuscript relating to these analyses except for the parts mentioned above.

4.3 The Murine mCLCA3 is a Zinc-dependent Metalloprotease with Autoproteolytic Activity

Authors: Bothe MK, Mundhenk L, Kaup M, Gruber AD
Year: 2011
Journal: Molecules and Cells Vol. 32, No. 6

DOI: 10.1007/s10059-011-0158-8

Declaration of own portion of work in the research publication:

Contributions of MK Bothe: Design, preparation, completion and evaluation of all presented experiments and subsequent creation of the entire manuscript except for the MALDI-TOF MS analyses and Edman-degradation

Contributions of all authors: Design and evaluation of experiments and completion and evaluation of MALDI-TOF MS analyses, subsequent compilation of the manuscript relating to the MALDI-TOF MS analyses

4.4 Impaired Autoproteolytic Cleavage of mCLCA6, a Murine Integral Membrane Protein Expressed in Enterocytes, Leads to Cleavage at the Plasma Membrane Instead of Cleavage in the Endoplasmic Reticulum

Authors: Bothe MK, Mundhenk L, Kaup M, Beck C, Gruber AD
Year: 2012
Journal: Molecules and Cells Vol. 33, No. 3

DOI: 10.1007/s10059-012-2217-1

Declaration of own portion of work in the research publication:

Contributions of MK Bothe: Design, preparation, completion and evaluation of all presented investigations and subsequent creation of the entire manuscript

Contributions of all authors: Design and evaluation of investigations

5 CONCLUDING DISCUSSION

To enlighten the role of CLCA proteins in diseases such as CF, the putative metalloprotease function of CLCA proteins was investigated (hypothesis I, chapter 3) and the cleavage processes of a secreted and a transmembrane CLCA protein were compared (hypothesis II, chapter 3). In this chapter, the hypotheses and aims of the study as well as the putative roles of CLCA proteins as proteases in diseases are discussed.

5.1 Hypothesis I: CLCA proteins show characteristics of metalloproteases

5.1.1 Aim no. 1: Characterization of the cleavage process of the secreted mCLCA3 protein

All CLCA proteins investigated so far possess a HEXXH amino-acid motif typical of metalloproteases in their amino-terminal subunit (Patel et al. 2009; Pawlowski et al. 2006). In this study, the cleavage was abrogated after introduction of the E157Q mutation in the HEXXH motif of mCLCA3. This has also been shown for hCLCA1 when introducing the same mutation (Pawlowski et al. 2006). However, in that study, only the cell lysates of transfected HEK293 cells and not the supernatants were analyzed, so the missing cleavage of the mutant protein may have been due to misfolding.

Misfolding of a protein often leads to impaired cellular transport as shown for the CFTR chloride channel (Brown et al. 1997). There, mutation F508 results in misfolding of the protein and thus it fails to exit from the endoplasmic reticulum (Brown et al. 1997). To exclude an impaired cellular passage due to improper folding, the cellular transport of mutant mCLCA3E157Q protein was investigated. The wild-type mCLCA3 protein undergoes high-mannose and complex glycosylation during its passage through the endoplasmic reticulum and Golgi apparatus before it is shed into the supernatant (Mundhenk et al. 2006). Same was true for the mCLCA3E157Q protein as shown in this study. Therefore, cleavage is not a prerequisite for cellular transport of the mCLCA3E157Q mutant. However, the remote possibility remains that the mutant protein is transported by the cell despite misfolding. Furthermore, proteolytic cleavage may be a critical prerequisite for protein function (Schlomann et al. 2002). Thus, future studies will have to exclude that the uncleaved mCLCA3E157Q protein has reduced or abolished protein function.

The HEXXH motif is responsible for zinc-binding (Hooper 1994). In this study, the cleavage of mCLCA3 was strictly dependent on the presence of zinc ions whereas other metal cations were dispensable for the cleavage process. Furthermore, the cleavage of the mCLCA3 precursor was inhibited by metalloprotease inhibitors or chelating agents but not by other protease inhibitors. It can therefore be concluded that cleavage of mCLCA3 itself is Zn^{2+}-dependent and inhibited by metalloprotease inhibitors only, indicating a metalloprotease as the cleaving agent.

To determine the number of metalloproteases involved in cleavage of mCLCA3, the exact cleavage site of the protein was identified via MALDI-TOF-MS and Edman degradation. A single cleavage site of a protein implicates the cleavage by a single protease whereas more than one cleavage sites would suggest more than one terminal protease processing mCLCA3. Of note, investigation of the cleavage site can only help to determine the number of terminal proteases processing the protein. A proteolytic cascade finally leading to activation of such a terminal protease and therefore cleavage of the protein cannot be excluded. The mCLCA3 protein is cleaved at a single cleavage site between R695 and A696 with the carboxy-terminal subunit starting with the amino acid sequence AMYID. A similar amino-terminus has been described for the carboxy-terminal subunit of the bovine endothelial cell homologue Lu ECAM-1 (Elble et al. 1997), suggesting that the cleavage process and probably the cleaving agent are highly conserved among homologous CLCA proteins of different species. Furthermore, the single cleavage site leads to the assumption that mCLCA3 is cleaved by a single cleaving agent. Nevertheless, the possibility remains that a proteolytic cascade of various cleaving agents in the end leads to mCLCA3 cleavage.

It has been proposed that the agent cleaving CLCA proteins must be universally expressed (Gruber et al. 2000). Furthermore, it was still elusive whether the cleavage is mediated by a non-CLCA protease or an autoproteolytic event. Abrogation of cleavage in the E157Q mutants of secreted CLCA proteins was a first hint towards autoproteolytic cleavage of CLCA proteins. In this study, the active wild-type mCLCA3 protein was able to process the cleavage-defective mutant E157Q via intermolecular cleavage. However, this cleavage appeared to be incomplete, leaving a small fraction of uncleaved precursor protein. A possible explanation is that the physiological cleavage process may be an intra- rather than intermolecular event. Of note, the remote possibility remains 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. However, as similar co-transfection experiments have previously proven autoproteolytic processing of other proteases (Schlomann et al. 2002), it is likely to assume that cleavage of mCLCA3 may also occur autoproteolytically.

