ABSTRACT

RAIFORD, KIMBERLY L. Functional Genomic Analysis of Mucin Secretion in Airway Epithelial Cells. (Under the direction of Dr. Kenneth B. Adler).

Mucin secretion in the airways is an exocytotic event that can cause substantial problems when it is imbalanced. Disproportionately large levels of mucin secretion can lead to impaired mucociliary function, increased susceptibility to bacterial pathogens, enhanced of inflammatory responses due to increases in inflammatory mediators and enzymes that induce MUC gene expression, mucin synthesis, and goblet cell hyperplasia, and, in extreme cases, death. The process of regulated exocytosis in the airway epithelium is still poorly understood, eventhough the results of an aberrent process are potentially so deleterious. Previous studies from our lab demonstrated that Myristoylated Alanine-Rich C Kinase substrate protein (*MARCKS*) regulated mucin secretion in *in vitro* cultures of human bronchial epithelial cells and in mice *in vivo.* It is our hypothesis that *MARCKS* mediates the exocytotic release of mucin from preformed membrane-bound mucin granules by facilitating granule movement from the cytosol to the plasma membrane where the granule docks, fuses and secretes its contents into the airway lumen. In the studies reported here, we use proteomics to elucidate novel proteins involved in the molecular mechanisms of mucin secretion that are associated with the mucin granules in the secretory cells. These studies will greatly contribute to our understanding of proteins involved in regulated exocytosis, suggesting new therapeutic targets.

In the first study, we elucidate some aspects of the regulated exocytosis mechanism whereby MARCKS, hCLCA1, and the chaperones cysteine string protein and heat shock

proteins interact in a complex. We found that these proteins form a complex that is associated with the mucin granule in normal bronchial epithelial cells grown using the in vitro air-liquid interface culture system. We further elucidated novel proteins that also associate with the mucin granule, most of which are cytoskeletal related.

The second study elucidates proteins associated with the mucin granules in unstimulated and stimulated conditions further expanding the study to include a diseased model, the cystic fibrosis cell line UNCN3T and the intestinal cell line HT29-18N2. We found that many of the proteins are conserved across these cell types, suggesting possible conservation in the regulated exocytosis machinery involved in mucin secretion.

Overall, these studies elucidate potential players in the mechanism of mucin secretion. These data provide the first proteomic analysis of mucin granule membrane-associated proteins in airway epithelial cells.

Functional Genomic Analysis of Mucin Secretion in Airway Epithelial Cells

by
Kimberly Lizell Raiford

A dissertation submitted to the Graduate Faculty of
North Carolina State University
In partial fulfillment of the
Requirements for the degree of
Doctor of Philosophy

Functional Genomics

Raleigh, North Carolina

2007

APPROVED BY:

_____	_____
Dr. Kenneth Adler Committee Chair	Dr. Matthew Breen
_____	_____
Dr. Anthony Blikslager	Dr. James Bonner

DEDICATION

To Emauni, my Mom, and my Husband

Emauni, dream big and go further than Mommy could ever imagine.

BIOGRAPHY

Kimberly Lizell Joyner Raiford was born December 3, 1976 in San Diego, California. She started her educational ascension at the age of 2 years old at Shiloh Christian School; advancing to public elementary school where she piloted the first second-grader in the academically gifted and talented program at Selma Elementary school. Kimberly remained in the AG Program through elementary school.

She excelled at Smithfield-Selma Senior High school and graduated with honors in the top 10% of her graduating class. Furthering her education, Kim attended and graduated from the University of North Carolina at Chapel Hill earning a BS degree in Biology. She decided to take a break from her educational agenda and joined the staff of her mentor, Dr Pericak-Vance at Duke University. While there are several articles were published in journals of medical research bearing her name.

Kim's aspiration to receive her PhD. degree encouraged her return to school at North Carolina State University. She became a doctoral candidate in the field of Functional Genomics under the direction of Dr. Kenneth Adler. A Pulmonary scholar, genetic research presenter, and award recipient reflects her studies at North Carolina State University.

Kimberly has always endeavored to be the best and top of all her undertakings. As a student her performance was to be outstanding; as a youth employee, she could be none other than the leader; as a professional, only as an asset to the profession; as a wife and mother, dedicated and devoted she is; but as a person in general, my very best friend who is always loving and caring.

She has numerous awards and honors worth mentioning, but being the humble person she is would cause her to blush at the congratulatory remarks. I can only say these things about her because I know her best being her mother for all these years. ~*Mom*~

ACKNOWLEDGMENTS

I would like to thank my advisor and mentor, Dr. Kenneth Adler, for everything!

I would like to thank all of my committee members and everyone else that has helped me along the way.

Thank you!

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

INTRODUCTION

Structure and function of airway epithelium

The airway epithelium serves as a physical barrier between the external and internal environment, however, it is not limited to this function. In fact, the airway epithelium also plays important roles in the clearance of inhaled agents, mediating inflammatory responses to injury, regulating lung fluid balance and anti-oxidant release, and overall pulmonary homeostasis (Knight et al., 2003). These functions are fundamental to the protection and maintenance of the airway epithelium, which may be rapidly disturbed after any infectious or inflammatory-related injury as seen in asthma, cystic fibrosis, and chronic obstructive pulmonary disease (Puchelle et al., 2006).

There are at least 8 ultrastructural, functional and biochemically diverse cell types making up the airway epithelium, which are divided into three main categories: basal, secretory, and ciliated (Spina et al., 1998). Airway cells can change their structure and functions either to adapt to changes in the local environment or to repair the epithelium after injury or inflammation.

Basal cells, the progenitors of epithelial cells, contain bundles of low molecular weight cytokeratins in their cytoplasm (Knight et al., 2003). In the upper and lower airways, the surface airway epithelium is normally pseudostratified, which means that all cells are attached to the basement membrane, but not all reach the airway lumen. Within the airway epithelium, basal cells are the only cell type that firmly attaches to the basement membrane and thereby mediate attachment of more superficial cells to the basement membrane (Knight

et al., 2003; Evans et al., 1988). Basal cells also mediate inflammatory responses and trans-epithelial water movement, as well as neutralize reactive oxygen species (Evans et al., 1988).

Secretory goblet cells produce and secrete a thin layer of mucus that coats the airway epithelium, trapping inhaled irritants, pathogens, and potentially damaging particles. Goblet cells, also called mucous cells, are characterized by the periodic-acid-Schiff positive membrane-bound mucin granules in their cytoplasm (Kim et al., 1997). Goblet cells are mainly found in the bronchi and larger bronchioles. Another secretory cell type, the Clara cells, express Clara cell secretory protein (CCSP) and surfactant (Knight et al., 2003). Secretory cells are less than half of the population of epithelial cells, where ciliated cells make up more than 50% (Knight et al., 2003).