The functional impact of this autoproteolytic cleavage will have to be the subject of future analysis.

5.1.2 Are CLCA proteins metalloproteases of the zincin family?

Metalloproteases or metallopeptidases represent a group of enzymes that cleave other proteins. Hydrolysis of the peptide bonds of substrate proteins by metalloproteases is performed with water acting as nucleophile and this water molecule is held in place by one or two metal ions (Pawlowski et al. 2006). In most families of metalloproteases, the metal ion is zinc bound by a HEXXH zinc-binding motif, although other metal ions including cobalt can be used to bind the water molecule (Odintsov et al. 2005). Enzymes of the HEXXH zinc metallopeptidase superfamily are also called zincins (Bode et al. 1993). Here, the evidences for CLCA proteins belonging to the zincin family of proteases are discussed. As in this study an (auto-)proteolytic activity was only proven for mCLCA3, the following paragraphs focus on the CLCA proteins of cluster 1.

5.1.2.1 HEXXH motif of metalloproteases

The catalytic mechanism of metalloproteases has been best studied for thermolysin, where the two histidines (H) serve as ligands for the catalytic zinc ion and the glutamic acid (E) serves as a general base to assist the nucleophilic attack of a water molecule (Nagase 2001). In this study, mutation E157Q of the HEXXH motif abrogated cleavage of mCLCA3 in the endoplasmic reticulum. This had also been shown for human hCLCA1 (Pawlowski et al. 2006). Of note, the HEXXH motif is highly conserved between CLCA homologues. Interestingly, the only exception to this conservation can be found in the human hCLCA3, a truncated family member that does not undergo cleavage (Gruber and Pauli 1999a). Only in hCLCA3, the E in the HEXXH motif is replaced by a glycine (G), leading to the amino acid sequence HGWAH instead of HEWAH. The functional significance of the hCLCA3 protein has not yet been established, but it would be interesting to test the effect of this natural amino acid switch in the catalytic region of hCLCA3 in other CLCA homologues.

Zinc-dependent proteases possessing the HEXXH motif are currently grouped into gluzincins, proteases bearing a glutamate as the third zinc ligand, and metzincins, bearing an extended HEXXH motif and a Met-turn carboxy-terminally to the HEXXH motif (Hooper 1994). The amino acid sequence of CLCA proteins at the HEXXH region is not exactly consistent with the proposed or analysed amino acid sequences for metzincins (Bode et al.

1993) or gluzincins (Hooper 1994). This indicates that CLCA proteins might represent a new, distinct family of zinc metalloproteases. However, both a catalytic and the structural zinc-binding site could be identified in hCLCA1 using amino acid alignments of hCLCA1 and **m**atrix **m**etallo**p**rotease 11 (MMP11), a metzincin (Pawlowski et al. 2006). This might suggest similarities between the CLCA proteins and the MMPs other than the common extended HEXXH motif in metzincins proposed by Bode and co-workers (Bode et al. 1993).

Although a HEXXH zinc-binding amino acid motif has been identified in many zinc-dependent proteases, this motif is not unique to zinc metallopeptidases (Jongeneel et al. 1989). The Bradykinin receptor 1, for example, also bears a HEXXH motif and no proteolytic activity has been reported for this receptor (Ignjatovic et al. 2002). Thus, the HEXXH motif is a hint towards metalloprotease activity but no prove and further evidence is required to prove the proteolytic activity of CLCA proteins.

5.1.2.2 Zinc-dependent cleavage mechanism

The cleavage process of the mCLCA3 precursor molecule has been shown to be zinc-dependent. Only zinc triggered the cleavage of the precursor molecule of these proteins while magnesium or calcium showed no significant effect on the cleavage of the molecule. Nevertheless, binding of zinc by the HEXXH motif of CLCA proteins has not yet explicitly been proven and future studies should include the zinc-binding mechanism of CLCA proteins. Of note, current data are restricted to the autoproteolytic cleavage of CLCA proteins. The zinc-dependency of the proteolysis of other proteins by the CLCA proteins will have to be investigated when substrates of the putative CLCA proteases have been identified.

In zinc metalloproteases, zinc plays both a structural and a catalytic role (O'Dell 1992). In hCLCA1, both a putative structural zinc binding site and a catalytic zinc binding site have been identified by alignment with MMP11 (Pawlowski et al. 2006). In the structural zinc binding site of hCLCA1, the amino acids Asp115, Glu116, Cys125, Glu127 and His133 have been suggested as metal coordinating ligands in both an alignment and a structural model (Pawlowski et al. 2006). But their explicit structural role in zinc binding and coordination will have to be established in future mutagenic approaches.

5.1.2.3 (Auto-)proteolytic activity

In co-transfected cells, a mCLCA3 protein containing the wild-type zinc-binding domain was capable of cleaving the mutant mCLCA3E157Q which served as a substrate. Of note, it is thinkable that co-transfection with wild-type mCLCA3 could also have led to activation of a distinct protease, which was then able to cleave the hydrolase-dead mutant. Only investigation of an isolated mCLCA3 protein would have fully excluded the implication of any other proteases in the cleavage process. But as all attempts to isolate the proteins after expression in E. coli failed and these proteins are too large for chemical synthesis, this study had to rely on co-expression experiments as one of the standard experiments to investigate intermolecular proteolysis (Cao et al. 2005).

Autoproteolysis can be distinguished in an intramolecular or intermolecular mechanism. Although only an intermolecular cleavage has been shown for mCLCA3, this cleavage was not fully effective, leaving uncleaved precursor of mCLCA3E157Q. This indicates that the natural mechanism is rather intramolecular. Of note, autoproteolytic cleavage has also been reported for proteins that do not represent proteases. Mucins, for example, undergo autoproteolytic processing (Levitin et al. 2005; Peng et al.) but the biological function of this autoproteolytic cleavage of a non-protease protein is still elusive. In proteases, autoproteolytic cleavage leads to activation of the protease (Guan et al. 1996). Activation of mCLCA3 by autoproteolytic cleavage will have to be investigated when substrates of CLCA proteins other than themselves have been identified.

In conclusion this study provides evidence that mCLCA3 represents a zinc-dependent, autoproteolytic metalloprotease and therefore supports hypothesis I. The metalloprotease functions of CLCA proteins other than mCLCA3 will have to be investigated. Furthermore, future investigations will have to focus on the determination of substrates of CLCA metalloproteases to substantiate the role of CLCA proteins as metalloproteases and set the stage for their possible roles in diseases.

5.2 Hypothesis II: The cleavage processes of transmembrane and secreted CLCA proteins differ in consequence of the transmembrane domain

Before comparison of the cleavage processes of a transmembrane and a secreted CLCA protein could be established, a murine transmembrane CLCA protein in tissues relevant for CF had to be identified.

5.2.1 Aim no. 2: Identification of a murine transmembrane CLCA protein in tissues relevant for cystic fibrosis

The recently discovered murine CLCA proteins mCLCA5 and mCLCA6 have been shown to be associated with the plasma membrane *in vitro* (Evans et al. 2004) and therefore were investigated with regard to expression in tissues relevant for CF and a transmembrane domain.

5.2.1.1 Expression pattern analysis of mCLCA5 and mCLCA6 with a focus on tissues relevant for cystic fibrosis

In CF patients, the secretory dysfunction predominantly affects intestine, lung, pancreas, gall bladder and testes (Schwiebert et al. 1998). In this study, mCLCA5 was detected on the mRNA and protein level in keratinizing layers of stratified squamous epithelia of various tissues including the skin, cervix, stomach, oesophagus and even keratinizing cells in Hassall's bodies in the thymus. The protein was associated with keratohyaline granules in these cells. This tissue expression pattern is similar to the tissue expression pattern reported recently for the human orthologue hCLCA2 (Connon et al. 2004) and the exclusive expression of mCLCA5 protein in keratinizing cells suggests a role in keratinisation, differentiation or maturation of these cells. Although mCLCA5 RNA was detected in many tissues, a clear threshold of 0.3 copies mCLCA5 mRNA per 100 copies of EF1a as housekeeping gene was only achieved and exceeded in organs consisting of stratified squamous epithelium (data provided by Josephine Braun), consistent with the results on the protein level. Upregulation of mCLCA5 RNA in CF relevant organs in tissues of a mCLCA3 -/- mouse model with regard to compensation of mCLCA3 was excluded by qRT-PCR (data provided by Josephine Braun). Its physiological tissue expression pattern in non-CF relevant tissues as well as the missing upregulation in CF relevant tissues of CF mouse models (Braun et al. 2011) made mCLCA5 a rather inapplicable candidate for comparison with mCLCA3 in CF relevant tissues.

In contrast, the mCLCA6 protein was exclusively detected in the intestine, particularly in the apical membrane of non-goblet cell enterocytes. This specifies the results of Evans and co-workers who detected mCLCA6 mRNA in the gastro-intestinal tract (Evans et al. 2004). In the crypts of the murine colon, mCLCA6 co-localizes with the murine CFTR protein. It might therefore be associated with CFTR function in the large intestine and its intestinal distribution makes it an applicable candidate for our study. Furthermore, both mCLCA3 and mCLCA6 are expressed in enterocytes, though mCLCA3 is expressed in goblet cells and mCLCA6 in non-goblet cell epithelial cells.

5.2.1.2 Transmembrane domains of mCLCA5 and mCLCA6

Computational analysis of the mCLCA5 and mCLCA6 protein predicted a single transmembrane domain in the carboxy-terminal subunits for both proteins. This transmembrane domain of mCLCA5 and mCLCA6 had to be proven biochemically. In a previous study, fused mCLCA5-YFP and mCLCA6-YFP proteins were detected at the plasma membrane of mCLCA5-YFP or mCLCA6-YFP overexpressing HEK293 cells (Evans et al. 2004), allowing for a transmembrane domain but not proving such a protein structure.

Concerning mCLCA5, only the complex-glycosylated amino-terminal subunit was detected in the supernatant of mCLCA5 overexpressing HEK293 cells in our study, though both complex-glycosylated subunits of the protein pass the Golgi apparatus after post-translational cleavage in the endoplasmic reticulum. This could be due to anchorage of the carboxy-terminal subunit to the plasma membrane or the carboxy-terminal subunit might degrade past the Golgi apparatus on its way through the cell. In immunohistochemical studies, the carboxy-terminal subunit of mCLCA5 was detected in association with keratohyaline granules of the granular layer of stratified squamous epithelium. An association with the plasma membrane could not be proven. This might be due to internalization of the carboxy-terminal subunit after shedding of the amino-terminal subunit or to insufficient sensitivity of the anti-mCLCA5-carboxy-terminal antibody. But as this study failed to localize the mCLCA5 protein at the plasma membrane as a basis for investigation of a true transmembrane domain, the subsequent experiments were performed on the mCLCA6 protein only.

The mCLCA6 protein was detected at the apical plasma membrane of non-goblet cell enterocytes with both antibodies against the amino- and the carboxy-terminal subunits, respectively. Only the complex-glycosylated amino-terminal subunit was shed into the supernatant of mCLCA6 overexpressing HEK293 cells. The carboxy-terminal subunit could not even be released after low pH treatment of the cells, indicating a true transmembrane domain in the carboxy-terminal subunit. Thus, in accordance with the computational data, the mCLCA6 protein possesses a true transmembrane domain in the carboxy-terminal subunit. In addition to the expression of mCLCA6 in a CF relevant tissue and its co-localization with the CFTR protein, these results make mCLCA6 a useful transmembrane murine CLCA protein to compare with mCLCA3 in regard of the cleavage process.