Ciliated cells are derived from both secretory Clara cells and basal cells. Their primary function is to maintain the process of mucociliary clearance. Mucociliary clearance is defined by the wave-like movement of the cilia towards the throat where particulates trapped in the mucus are removed from the airway. On average, there is a density of 300 cilia/cell. Ciliated cells were thought to be terminally differentiated, however, more recent evidence suggests that they can trans-differentiate into a secretory cell phenotype (Park et al., 2006).

Airway mucus and mucin

Mucus is a viscoelastic mixture of water, cell derived debris, cell mediators, and proteins; more specifically electrolytes, oxidants and antioxidants, and mucins (Rogers, 2007). Airway mucus is believed to be a liquid bi-layer with a thicker upper gel that floats

atop a thin watery layer. The accepted function of the upper layer is to entrap invading particulates while the thinner periciliary layer facilitates the lubrication of the beating cilia in the process of mucociliary clearance (Rogers, 2007). The mucus-entrapped irritants are transported to the throat where they are either swallowed and digested by the gastrointestinal tract or coughed up and expelled as sputum.

Mucins, the highly glycosolated protein structural components of mucus, are stored in membrane-bound granules in the cytoplasm of respiratory epithelial goblet cells. During exocytosis, they are hydrated to form a gel, which is secreted into the airway lumen (Verdugo et al., 1990). To date there are at least 20 mucin genes with at least nine (MUC 1, 2, 4, 5AC, 5B, 7,8,13, and 19) being expressed in human airways (Wu et al., 2007). Mucins are subclassified as gel-forming mucins (MUC2, 5AC, 5B, 6, and 19), non gel-forming mucins (MUC7, 8, and 9), and membrane-associated mucins (MUC1, 3A, 3B, 4, 11, 12, 13, 14, 15, 16, 17, 18, and 20) (Rose et al., 2006; Williams et al., 2006). MUC5AC, MUC2, and MUC5B gene and protein are most associated with airway inflammatory diseases, with MUC5AC prominently seen in goblet cells. MUC2 and MUC5B are present in submucosal gland mucous cells (Groneberg et al., 2002; Wang et al., 2007; Leikauf et al., 2002).

The mucins are high molecular weight glycoproteins (2~40mDa), rich in proline and serine/ threonine regions (Rose et al., 2006). The serine/threonine residues are heavily glycosylated, and 40–80% of the mass of such mucins consists of O-linked oligosaccharides (Rose et al., 2006). Cysteines at the N- and C- terminus link mucin monomers by disulfide bridges to form linear mucin oligomers (Rose et al., 2006).

Chronic inflammatory diseases and mucin hypersecretion

Chronic airway inflammation can result in morphologic and functional changes of the airway known as airway remodeling. Pathophysiological characteristics associated with this phenomenon are airway hyperresponsiveness, mucous cell hyperplasia, smooth muscle hypertrophy, and subepithelial fibrosis. The helper-T type 2 (Th2) cytokine interleukin 13 (IL-13) has been implicated as the key mediator (Wills-Karp et al., 1998; Kuperman et al., 2002; Black JL et al., 2001). Chronic inflammatory diseases such as asthma, cystic fibrosis, and chronic bronchitis are associated with hypersecretion of mucin resulting in excess mucus in the airways impairing mucociliary function, increased susceptibility to bacterial pathogens, vivification of inflammatory responses, and, in extreme cases, death (Rogers, 2007).

Asthma and mucin hypersecretion

Asthma is characterized by airway hyperresponsiveness, chronic pulmonary eosinophilia, elevated serum immunoglobulin E, and excessive mucus production (Wills-Karp et al., 1998) where there is a propensity to develop acute episodes of airway obstruction and wheezing (Barnes, 2002). It is possible that abnormalities in goblet cells, submucosal gland cells, and vascular endothelial cells could all contribute to the result of excess airway mucus. In asthma, blood vessel number in the submucosa and stimulation of inflammatory mediators, including histamine and leukotrienes, which are known to promote vascular permeability, are increased (Fahy, 2001). Excessive plasma leakage from blood vessels in the submucosa could contribute to acute or chronic mucus hypersecretion in asthma by increasing the availability of mucus contents. Fatal asthma is nearly always associated with

airway occlusion from mucous plugs (Houston et al., 1953). In addition, sputum production is a common symptom in asthma, especially during asthma exacerbations (Oppenshaw et al., 1989).

Cystic fibrosis and mucus hypersecretion

A defect in the cystic fibrosis transmembrane conductance regulator (CFTR) in airway epithelial cells is associated with mucus hypersecretion, inflammation and infection with marked airway obstruction responsible for the morbidity and mortality in patients with cystic fibrosis. There is a decreased capacity of mucins packed inside the mucin granules to swell from the condensed phase inside the granules into an expanded gel phase (Puchelle et al., 2002) due to abnormal regulation of the flow of salts and water (Vasconcellos et al., 1994). In part, this may decrease mucus transportability thus increasing its accumulation in the airway lumen (Puchelle et al., 2002). Sputum samples from cystic fibrosis patients were shown to contain an increased amount of filamentous Actin, which may also contribute to cystic fibrosis mucus hyperviscosity (Vasconcellos et al., 1994).

Exocytosis machinery

Exocytosis of mucin granules can be either unregulated, where constitutive low levels of mucus are secreted to coat and protect the airways, or regulated, where extracellular stimuli provoke the process. In either case, mucin granule contents discharged into the airway lumen results from the translocation and fusion of the granule to the plasma membrane. The molecular mechanisms of this regulated process, and the unregulated

process, are still poorly understood, thereby leaving few potential therapeutic targets to control excessive airway mucus secretion.

Even though the mechanism controlling mucin secretion has not been fully resolved, there are many secretagogues that have been found to provoke mucin hypersecretion, including oxidants (Wright et al., 1996), cytokines (Kuperman et al., 2002), human neutrophil elastase (Park JA et al., 2005), and purinergic agonists (Rossi et al., 2006). In most cases, the secretagogues are believed to induce mucin secretion via PKC activation (Li et al., 2001). A large number of studies have implicated protein phoshorylation and the Actin cytoskeleton in regulated exocytosis (reviewed in Burgoyne et al., 2003). Regulated exocytosis of secretory granules has been examined in many well-characterized cell types where the mechanisms are similar to synaptic vesicle exocytosis (Burgoyne et al., 2003). Most secretory granules have the same basic protein components: soluble N-ethylmaleimide-sensitive fusion attachment protein (SNAP) receptor (SNARE) (i.e. SYNTAXIN or SNAP-25), SNARE regulators (i.e. N-ethylmaleimide-sensitive fusion and α-SNAP), Rab proteins and effectors (i.e. Rab 3 and Noc2), calcium binding proteins (i.e. calmodulin) (Burgoyne et al., 2003), and Sec1/Munc18-like proteins (SM proteins). Despite the conservation of core machinery, many variations occur in the control of secretory granule exocytosis that are related to the specialized physiological role of particular cell types.