5.2.2 Aim no. 3: Comparison of the cleavage processes of the secreted mCLCA3 and the transmembrane mCLCA6 proteins

Cleavage of CLCA proteins was reported to be located either in the endoplasmic reticulum as shown for secreted CLCA family members (Mundhenk et al. 2006; Plog et al. 2009; Range et al. 2007) or at the plasma membrane as shown for transmembrane hCLCA2 (Elble et al. 2006). In this study, it was tested whether cleavage at the plasma membrane is true for all CLCA proteins possessing a transmembrane domain or whether it is special for hCLCA2. The transmembrane CLCA family member mCLCA6 turned out to be cleaved in the endoplasmic reticulum or early Golgi apparatus consistent with the findings for secreted mCLCA3 (Mundhenk et al. 2006). Thus, although both hCLCA2 and mCLCA6 possess a transmembrane domain, their cleavage occurs in different cellular compartments. It had previously been shown that cleavage in the endoplasmic reticulum of CLCA proteins of cluster 1 is conserved between porcine, equine and murine orthologues (Mundhenk et al. 2006; Plog et al. 2009; Range et al. 2007). However, nothing is known yet about the cellular compartment where cleavage of human hCLCA1 occurs. Therefore, cleavage at the plasma membrane might be a specific phenomenon of human CLCA proteins.

The cellular compartment where cleavage occurs does not only differ between the clusters of CLCA proteins but between CLCA orthologues of the same cluster as well. CLCA proteins of cluster 2, namely hCLCA2 and mCLCA5, are also cleaved in different cellular compartments. As mentioned, human hCLCA2 undergoes cleavage at the plasma membrane (Elble et al. 2006) while it has been shown in this study that its murine orthologue mCLCA5 is cleaved in the endoplasmic reticulum or early Golgi apparatus. The significance of the missing interspecies conservation of the cleavage location of hCLCA2 and mCLCA5 is barely understood. It might be due to differences between human and murine CLCA, but as long as nothing is known about the location of cleavage of other human CLCA, additional experiments are required to shed light on this issue.

Cleavage in the endoplasmic reticulum was abrogated after mutation E157Q in the HEXXH motif of both the secreted mCLCA3 and the transmembrane mCLCA6. The uncleaved precursor of mCLCA3E157Q underwent complex glycosylation in the Golgi apparatus and the mature, uncleaved precursor was shed into the supernatant. The uncleaved precursor molecule of mCLCA6E157Q also underwent complex glycosylation in the Golgi apparatus. But in contrast to the mCLCA3 precursor, it reached the plasma membrane where it was cleaved and only the amino-terminal subunit was shed into the supernatant. Thus, despite abrogation of the original cleavage mechanism via mutation of the HEXXH motif, the mCLCA6E157Q protein can be cleaved in a distinct cellular compartment by a still unknown cleaving agent.

First attempts to characterize the cleavage process of mCLCA6E157Q protein at the plasma membrane revealed that the delayed cleavage of the mCLCA6E157Q protein was zinc dependent and could be inhibited by chelating agents. Interestingly, the autoproteolytic cleavage of mCLCA3 in the endoplasmic reticulum shows the same characteristics. Thus, the mCLCA6E157Q mutant can either be cleaved by itself at the plasma membrane or a distinct metalloprotease is capable of mCLCA6E157Q cleavage.
Cleavage of mCLCA3 in the endoplasmic reticulum is most likely an autoproteolytic event as shown by co-expression experiments in this study. In other proteins, introduction of mutation E versus Q in a HEXXH motif has been reported to reduce or abrogate the proteolytic activity (Cha and Auld 1997; Fushimi et al. 1999; Li et al. 2000). Thus, both a self-cleavage mechanism of mCLCA6E157Q in a distinct cellular compartment and cleavage by a metalloprotease are possible. Interestingly, hCLCA2 has been shown to be cleaved at the plasma membrane instead of the endoplasmic reticulum (Elble et al. 2006), calling into question whether hCLCA2 and mCLCA6E157Q could be cleaved at the plasma membrane by a similar or the same metalloprotease.

Of note, the delayed cleavage of mCLCA6E157Q also took place in a truncated mCLCA6 protein lacking the transmembrane domain. Thus, differences other than the transmembrane domain might be responsible for the differences in the cleavage process, for example the structure of the cleavage site or the HEXXH motif of the proteins. But without structural data the exact role of conserved amino acid residues at the cleavage site in CLCA protein function or stability can only be assumed. But the fact that mCLCA6$^{\Delta TM}$E157Q undergoes delayed cleavage despite lacking the transmembrane domain calls into question whether CLCA proteins can really be divided in two simple groups with and without a transmembrane domain. Maybe distinct subgroups based on yet unidentified CLCA protein characteristics will have to be employed for future investigations.

In conclusion the cleavage process of the transmembrane CLCA protein mCLCA6 differs from the cleavage process of secreted mCLCA3. In contrast to the mCLCA3E157Q mutant protein a rescue cleavage of the mCLCA6E157Q mutant protein has been shown to occur in a post-Golgi compartment. Future studies will have to test whether this rescue cleavage only takes place in mCLCA6 or in other transmembrane CLCA proteins as well. However, the rescue cleavage was not depending on the transmembrane domain of the protein, therefore disproving hypothesis II. Differences other than the transmembrane domain might lead to the rescue cleavage and will have to be discovered in future analyses.

5.3 Possible role of cluster 1 CLCA proteins as metalloproteases in disease

As an intermolecular autoproteolytic function was described for mCLCA3 in this study, it seems likely to assume that same is true for other CLCA proteins of cluster 1. It remains to be proven whether CLCA proteins of other clusters represent metalloproteases as well. Functionally heterogenous gene families have been known in other families of proteases, i.e. the ADAMS protease family consists of proteases and putative non-proteases (for review, see (Klein and Bischoff 2011). But in ADAMS, none of the putative non-protease proteins possesses the HEXXH motif, corroborating the key role of this motif in metalloprotease function. In contrast, every CLCA protein except for hCLCA3 possesses the HEXXH motif and it will be merely a matter of time until protease function for CLCA proteins of other clusters will be proven or disproven. Here, the possible roles of mCLCA3 and cluster 1 CLCA proteins in diseases are discussed in detail.