Docking and Fusion

It has been well documented that vesicle docking and fusion is regulated by SNAREs (soluble N-ethylmaleimide-sensitive fusion protein attachment protein) receptors of the

transport vesicle and target membranes. The location of the SNARE dictates its classification as either a vesicle (v-) SNARE or target (t-) SNARE. SNARE proteins form coiled-coil aggregates that help link two opposing membranes for fusion (Sollner et al., 1993). Knockout mice of v-SNARE VAMP-8 have a significant defect in agonist-induced secretion (Ren et al., 2007).

The Rab GTPase family is also critical to this process by positioning granules appropriately for the v-SNARE on the granule (i.e. VAMP2) and t- SNARES on the plasma membrane (i.e. SYNTAXIN) to interact. There are over 50 protein members of the Rab GTPase family, and they have been implicated in the formation, targeting, and fusion of transport vesicles. Rab proteins play a well-established role in docking of vesicles to their target compartment and in vesicle association with the Actin cytoskeleton (Lapierre et al., 2001). Fusion and docking proteins are cell-specific. Using transfected HEK293 and HeLa cells as an in vitro assay system, it was demonstrated that truncated syntaxins lacking a transmembrane region attenuated the exocytosis of human growth hormone (Khvotchev et al., 2007). This attenuation was enhanced by co-expression of either Munc18-1 or SNAP-25 suggesting that truncated syntaxins block exocytosis by forming an inhibitory SNARE complex/Munc18-1 (Khvotchev et al., 2007).

There are other proteins involved in docking and fusion such as α-SNAP and NSF. Both α-SNAP and NSF are involved in disassembling SNARE complexes, which requires large amounts of energy. NSF, an ATPase that provides the necessary energy, is recruited to the complex by α-SNAP (Burgoyne et al., 2003).

Myristoylated alanine-rich C-kinase substrate

MARCKS is a PKC substrate that has been implicated in biological processes including secretion, cell motility, phagocytosis, membrane trafficking, and Actin cytoskeletal involvement (Hartwig et al., 1992; Seykora et al., 1996). MARCKS has three distinct domains: the myristoylated N terminus; the Multiple Homology 2 (MH2) domain; and the Phosphorylation Site Domain (PSD). MARCKS associates tightly with membranes by insertion of the myristic acid moiety at the N terminus into the lipid bilayer and ionic interactions between the PSD and polar head groups of membrane phospholipids. A synthetic peptide corresponding to the first 24 sequences of the N-terminus inhibit constitutive and cholinergically stimulated mucin secretion in ovalbumin sensitized mice (Singer et al., 2004). When PKC phosphorylates the PSD of MARCKS, it can no longer insert into membranes, as the myristic acid insertion is not strong enough to keep it attached. Phosphorylated MARCKS cannot bind to actin, myosin, or Ca2+/calmodulin via the PSD (Thelen et al., 1991; Seykora et al., 1996). Therefore, MARCKS cycles between the plasma membrane and the cytosol as a result of PKC phosphorylation of its PSD and perhaps, subsequent dephosphorylation by protein phosphatases (Thelen et al., 1991; Clarke et al., 1993). There is no known function for the MH2 domain.

Several studies suggest that MARCKS has a role in exocytosis where phosphorylation of MARCKS by PKC, or interaction of MARCKS with calmodulin (CaM), causes its translocation from the plasma membrane to the cytoplasm and its disassembly from the Actin filaments, thus allowing the secretory vesicles to fuse with the plasma membrane (Porumb et al., 1997.; Arbuzova et al., 1998; Arbuzova et al., 2000; Dank et al.,

1999; Vaaraniemi et al., 1999; Wohnsland et al., 2000; Eliyahu et al., 2006). Binding of MARCKS to CaM can trigger translocation of MARCKS from the plasma membrane to the cytosol in cortical granule exocytosis (Eliyahu et al., 2006) and a CaM inhibitor nullifies this effect. When the PSD of MARCKS was deleted in the human bronchial epithelial cell line HBE-1, mucin secretion stimulated by PKC agonists was attenuated (Li et al., 2001) Furthermore, pre-incubation of HBE-1 cells with a PKCδ-specific inhibitor, Rottlerin, also reduced mucin secretion as well as phosphorylation of MARCKS in these cells. These effects were significantly enhanced by the PKCδ activator bryostatin 1 (Park JA et al., 2005). MARCKS may serves as the mediator of mucin secretion by facilitating the translocation of the granule from the cytosol to the plasma membrane via an Actin/myosin contractile events.

Heat shock protein 70 and Cysteine string protein

Heat shock proteins are a family of proteins activated to respond to a wide range of stressors. HSP70 is specifically involved in maintenance of cellular integrity via protein translocation folding (reviewed in Pilon M et al., 1999). HSP70, the inducible form of HSC70, is a molecular chaperone that can be activated by CSP (Evans et al., 2003). HSC70/HSP70 have two distinct domains: an ATPase domain; and a substrate-binding domain. Both domains must cooperate to create a binding site for CSP. The C-terminal domain of HSC70 seems to function as a regulator for the formation of the HSC70/CSP complex (Stahl et al., 1999). Interaction between constitutively expressed heat shock protein (Hsc 70) and CSP is important for cortical granule exocytosis in Xenopus oocytes (Smith et al.,2005). Point mutations in the J-domain of CSP interfere with binding to Hsc 70 and

cortical granule exocytosis (Smith et al.,2005).