5.3.1 Diseases with secretory dysfunction (cystic fibrosis, asthma, chronic obstructive pulmonary disease)

Expression of CLCA proteins in tissues and cell types relevant for CF, asthma or COPD makes these proteins likely to be involved in the disease and several studies have linked CLCA proteins to these diseases (Hegab et al. 2004; Kamada et al. 2004; Ritzka et al. 2004; van der Doef et al. 2010). In diseases with secretory dysfunctions, a protease function of CLCA proteins could lead to both modulation of chloride conductances or regulation of mucus hypersecretion.

5.3.1.1 CLCA proteases in modulation of chloride secretion

CLCA proteins have been shown to modulate endogenous chloride conductances (Hamann et al. 2009) but besides the fact that these proteins do not form ion channels (Gibson et al. 2005; Mundhenk et al. 2006), their modulatory role has not been characterized any further. The data of this study suggest that CLCA proteins, especially of cluster 1, might regulate chloride conductances as proteases.

In CF, regulation of the chloride conductance has been reported for various proteases. Serine proteases activate CFTR chloride conductances in an indirect way via protease-activated receptors (Palmer et al. 2006). Interestingly, inhibition of **m**atrix **m**etallo**p**rotease **2** (MMP2) also activates CFTR chloride conductance (Duszyk et al. 1999), indicating a

complex mechanism of CFTR regulation by different proteases. Besides the regulation of CFTR activity, proteases like calpain are involved in the cellular turnover of the CFTR, which is defective in many CF patients (Averna et al. 2011). Additionally, the sodium channel ENaC, which is involved in sodium hyperabsorption in CF, is also regulated by complex intra- and extracellular proteolytic cleavage (Bhalla and Hallows 2008).

Of note, for the porcine orthologue of mCLCA3, named pCLCA1, intracellular chelation of cations by 1,2-**b**is(2-**a**mino**p**henoxy)ethane-*N,N,N′,N′*-**t**etraacetic **a**cid-**a**cetoxy**m**ethyl (BAPTA-AM) inhibited calcium-activated chloride conductances in pCLCA1 transfected 3T3 cells (Loewen et al. 2002). This effect was supposed to be due to chelation of calcium cations or inhibition of protein kinase C, which was suggested to activate CLCA proteins (Loewen et al. 2002). But BAPTA-AM has also been shown to be a zinc chelator (Wie et al. 2001) and in this study, mutation E157Q of pCLCA1 also led to elimination of cleavage similar to mCLCA3, indicating a similar, zinc-dependent cleavage process. This leads to the question whether the effect of BAPTA-AM on pCLCA1 modulated chloride currents was really due to inhibition of protein kinase C, chelation of calcium cations or inhibition of the protease function of pCLCA1 by chelation of zinc.

5.3.1.2 CLCA proteases in mucus hypersecretion

Of note, modulation of chloride currents by CLCA requires high, non-physiological Ca^{2+} concentrations (Eggermont 2004), indicating that this modulation might not be the physiological function of CLCA proteins. In CF tissues, modulation of mucus hypersecretion apart from regulation of chloride secretion might just as well be the functional role of CLCA proteins. Several proteases enhance mucus production in the lung, for example neutrophil elastase or ADAMs (for review, see (Garcia-Verdugo et al. 2011). Similar to hCLCA1, neutrophil elastase increases MUC5AC mRNA levels in respiratory epithelial cells and induces mucus cell metaplasia (Voynow et al. 2004; Voynow et al. 1999). Human hCLCA1 is supposed to regulate MUC5AC in an IL-13-dependent pathway (Patel et al. 2009). Interestingly, IL-13 has also been shown to be a protease regulating cytokine in the lung (Chen et al. 2005).

Because proteases cleave and degrade other proteins, a further potential role of proteases in diseases with mucus hypersecretion is the degradation of the mucus and the remodelling of the extracellular matrix. Several proteases including matrix metalloproteases were discussed to play a role in modulating the phenotypes of CF (Gaggar et al. 2010), asthma (Gueders et al. 2006) and COPD (Owen 2005) by remodelling of extracellular matrix. Upregulation of

cluster 1 CLCA proteins in lungs of CF, asthma or COPD patients in addition to their protease nature might be a first hint towards a possible role of these proteins in matrix degradation. This makes the investigation of the substrate requirements and the identification of possible substrates of CLCA even more important.

5.3.2 CLCA proteases in adhesion of metastasizing tumor cells and lymphocytes

The role of CLCA proteins in tumor invasion might be based on their function as adhesion molecules of metastasizing tumor cells and lymphocytes. Interestingly, a combined role as both adhesion molecule and protease has also been proven for family members of the ADAM family of metalloproteases (Edwards et al. 2008). These proteases modulate cell adhesion and metastasis of tumor cells via integrins (Arribas et al. 2006), a mechanism also proposed for CLCA proteins (Abdel-Ghany et al. 2003; Abdel-Ghany et al. 2002, 2001). The interaction between the integrin binding domain and the protease domain in ADAMs is still under investigation (White 2003), but future research on CLCA proteins may consider the similarities between CLCA proteins and ADAMs.