Cysteine string proteins (CSPs) belong to the DnaJ-like chaperone family and play an important role in regulated exocytosis in neurons and endocrine cells. (Boal et al., 2007). CSP is a PKA substrate believed to stabilize exocytosis proteins and their complexes (Evans et al., 2003). CSPs have several conserved functional domains: a cysteine string which can be extensively palmitoylated (Boal et al.); an N-terminal J domain (the defining domain of the dnaJ family of chaperones); a linker domain and the C-terminal domain. CSP is localized to the membranes of secretory vesicles of various cell types due to its heavily palmitoylated cysteine string (Chamberlain et al., 1998), and the J domain mediates CSP binding and activation of HSC70/HSP70 (Chamberlain et al., 1998). The linker domain is important to secretion, as demonstrated by a mutation in this domain attenuates insulin secretion in hamster pancreatic β cells (Zhang et al., 2002). In a study adrenal chromaffin cells, CSP is phosphorylated on the Ser 10 residue by PKA, or more recently, by Akt increased the exocytosis event that was catecholamine release (Evans et al., 2001). It has been hypothesized that CSP interacts with syntaxin and synaptotagmin, depending on its phosphorylation state; phosphorylation of CSP at Ser 10 reduces its binding affinity 10 fold (Evans et al., 2001). Through its interaction with the ubiquitous chaperone, HSC70, it is thought that cysteine string protein targets chaperone complexes to the exocytosis machinery to facilitate the correct folding of polypeptides or to regulate the assembly of protein complexes in synaptic vesicles (SV) (Evans et al., 2003). In a recent study, CSP was identified as a component of a chaperone complex containing α-GDP-dissociation inhibitor (αGDI), which is involved in removing Rab3A-GDP from the synaptic vesicle membrane,

Hsp90 and HSC70 further demonstrating a role for CSP and HSP70 in exocytosis (Sakisaka et al., 2002).

Calcium activated chloride channel 1

Calcium activated chloride channel proteins are a large family that were originally identified as integral membrane proteins. It is known that hCLCA1 is linked to mucus hypersecretion, as inhibiting this protein reduces mucus secretion. To determine the effect of niflumic acid (NFA), an inhibitor of mCLCA3, on mucin expression and mucus secretion Hauber et al. (2004) used LPS to stimulate human lung carcinoma cells and explanted human airway tissue. LPS-induced hCLCA1 and muc5AC expression was attenuated by NFA in a concentration-dependent manner, as shown by quantitative real time PCR and PAS staining (Hauber et al., 2004). In another study cigarette smoke-induced mucus production was inhibited by NFA (Hegad AE et al.). Mice exposed to cigarette smoke, or a human bronchial epithelial cell line (NCI-H292) stimulated with cigarette smoke-extract, both showed an upregulation of hCLCA1, Muc5AC expression, and mucin synthesis, a phenomenon completely inhibited by NFA. Taken together, these reports suggest an important role for hCLCA1 in pathological mucin production.

Proteomics

Proteomics can be described as the identification, characterization and quantification of all proteins involved in a particular pathway of an organism that can be studied in concert,

thereby providing a more accurate and comprehensive representation of the system at that time. The goal of many proteomic studies is to identify the protein signatures of various diseases, especially at their onset to help in the diagnosis and treatment. Protein signatures are particularly important in drug design and clinical studies, and also important to basic scientists who are atrying to understand how proteins change to facilitate an overall function such as growth or exocytosis of secretory vesicles.

REFERENCES

Arbuzova A, Murray D, McLaughlin S. MARCKS, membranes, and calmodulin: kinetics of their interaction. Biochim Biophys Acta. 1998 Nov 10;1376(3):369-79. Review.

Arbuzova A, Schmitz AA, Vergères G. Cross-talk unfolded: MARCKS proteins. Biochem J. 2002 Feb 15;362(Pt 1):1-12. Review.

Barnes PJ. Cytokine modulators as novel therapies for asthma. Annu Rev Pharmacol Toxicol. 2002; 42:81-98. Review.

Black JL, Roth M, Lee J, Carlin S, Johnson PR.Mechanisms of airway remodeling. Airway smooth muscle. Am J Respir Crit Care Med. 2001 Nov 15;164(10 Pt 2):S63-6.

Boal F, Le Pevelen S, Ciepluch C, Scotti P, Lang J. Cysteine-string protein isoform beta (Cspbeta) is targeted to the trans-Golgi network as a non-palmitoylated CSP in clonal beta-cells. Biochim Biophys Acta. 2007 Feb;1773(2):109-19.

Burgoyne RD, Morgan A. Secretory granule exocytosis. Physiol Rev. 2003 Apr;83(2):581-632. Review.

Chamberlain LH, Burgoyne RD. Activation of the ATPase activity of heat-shock proteins Hsc70/Hsp70 by cysteine-string protein. Biochem J. 1997 Mar 15;322 (Pt 3):853-8.

Chamberlain LH, Burgoyne RD. The cysteine-string domain of the secretory vesicle cysteine-string protein is required for membrane targeting. Biochem J. 1998 Oct 15;335 (Pt 2):205-9.

Clarke PR, Siddhanti SR, Cohen P, Blackshear PJ. Okadaic acid-sensitive protein phosphatases dephosphorylate MARCKS, a major protein kinase C substrate. FEBS Lett. 1993 Dec 20;336(1):37-42.

Danks K, Wade JA, Batten TF, Walker JH, Ball SG, Vaughan PF. Redistribution of F-actin and large dense-cored vesicles in the human neuroblastoma SH-SY5Y in response to secretagogues and protein kinase Calpha activation. Brain Res Mol Brain Res. 1999 Feb 5;64(2):236-45.

Eliyahu E, Shtraizent N, Tsaadon A, Shalgi R. Association between myristoylated alanin-rich C kinase substrate (MARCKS) translocation and cortical granule exocytosis in rat eggs. Reproduction. 2006 Feb;131(2):221-31.

Evans GJ, Morgan A, Burgoyne RD. Tying everything together: the multiple roles of cysteine string protein (CSP) in regulated exocytosis. Traffic. 2003 Oct;4(10):653-9. Review.

Evans GJ, Wilkinson MC, Graham ME, Turner KM, Chamberlain LH, Burgoyne RD, Morgan A. Phosphorylation of cysteine string protein by protein kinase A. Implications for the modulation of exocytosis. J Biol Chem. 2001 Dec 21;276(51):47877-85.

Evans MJ, Plopper CG. The role of basal cells in adhesion of columnar epithelium to airway basement membrane. Am. Rev. Respir. Dis. 1988; 138: 481–3.

Fahy JV. Remodeling of the airway epithelium in asthma. Am J Respir Crit Care Med. 2001 Nov 15;164(10 Pt 2):S46-51. Review.

Groneberg DA, Eynott PR, Oates T, Lim S, Wu R, Carlstedt I, Nicholson AG, Chung KF. Expression of MUC5AC and MUC5B mucins in normal and cystic fibrosis lung. Respir Med. 2002 Feb;96(2):81-6.

Hartwig JH, Thelen M, Rosen A, Janmey PA, Nairn AC, Aderem A. MARCKS is an actin filament crosslinking protein regulated by protein kinase C and calcium-calmodulin. Nature. 1992 Apr 16;356(6370):618-22.