5.3.3 CLCA proteases in tumor growth / apoptosis

Down-regulation of members of the CLCA protein family in several tumors and their involvement in inhibition of tumor cell growth has raised the hypothesis of CLCA proteins as tumor suppressors (see chapter 2.2.3). Interestingly, initial studies reported other metalloproteases like MMPs and ADAMs rather to be up-regulated in tumor cells and thus research attempts focussed on inhibition of these proteases to inhibit tumor growth (Coussens et al. 2002). But in recent years, re-evaluation of the role of MMPs in cancer has shown that similar to CLCA proteins some metalloproteases function as tumor suppressors (Lopez-Otin et al. 2009). The functional roles of such anti-tumor proteases include inhibition of tumor growth, tumor survival, angiogenesis, tumor invasion or inflammation (Lopez-Otin and Matrisian 2007). For members of the CLCA protein family, both an inhibitory role of tumor growth and an inhibitory role on tumor invasion have been described (Beckley et al. 2004; Walia et al. 2009; Zhu et al. 1992; Zhu et al. 1991). The exact molecular pathway of CLCA proteins possibly leading to inhibition of tumor growth will have to be identified in future studies.

5.4 Conclusion and outlook

The exact modulatory role of CLCA proteins in diseases such as CF is not yet fully understood. Secreted CLCA proteins may be capable of intermolecular autoproteolysis and little doubt remains that CLCA proteins of cluster 1 represent metalloproteases. For CLCA proteins of other clusters the protease function will have to be tested. To determine the role of CLCA proteases of cluster 1 in diseased tissues, future investigations should aim on identification of the substrates of CLCA proteases. This will gain insight into the molecular pathways in which CLCA proteins are involved and set the stage for potential future in therapeutic interventions.

Furthermore, in this study the cleavage processes of a secreted and a transmembrane CLCA protein were compared in order to shed light on the mechanism of action of CLCA proteins. Murine mCLCA6 was identified as the first murine CLCA family member to possess a transmembrane domain. The cleavage of transmembrane mCLCA6, though a general feature of all CLCA proteins, differs from the cleavage of secreted mCLCA3 when the E157Q mutation was introduced into the HEXXH motif of the CLCA family members. However, these differences were not depending on the transmembrane domain. The fact that such a general feature of CLCA proteins bears differences independent on the transmembrane domain raises the question of whether classification of CLCA proteins into transmembrane proteins and secreted proteins is an oversimplification. Maybe more complex characteristics will have to be used for differentiation between CLCA homologues in the future.

6 SUMMARY

Are CLCA Proteins Metalloproteases?
Characterization of the Cleavage Processes of the Secreted mCLCA3 and Transmembrane mCLCA6 Proteins
Melanie K. Bothe

CLCA proteins, originally termed chloride channels, calcium activated, have on the one hand been described to play a modulatory role in diseases with secretory dysfunctions, predominantly cystic fibrosis (CF), asthma or chronic obstructive pulmonary disease, and, on the other hand, in cancer. They possess a broad tissue expression pattern including mucous membranes of various organs. Members of the CLCA protein family modulate endogenous chloride conductances in cultured cells in a still elusive way. The cellular processing of CLCA proteins may indicate their role as signalling molecules because CLCA proteins are either fully secreted proteins or possess one single transmembrane domain in the carboxy-terminal subunit, while the amino-terminal subunit undergoes ectodomain shedding. A recently identified HEXXH zinc binding motif indicates that CLCA proteins might act as metalloproteases, implying a putative channel activating function.

This study addresses the question of whether CLCA proteins might indeed represent metalloproteases. As all CLCA proteins undergo post-translational cleavage, the cleavage process of murine mCLCA3, a secreted CLCA family member relevant for CF as an example for secretory diseases, was investigated with regard to an autoproteolytic activity. Furthermore, this study includes the comparison of the cleavage processes of a secreted murine CLCA family member with a murine CLCA family member of CF relevant tissues possessing a transmembrane domain. The results were supposed to either corroborate or neglect the hypotheses that CLCA proteins are metalloproteases (hypothesis I) and that the cleavage processes of secreted CLCA proteins differ from those of transmembrane CLCA proteins due to the transmembrane domain (hypothesis II). Therefore, this study will set the stage for investigating the proteolytic role of the putative CLCA proteases in the fields of secretory disorders or cancer.

The tissue expression pattern and the role of the secreted murine CLCA family member mCLCA3 in CF mouse models have been intensively studied in the past. To identify a murine CLCA family member with a transmembrane domain in CF relevant tissues, the tissue expression patterns and cellular processing of murine mCLCA5 and mCLCA6 were investigated. The mCLCA5 protein was expressed in keratinizing keratinocytes of stratified squamous epithelium of skin, cervix, stomach and other organs. Though cell culture

experiments and computational analyses suggested a transmembrane domain in the carboxy-terminal subunit, this study failed to identify the carboxy-terminal subunit associated with the plasma membrane in immunohistochemical analyses. The mCLCA5 protein was rather associated with keratohyaline granules. In contrast, the mCLCA6 protein was identified at the apical plasma membrane of non-goblet cell enterocytes in both the small and large intestine but in no other organs. In addition, the presence of a transmembrane domain of mCLCA6 was corroborated via acid treatment. Thus, mCLCA6 was used as a murine transmembrane CLCA family member expressed in intestine as a CF relevant tissue and its cleavage process was compared with that of the secreted mCLCA3 expressed in intestinal goblet cells.

In metalloproteases, the HEXXH zinc-binding amino acid motif is involved in the catalytic process. Mutation E157Q of the HEXXH motif of mCLCA3 or mCLCA6 abrogated cleavage of both proteins in the endoplasmic reticulum, consistent with the previously reported data for hCLCA1. In contrast to mCLCA3E157Q whose uncleaved precursor was fully secreted similar to the wild-type protein, the precursor molecule of mCLCA6E157Q was cleaved at the plasma membrane instead of the endoplasmic reticulum. Both the cleavage of mCLCA3 in the endoplasmic reticulum and the cleavage of mCLCA6E157Q at the plasma membrane were zinc-dependent. In contrast to mCLCA3, however, which was capable of intermolecular autoproteolytic cleavage, the cleaving agent of mCLCA6E157Q at the plasma membrane remains unidentified. The cleavage may therefore be performed by a metalloprotease or represents an autoproteolytic process. Interestingly, the delayed cleavage of mCLCA6E157Q does not require membrane association via a transmembrane domain, raising the question of whether the same is true for other transmembrane CLCA or whether it is specific for mCLCA6.

The results of this study support the hypothesis that at least secreted CLCA proteins represent metalloproteases because their cleavage is zinc-dependent, abrogated after mutation of the HEXXH motif and they are capable of intermolecular proteolysis, possibly even intramolecular cleavage. The *in vivo* substrates of CLCA proteases of cluster 1 as well as their role in secretory disorders or cancer remain to be established. Furthermore, the mutant mCLCA6E157Q protein underwent rescue cleavage while the mutant mCLCA3E157Q protein did not. This rescue cleavage was not dependent on the transmembrane domain of the protein. Protein characteristics other than the transmembrane domain might therefore be responsible for the different cleavage processes of the mCLCA3E157Q and the mCLCA6E157Q proteins. These protein characteristics should be addressed in the future.

CLCA proteases of cluster 1 may play a role in diseases with secretory dysfunctions including CF as well as in tumor biology. Roles of other metalloproteases in CF include activation of chloride channels, hypersecretion and degradation of mucus. The exact role of CLCA proteases in CF and other diseases requires knowledge on potential substrates and the substrate specificity of CLCA proteases. Investigation of the conserved amino acids at the cleavage site of mCLCA3 could give a first hint towards the substrate specificity of CLCA proteases of cluster 1 and might provide the basis for potential therapeutic interventions in the future.

7 ZUSAMMENFASSUNG

Sind CLCA Proteine Metalloproteasen?
Vergleich des Spaltungsprozesses des sezernierten mCLCA3 und des transmembranären mCLCA6 Proteins
Melanie K. Bothe

Für CLCA Proteine, ursprünglich als Calcium-aktivierbare Chloridkanäle bezeichnet, wurden modulatorische Funktionen bei verschiedenen Krankheitsbildern mit sekretorischer Dysfunktion wie CF, Asthma und COPD oder auch bei Tumoren beschrieben. Ihr breites Expressionsspektrum umfasst vor allem Schleimhäute in verschiedenen Organen. Zellkulturexperimente wiesen für einige CLCA Vertreter nach, dass sie endogene Calcium-aktivierbare Chloridionenströme modulieren. Der genaue Funktionsmechanismus dieser Modulation ist bisher unbekannt, die zelluläre Prozessierung gibt jedoch Hinweise auf eine mögliche Funktion als Signalmoleküle. CLCA Proteine werden entweder vollständig sezerniert oder sind mit einer einzigen Transmembrandomäne in der Plasmamembran verankert, so dass nur die amino-terminale Untereinheit abgegeben wird. Daher ist eine Funktion als eigenständiger Kanal unwahrscheinlich. Eine aktuelle Studie identifizierte ein HEXXH Aminosäuremotiv in CLCA Proteinen. Da dieses HEXXH Motiv vor allem bei Zink-bindenden Metalloproteasen beschrieben wurde, ist eine möglicherweise aktivierende Proteasefunktion von CLCA Proteinen durchaus denkbar.

Diese Arbeit beschäftigt sich mit der Frage, ob CLCA Proteine tatsächlich eine Gruppe von Metalloproteasen darstellen. Alle CLCA Proteine werden post-translational gespalten. Dieser proteolytische Prozessierungsschritt wurde in dieser Studie auf eine auto-proteolytische Aktivität von CLCA Proteinen untersucht. Dabei wurden ein sezerniertes und ein transmembranäres CLCA Protein verglichen mit Fokussierung auf CF-relevante murine CLCA Vertreter als Beispiel für eine Rolle von CLCA Proteinen in Dyskrinien. Die Ergebnisse sollten die Hypothese, dass CLCA Proteine Metalloproteasen sind, entweder unterstützen oder widerlegen und somit Möglichkeiten für die gezielte Untersuchung der Rolle von CLCA Proteinen bei Krankheiten wie CF, Asthma, COPD oder Krebs bieten.

Das Gewebsexpressionsmuster von sezernierten CLCA Proteinen und besonders die Rolle des murinen mCLCA3 in CF Mausmodellen wurden in der Vergangenheit bereits intensiv erforscht. Zunächst befasste sich diese Studie daher mit der Charakterisierung eines membrangebundenen, murinen CLCA Proteins in CF-relevanten Geweben. Zu diesem Zweck wurden das Expressionsspektrum und die zelluläre Prozessierung der CLCA Vertreter mCLCA5 und mCLCA6 untersucht.

Das mCLCA5 Protein wird von Keratinozyten des Stratum granulosum in mehrschichtigen Plattenepithelien exprimiert, ein Zelltyp, der für CF keine bedeutsame Rolle spielt. Obgleich in Zellkulturexperimenten mit transfizierten HEK293 Zellen beide Untereinheiten den Golgi Apparat passieren, wird nur das amino-terminale Spaltprodukt von mCLCA5 abgegeben. Eine Lokalisation der carboxy-terminalen Untereinheit an der Plasmamembran von Keratinozyten konnte jedoch nicht nachgewiesen werden, in diesen Zellen ist die carboxy-terminale Untereinheit assoziiert mit keratohyalinen Granula. Eine Transmembrandomäne erscheint somit *in vitro* nicht eindeutig.

Das mCLCA6 Protein befindet sich in der apikalen Plasmamembran von Enterozyten des Dick- und Dünndarms, es ist in den Krypten des Dickdarms mit dem CFTR Protein co-lokalisiert. Eine Transmembrandomäne in der carboxy-terminalen Untereinheit konnte mittels Acid Release nachgewiesen werden. Das mCLCA6 Protein ist somit der vielversprechendere Kandidat für eine Modulation bei CF und auch aufgrund der bewiesenen Transmembrandomäne besser für die geplante Studie geeignet. Daher lag der Fokus dieser Studie im Folgenden auf dem Vergleich des Spaltungsprozesses von mCLCA3, dem sezernierten CLCA Protein aus Becherzellen, und mCLCA6, dem transmembranären CLCA Protein aus Nicht-Becherzell Enterozyten.