Hauber HP, Tsicopoulos A, Wallaert B, Griffin S, McElvaney NG, Daigneault P, Mueller Z, Olivenstein R, Holroyd KJ, Levitt RC, Hamid Q. Expression of HCLCA1 in cystic fibrosis lungs is associated with mucus overproduction. Eur Respir J. 2004 Jun;23(6):846-50.

Hegab AE, Sakamoto T, Nomura A, Ishii Y, Morishima Y, Iizuka T, Kiwamoto T, Matsuno Y, Homma S, Sekizawa K. Niflumic acid and AG-1478 reduce cigarette smoke-induced mucin synthesis: the role of hCLCA1. Chest. 2007 Apr;131(4):1149-5.

Houston JC, De Navasquez S, Trounce JR. A clinical and pathological study of fatal cases of status asthmaticus. Thorax 1953; 8: 207-213.

Khvotchev M, Dulubova I, Sun J, Dai H, Rizo J, Südhof TC. Dual modes of Munc18-1/SNARE interactions are coupled by functionally critical binding to syntaxin-1 N terminus. J Neurosci. 2007 Nov 7;27(45):12147-55.

Kim KC, McCracken K, Lee BC, Shin CY, Jo MJ, Lee CJ, Ko KH.Airway goblet cell mucin: its structure and regulation of secretion. Eur Respir J. 1997 Nov;10(11):2644-9. Review.

Knight DA, Holgate ST. The airway epithelium: structural and functional properties in health and disease. Respirology. 2003 Dec;8(4):432-46. Review.

Kuperman DA, Huang X, Koth LL, Chang GH, Dolganov GM, Zhu Z, Elias JA, Sheppard D, Erle DJ. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. Nat Med. 2002 Aug;8(8):885-9.

Lapierre L, Kumar R, Hales C, Navarre J, Bhartur S, Burnette J, Provance D, Mercer J, Bahler M, Goldenring J. Myosin vb is associated with plasma membrane recycling systems. Mol. Biol. Cell. 2001;12:1843–1857.

Leikauf GD, Borchers MT, Prows DR, Simpson LG. Mucin apoprotein expression in COPD. Chest. 2002 May;121(5 Suppl):166S-182S. Review.

Li Y, Martin LD, Spizz G, Adler KB. MARCKS protein is a key molecule regulating mucin secretion by human airway epithelial cells in vitro. J Biol Chem. 2001 Nov 2;276(44):40982-90.

Oppenshaw PJM, Turner-Warwick M. Observations on sputum production in patients with variable airflow obstruction; implications for the diagnosis of asthma and chronic bronchitis. Respir Med 1989; 83: 25-31.

Park JA, Crews AL, Lampe WR, Fang S, Park J, Adler KB. Protein kinase C delta regulates airway mucin secretion via phosphorylation of MARCKS protein. Am J Pathol. 2007 Dec;171(6):1822-30.

Park JA, He F, Martin LD, Li Y, Chorley BN, Adler KB. Human neutrophil elastase induces hypersecretion of mucin from well-differentiated human bronchial epithelial cells in vitro via a protein kinase C{delta}-mediated mechanism. Am J Pathol. 2005 Sep;167(3):651-61.

Park KS, Wells JM, Zorn AM, Wert SE, Laubach VE, Fernandez LG, Whitsett JA. Transdifferentiation of ciliated cells during repair of the respiratory epithelium. Am J Respir Cell Mol Biol. 2006 Feb;34(2):151-7.

Pilon M, Schekman R. Protein translocation: how Hsp70 pulls it off. Cell. 1999 Jun 11;97(6):679-82. Review.

Porumb T, Crivici A, Blackshear PJ, Ikura M. Calcium binding and conformational properties of calmodulin complexed with peptides derived from myristoylated alanine-rich C kinase substrate (MARCKS) and MARCKS-related protein (MRP). Eur Biophys J. 1997;25(4):239-47.

Puchelle E, Bajolet O, Abély M. Airway mucus in cystic fibrosis. Paediatr Respir Rev. 2002 Jun;3(2):115-9.

Puchelle E, Zahm JM, Tournier JM, Coraux C. Airway epithelial repair, regeneration, and remodeling after injury in chronic obstructive pulmonary disease. Proc Am Thorac Soc. 2006 Nov;3(8):726-33. Review.

Ren Q, Barber HK, Crawford GL, Karim ZA, Zhao C, Choi W, Wang CC, Hong W, Whiteheart SW. Endobrevin/VAMP-8 is the primary v-SNARE for the platelet release reaction. Mol Biol Cell. 2007 Jan;18(1):24-33.

Rogers DF. Physiology of airway mucus secretion and pathophysiology of hypersecretion. Respir Care. 2007 . Sep;52(9):1134-46; discussion 1146-9. Review.

Rose MC, Voynow JA. Respiratory tract mucin genes and mucin glycoproteins in health and disease. Physiol Rev. 2006 Jan;86(1):245-78. Review.

Rose MC. Mucins: structure, function, and role in pulmonary diseases. Am J Physiol. 1992 Oct;263(4 Pt 1):L413-29. Review.

Rossi AH, Salmon WC, Chua M, Davis CW. 2006. Calcium Signaling In Human Airway Goblet Cells Following Purinergic Activation. Am J Physiol Lung Cell Mol Physiol.

Sakisaka T, Meerlo T, Matteson J, Plutner H, Balch WE. Rab-αGDI activity is regulated by a Hsp90 chaperone complex. EMBO J 2002;21: 6125–6135.

Seykora JT, Myat MM, Allen LA, Ravetch JV, Aderem A. 1996. Molecular determinants of the myristoyl-electrostatic switch of MARCKS. J Biol Chem. 271:18797-802.

Singer M, Martin LD, Vargaftig BB, Park J, Gruber AD, Li Y, Adler KB. A MARCKS-related peptide blocks mucus hypersecretion in a mouse model of asthma. Nat Med. 2004 Feb;10(2):193-6.

Smith GB, Umbach JA, Hirano A, Gundersen CB.Interaction between constitutively expressed heat shock protein, Hsc 70, and cysteine string protein is important for cortical granule exocytosis in Xenopus oocytes. J Biol Chem. 2005 Sep 23;280(38):32669-75.

Söllner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, Rothman JE. SNAP receptors implicated in vesicle targeting and fusion. Nature. 1993 Mar 25;362(6418):318-24.

Spina D. Epithelium smooth muscle regulation and interactions. Am. J. Respir. Crit. Care Med. 1998; 158: S141–5.