Die Spaltung von mCLCA3 und mCLCA6 findet im endoplasmatischen Retikulum statt. Beide Proteine tragen das HEXXH Motiv, und nach Mutation E157Q dieses HEXXH Motives bleibt die Spaltung aus. Das mutierte mCLCA3E157Q Protein passiert dann den Golgi Apparat und wird als komplex glykosyliertes, ungespaltenes Protein in den Überstand sezerniert. Im Gegensatz dazu wird das mutierte mCLCA6E157Q Protein zwar ebenfalls durch den Golgi Apparat transportiert, es gelangt jedoch an die Plasmamembran, wird dort mittels Transmembrandomäne verankert und gespalten, so dass nur die amino-terminale Untereinheit abgegeben wird. Die Spaltung an der Plasmamembran ist zinkabhängig, es handelt sich entweder um eine verspätete Selbstspaltung des Proteins oder um Proteolyse durch eine Metalloprotease. Der Spaltungsmechanismus ist jedoch nicht abhängig von der Verankerung des mCLCA6E157Q Proteins in der Plasmamembran. Auch ein sezerniertes Trunkat, dem die Transmembrandomäne fehlt, wurde nach der E157Q Mutation noch gespalten. Offensichtlich spielen andere Faktoren bei der unterschiedlichen Prozessierung von mCLCA6 eine Rolle. Dies wirft die Frage auf, ob eine Unterteilung von CLCA Proteinen in sezernierte Proteine und membranständige Proteine ausreichend ist oder ob eine andersartige Unterteilung möglich und sinnvoll wäre.

Das mCLCA3 Protein spaltet sich im endoplasmatischen Retikulum mittels intermolekularer Auto-Proteolyse in Abhängigkeit von Zink. Die Spaltung erfolgt zwischen Aminosäure R695 und A696. Das mCLCA3 Protein ist somit in der Lage, ein anderes Protein zu erkennen und zu spalten. Nachgewiesen wurde dies zwar bisher nur für das mCLCA3 Protein selbst, aber zukünftige Studien werden sich mit der Identifizierung von Substraten der CLCA Proteine befassen. Neben der Fähigkeit zur intermolekularen Autoproteolyse sind die Zinkabhängigkeit des Spaltungsprozesses und das katalytisch aktive HEXXH Motiv Hinweise, die die Hypothese untermauern, dass CLCA Proteine des Cluster 1 tatsächlich Metalloproteasen sind. Für die anderen CLCA Cluster spricht zwar bisher nichts gegen eine Metalloproteaseaktivität, der endgültige Beweis muss jedoch noch erbracht werden.

CLCA Proteasen des Cluster 1 könnten sowohl bei Krankheiten mit sekretorischer Dysfunktion wie Cystischer Fibrose als auch bei Tumoren eine Rolle spielen. Beispielsweise wurde für andere Proteasen bei CF eine Rolle sowohl bei der Aktivierung von Chloridkanälen als auch bei der Hypersekretion von Mucus oder sogar beim Abbau des vermehrten Mucus propagiert. Die genaue Rolle von CLCA Proteasen bei den genannten Krankheiten erfordert Kenntnisse der Substrate und der Substratspezifität dieser Proteasen. Einen ersten Hinweis auf die Substratspezifität kann die Untersuchung der konservierten Aminosäuren der CLCA Spaltstelle liefern. Damit wird die Erforschung der modulatorischen Funktion von CLCA Proteasen des Cluster 1 bei den genannten Krankheitsfeldern möglich sein und somit die Basis für zukünftige therapeutische Anwendungsgebiete darstellen.

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9 TALKS AND POSTER PRESENTATIONS

Talks:

Melanie K. Bothe, Lars Mundhenk, Achim D. Gruber: *CLCA proteins – proteases or chloride channels or both?*
Ph.D. Symposium of the Department of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany, 01.07.2011

Melanie K. Bothe, Lars Mundhenk, Matthias Kaup, Christoph Weise, Achim D. Gruber: *Sind CLCA Proteine Metalloproteasen?*
54th Meeting of the Pathology Group of the German Veterinary Society, Fulda, Germany, 12.-13.03.2011

Melanie K. Bothe, Josephine Braun, Lars Mundhenk, Achim D. Gruber: *CLCA proteins as chloride channel mediators and their significance for cystic fibrosis*
Symposium of the Mukoviszidose Institute, Schloss Eringerfeld, Germany, 30.09.-01.10.2010

Melanie K. Bothe, Lars Mundhenk, Achim D. Gruber: *Cleavage of CLCA proteins – necessity or accident?*
Ph.D. Symposium of the Department of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany, 02.07.2010

Melanie K. Bothe, Achim D. Gruber: *CLCA proteins – role in mucus secretion or hydration?*
Basic Science Conference of the European Cystic Fibrosis Society, Tavira, Portugal, 15.04.-19.04.2009

Melanie K. Bothe, Josephine Braun, Lars Mundhenk, Achim D. Gruber: *Das mCLCA6-Protein der Maus als potentieller Modulator der Mukoviszidose im Mausmodell*
51st Meeting of the Pathology Group of the German Veterinary Society, Fulda, Germany, 06.03.-09.03.2008

Poster presentations:

Melanie K. Bothe, Josephine Braun, Friederike Range, Lars Mundhenk, Achim D. Gruber: *Role of CLCA proteins in mucus homeostasis in diseases with secretory dysfunctions*
26th Meeting of the European Society of Veterinary Pathology, Dubrovnik, Croatia, 17.09.-21.09.2008

Melanie K. Bothe, Achim D. Gruber: *Charakterisierung der carboxy-terminalen Spaltprodukte relevanter CLCA Proteine*
10th German Mukoviszidose Conference, Würzburg, Germany, 09.11.-10.11.2007
(Poster award of the Mukoviszidose e.V.)

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Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, 29.12.2011

Melanie Bothe