Stahl B, Tobaben S, Südhof TC. Two distinct domains in hsc70 are essential for the interaction with the synaptic vesicle cysteine string protein. Eur J Cell Biol. 1999 Jun;78(6):375-81.

Thelen M, Rosen A, Nairn AC, Aderem A. Regulation by phosphorylation of reversible association of a myristoylated protein kinase C substrate with the plasma membrane. Nature. 1991 May 23;351(6324):320-2.

Vääräniemi J, Palovuori R, Lehto VP, Eskelinen S. Translocation of MARCKS and reorganization of the cytoskeleton by PMA correlates with the ion selectivity, the confluence, and transformation state of kidney epithelial cell lines. J Cell Physiol. 1999 Oct;181(1):83-95.

Vasconcellos CA, Allen PG, Whol ME, Drazen JM, Janmeuy PA, Stossel TP. Reduction in viscosity of cystic fibrosis sputum in vitro by gelsolin. Science 1994(263):969–971.

Verdugo P. Goblet cells secretion and mucogenesis. Annu Rev Physiol. 1990;52:157-76. Review.

Wang K, Wen FQ, Feng YL, Ou XM, Xu D, Yang J, Deng ZP. Increased expression of human calcium-activated chloride channel 1 gene is correlated with mucus overproduction in Chinese asthmatic airway. Cell Biol Int. 2007 Nov;31(11):1388-95.

Williams OW, Sharafkhaneh A, Kim V, Dickey BF, Evans CM. Airway mucus: From production to secretion. Am J Respir Cell Mol Biol. 2006 May;34(5):527-36. Review.

Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, Donaldson DD. Interleukin-13: central mediator of allergic asthma. Science. 1998 Dec 18;282(5397):2258-61.

Wohnsland F, Schmitz AA, Steinmetz MO, Aebi U, Vergéres G. Interaction between actin and the effector peptide of MARCKS-related protein. Identification of functional amino acid segments. J Biol Chem. 2000 Jul 7;275(27):20873-9.

Wright DT, Fischer BM, Li C, Rochelle LG, Akley NJ, Adler KB. Oxidant stress stimulates mucin secretion and PLC in airway epithelium via a nitric oxide-dependent mechanism. Am J Physiol. 1996 Nov;271(5 Pt 1):L854-61.

Wu DY, Wu R, Chen Y, Tarasova N, Chang MM. PMA stimulates MUC5B gene expression through an Sp1-based mechanism in airway epithelial cells. Am J Respir Cell Mol Biol. 2007 Nov;37(5):589-97.

Zhang H, Kelley WL, Chamberlain LH, Burgoyne RD, Lang J. Mutational analysis of cysteine-string protein function in insulin exocytosis. J Cell Sci. 1999 May;112 (Pt 9):1345-51.

SUMMARY AND HYPOTHESIS

Mucin secretion, exocytosis of mucin granules, involves the translocation of the granule from the cytoplasm of the goblet cells to the plasma membrane where it docks and fuses thus secreting its mucin contents into the airway lumen. This process is regulated response to stimulation; however, constitutively low levels facilitate the normal mucociliary clearance mechanism. Mucin hypersecretion results in excess mucus in the airways and is a phenotype associated with chronic inflammatory diseases such as chronic bronchitis, asthma, and cystic fibrosis that (Wills-Karp et al., 1998, Kuperman et al., 2002; Rogers, 2007). Several proteins have been associated with the mucin hypersecretory phenotype, including myristoylated alanine-rich C kinase substrate (MARCKS), calcium activated chloride channel (hCLCA1), and chaperones cysteine string protein (CSP) and heat shock protein 70 (HSP70). The molecular mechanisms of this regulated exocytosis process is still poorly understood thereby leaving few potential therapeutic targets to control excessive airway mucus secretion.

The aims of the first study were:

1) to determine whether or not MARCKS, HSP70, and CSP interact as mucin granule associated proteins in normal bronchial epithelial cells;
2) to elucidate other proteins associated with the mucin granule membranes;
3) to determine whether or not Protein kinase C activation alters the proteins associated with the mucin granule membrane.

We elucidate some aspects of the regulated exocytosis mechanism whereby MARCKS and chaperones cysteine string protein and heat shock proteins interact in a complex under

stimulated and unstimulated conditions. We found that these proteins form a complex that is associated with the mucin granule in normal bronchial epithelial cells grown using the in vitro air-liquid interface culture system. Another novel finding was that hCLCA1 is also found in complex with MARCKS, CSP, and HSP70 on the mucin granule membranes. We further elucidated novel proteins that associate with the mucin granule membrane, most of which are cytoskeletal related. PMA stimulation did not appear to alter these interactions.

The aims of the second study were:

1) to determine whether or not MARCKS, CSP, HSP70, and hCLCA1 interactions were conserved in diseased cystic fibrosis cells and the intestinal colon cancer cells;
2) to determine whether or not novel protein associations with the mucin granule membrane could be identified.

We elucidates proteins associated with the mucin granules in unstimulated and stimulated conditions further expanding the study to include a diseased model, cystic fibrosis cell line UNCN3T, and the intestinal cell line HT29-18N2. We found that the key proteins, MARCKS, CSP, and HSP70 along with many other proteins are identified in all cell types suggesting possible conservation in regulated exocytosis machinery. However, the intestinal cells were dissimilar with the regulation of these proteins from the bronchial epithelial cells.

Overall these studies further validate key players and mechanisms in our hypothetical model of mucin secretion in airway epithelial cells. These data provide identification of numerous proteins that may further be identified as core regulated exocytosis machinery.

CHAPTER II

MUCIN GRANULE ASSOCIATED PROTEINS AND A LINK BETWEEN MARCKS AND hCLCA1 IN NORMAL BRONCHIAL EPITHELIAL CELLS

Authors: **Kimberly L Raiford, Jougjoa Park, Ko-Wei Lin, and Kenneth B Adler.**

North Carolina State University, Raleigh, NC, United States.

Institution: **Department of Molecular Biomedical Sciences,**

College of veterinary medicine

North Carolina State University,

Raleigh, NC 27606.

ABSTRACT

Dysregulation of mucin secretion causing excess mucus in the airways can result in obstruction in patients with diseases such as chronic bronchitis, asthma, and cystic fibrosis. Mucins, the highly glycosolated protein components of mucus, are stored in membrane-bound granules housed in the cytoplasm of mucous and goblet cells until they are secreted into the airway lumen during the process of regulated exocytosis. The precise mechanism of mucin secretion, including those proteins involved in the process, has yet to be elucidated. Previously, we have shown that the Myristoylated Alanine-Rich C Kinase Substrate (MARCKS) regulates mucin secretion by controlling mucin granule movement from the cytosol to the plasma membrane where the granule docks, fuses and releases its contents into the airway lumen (Li et al., 2001). A calcium-activated chloride channel (mCLCA3 alias Gob-5) known to associate with the mucin granule membranes has also been linked with mucus overproduction and airway hyperresponsiveness in mouse models of pulmonary inflammation (Wills-Karp et al., 1998; Kuperman et al., 2002). Link(s) between MARCKS and hCLCA1 (the human ortholog of Gob-5) have not yet been shown. Here, analysis of mucin granules isolated from well-differentiated normal human bronchial epithelial (NHBE) cells maintained in air/liquid interface culture revealed that MARCKS and hCLCA1, together with the chaperones Cysteine String Protein (CSP) and Heat Shock Protein 70 (HSP70), appear to all associate together on the mucin granule membrane. These four proteins appear to complex together and to interact to regulate the mucin secretion process, as inhibition of any one of these via different techniques attenuates mucin secretion (Li et al., 2001; Wills-Karp et al., 1998; Evans et al., 2003).

INTRODUCTION

The role of the airway epithelium extends well beyond being the physical barrier between the external and internal milieu. The airway epithelium functions in providing overall pulmonary homeostasis mediating inflammatory responses to injury, regulating lung fluid balance and anti-oxidant release, and mucociliary clearance of inhaled agents (Knight et al., 2003). Mucins, the highly glycosolated protein components of mucus are stored in membrane-bound granules in the cytoplasm of airway epithelial goblet cells. When secreted, a thin layer of mucus forms that protects the airways from inhaled pathogens and particulates, while the ciliated epithelial cells transport the contaminated mucus out of the airways, defining the process of mucociliary clearance (Kim et al., 1997; Rogers, 2007).

Mucin secretion, exocytosis of mucin granules, involves the translocation of the granule from the cytoplasm of the goblet cells to the plasma membrane where it docks and fuses, thus allowing for secretion of its mucin contents into the airway lumen. This process is a regulated response to stimulation; however, constitutively low levels of mucin secretion facilitate the normal mucociliary clearance mechanism. Mucin hypersecretion results in excess mucus in the airways and is a phenotype associated with chronic inflammatory diseases such as chronic bronchitis, asthma, and cystic fibrosis that (Wills-Karp et al., 1998; Kuperman et al., 2002; Rogers, 2007). Several proteins have been associated with the mucin hypersecretory phenotype, including myristoylated alanine-rich C kinase substrate (MARCKS), calcium activated chloride channel (hCLCA1), and chaperones cysteine string protein (CSP) and heat shock protein 70 (HSP70). The molecular mechanisms of this

regulated exocytosis process are still poorly understood thereby leaving few potential therapeutic targets to control excessive airway mucus secretion.

MARCKS is a protein kinase C substrate that has been implicated in biological processes including secretion, cell motility, and Actin cytoskeletal function (Wright et al., 1996; Rossi et al., 2006). MARCKS associates with membranes by insertion of its myristic acid moiety at the N terminus into the lipid bilayer and ionic interactions between the phosphorylation site domain (PSD) and polar head groups of membrane phospholipids. A synthetic peptide corresponding to the first 24 sequences of the N-terminus inhibits constitutive and cholinergically-stimulated mucin secretion in ovalbumin sensitized mice (Singer et al., 2004). Phosphorylation of MARCKS renders it incapable of binding Actin, myosin, or Ca2+/calmodulin since these proteins also associate with MARCKS via the PSD (Thelen et al., 1991; Seykora et al., 1996). Numerous studies have shown that MARCKS cycles between the plasma membrane and the cytosol as a result of Protein Kinase C phosphorylation of its PSD and subsequent dephosphorylation by protein phosphatases (Park JA et al., 2005; Hartwig et al., 1992). Ultrastructural immunohistochemistry of isolated mucin granule from murine bronchial epithelial cells show an association of MARCKS with the granules (Singer et al., 2004). These findings suggest that MARCKS may serves as a mediator of mucin secretion by facilitating the translocation of the granule from the cytosol to the plasma membrane via Actin/myosin based contractile system.

The molecular chaperones HSP70 and the Protein Kinase A substrate CSP have been implicated in regulated exocytosis of various cell types (Evans et al., 2003; Smith et al.,2005, Sakisaka et al., 2002). CSP is localized to the membranes of secretory vesicles by way of its

heavily palmitoylated cysteine string, and it is believed to stabilize exocytosis proteins and their complexes (Chamberlain et al., 1998). HSP70, the inducible form of HSC70, is a molecular chaperone that can be activated by CSP (Chamberlain et al., 1998). Studies from our lab illustrated that both CSP and HSP70 may regulate mucin secretion, because of either protein via blocking peptides or siRNA attenuated mucin secretion (Park et al., 2006). Interaction between constitutively expressed heat shock protein, Hsc 70, and cysteine string protein is also important for cortical granule exocytosis in Xenopus oocytes where point mutations in the J-domain of CSP interfere with binding to Hsc 70 and cortical granule exocytosis (Smith et al., 2005). It was also reported that CSP/HSC70 complexes targets chaperone complexes to the exocytosis machinery to regulate the assembly of protein complexes in synaptic vesicles (Evans et al., 2003).

Human calcium-activated chloride channel and its murine ortholog, mCLCA3 (alias Gob-5) have been shown to play a role in goblet cell hyperplasia and mucus overproduction (Wills-Karp et al., 1998; Kuperman et al., 2002). Recent studies identified hCLCA1 as a secreted protein, not a channel, more likely a regulator of chloride channels (Gibson et al., 2005; Mundhenk et al., 2006). Subsequent bioinformatics analysis and immunoprecipitation experiments from the same group (Gruber et al., 1998) identified mCLCA3/hCLCA1 as a strongly associated mucin granule protein (Gibson et al., 2005, Mundhenk et al., 2006). Immune transmission electron microscopy using gold-labeled secondary antibodies of murine intestinal mucosa localized mCLCA3 to mucin granule membranes (Leverkoehne et al., 2002). mCLCA3 was identified as exclusively associated with mucin granule membranes of gastrointestinal, respiratory, and uterine goblet cells and other mucin-

producing cells (Leverkoehne et al., 2002), so it has been used as a biomarker in mucin granule isolations (Singer et al., 2004) .

MARCKS, hCLCA1, CSP, and HSC70/HSP70 have all been implicated in mucin hypersecretion (Li et al., 2001; Park et al., 2006; Nakanishi et al., 2001). Here we evaluated whether or not MARCKS, CSP, and HSP70 proteins are associated with the membranes of mucin granules in both constitutive and stimulated conditions. We further expand our scope to identify a proteome in normal human bronchial epithelial (NHBE) cells that is associated with the membranes of mucin granules. The results confirm that these proteins do associate with the mucin granules, along with many other cytoskeletal, signaling, and accessory proteins. Interestingly, we found that MARCKS, CSP, and HSP70 also form a complex with hCLCA1. This could aid in our understanding of the mechanisms of mucin secretion and suggest potential new functions of these proteins.

MATERIALS AND METHODS

Cell Culture

Primary culture of NHBE cells in an air/liquid interface system has been described previously (Krunkosky et al., 2000). Briefly, commercially available normal human bronchial epithelial (NHBE) cells (Lonza, Cambridge, MA) were seeded into vented T75 tissue culture flasks at a density of 500 cells/cm^2. The cells were expanded in growth medium at 5% CO_2 at 37ºC to a confluence of 85-90%, dissociated from the flasks using 0.25% trypsin/EDTA, and frozen in liquid nitrogen as passage-2 cells (2 X 10^6 cells/ml).

Air/liquid interface cultures of NHBE cells were established on Transwell®-Clear culture 0.4μm pore polyester inserts (Costar, Cambridge, MA) thinly coated with rat-tail collagen type I (Collaborative Biomedical, Bedford, MA). Frozen NHBE cells were recovered and seeded at a density of 2 x 10^4 cells/cm^2 onto the apical surface of the inserts with complete media alone in the basolateral compartment. The complete medium was composed a 50:50 mixture of bronchial epithelial growth medium and high glucose (4.5 g/L) Dulbecco's modified Eagle's medium containing a final concentration of 50 μg/ml gentamicin, 5 μg/ml insulin, 10 μg/ml transferrin, 0.5 μg/ml epinephrine, 6.5 ng/ml triiodothyronine, 0.5 ng/ml human recombinant epidermal growth factor, 0.5 μg/ml hydrocortisone, 50 ng/ml amphotericin-B (Lonza), 0.13 mg/ml bovine pituitary extract, 5 x 10–8 mol/L all-trans retinoic acid, 1.5 μg/ml bovine serum albumin (Sigma, St. Louis, MO), and 20 U/ml nystatin (Ameresco, Solon, OH). Cells were grown submerged in a 5% CO_2 atmosphere at 37°C, and medium was changed the next day, then every other day until cells reached 90% confluence. At this point, air/liquid interface (ALI) was established by

removing the apical media (Day 0 ALI), whereas basolateral media was changed daily for 14 days. Mucin was observed at 14 days in culture (Day 7 ALI) and cilia were apparent by 18 days in culture (Day 11 ALI), and experiments were conducted on cells at 21 days in culture (Day 14 ALI) ensuring that the culture was well differentiated. An immortalized cystic fibrosis cell line, UNCN3T, grown in the same manner as the NHBE cells was also utilized.

When stimulating NHBE cells, the apical surface of the cells was washed in phosphate buffered saline (PBS), pH 7 using gentle agitation for 5 minutes prior to treatment to remove the accumulated mucus. Cells were exposed to 100nM of the protein kinase C (PKC) agonist phorbol-12-myristate-13-acetate (PMA) doped medium or medium alone for 2, 5, 10, and 15 minutes.

Immuno-isolations of mucin granules

Granule immuno-isolations were performed using a modified version of a protocol from Wu et al. (1997). After treatments, cells were washed in PBS then collected in isolation buffer (PBS, 1 mM phenylmethyl sulfonyl fluoride, protease inhibitor cocktail 1, phosphatase inhibitor cocktail (Sigma, St. Louis, MO) using a rubber policemen. Cell lysates were pooled into control or PMA stimulated samples (due to the low yield of mucin granules). The collected cells were lysed by brief sonication, and the lysates were spun at 600 x g for 10 minutes. The supernatants were added to 1.9 volumes of 86% Percoll, 0.3M sucrose, 5mM MOPS (4-Morpholinepropanesulfonic acid), 1mM EDTA, and 0.2μg/ml DPPD (N,N'-

diphenyl-4-phenylenediamine) (Sigma), ph 6.8, and centrifuged for 30 minutes at 17,000 x g in a Sorvall Discovery 100S ultracentrifuge (Sorvall, Inc. Newtown, CT). The crude granules were transferred from the bottom of the self-formed gradient into a new tube, diluted with 3 volumes of 0.3M sucrose containing 2mM MOPS, 1mM EDTA, and 0.2µg/ml DPPD, and centrifuged for 15 minutes at 2000 x g. The pellet was reconstituted in PBS, incubated with an antibody to gob-5/mclca3 (ortholog to human clca1) overnight at 4°C on a nutator. The rabbit polyclonal gob-5 antibody used was generated to the mclca3 peptide epitope ESWKAKPEYTRPKLE (Covance, Denver, PA). After incubation, the antibody-granule complex was applied to protein G coated Dynal beads. The beads were washed thoroughly and the complex was eluted with Na-citrate pH 2.5 or loading dye.

Protein subcellular fractionation

After treatments, cells were washed with ice-cold PBS containing a phosphatase inhibitor (Active Motif Inc, Carlsbad, CA) and then scraped into lysis buffer (50 mM Tris, pH 7.5, 1 mM ethylenediamine tetraacetic acid, 100 mM NaCl, 1 mM phenylmethyl sulfonyl fluoride) using a rubber policemen. The collected cellular mixture was lysed by brief sonication. The lysates were spun at 14,000 x g at 4°C in an Eppendorf 5417R centrifuge (Eppendorf Corp., Hamburg, Germany) for 30 minutes to separate the cytosolic and membrane fractions. The supernatant was kept as the cytosolic sample while the pellet was resuspended in lysis buffer containing 0.01% Triton-100, dissolved by sonication, and incubated on ice for 30 minutes. Following incubation, the samples were centrifuged again at 14,000 x g at 4°C for 30 minutes, and the supernatant separated from the pellet mixture was

kept as the membrane fraction.

For preparation of whole cell crude lysates, the disrupted cellular mixture was centrifuged at 15,000 rpm in an Eppendorf 5417R centrifuge (Eppendorf Corp., Hamburg, Germany) for 15minutes at 4°C. The supernatant was collected as the whole crude cell lysate.

The protein concentrations of all cell lysate samples were quantified by a Bradford assay (BioRad Laboratories, Hercules, CA). Bovine serum albumin (Sigma) was used as the standard and serial dilutions were made from the initial stock concentration of 400 ng/ml. Absorbance values were determined by the microplate reader system, and the linear regression and protein concentrations calculated by SoftMax Pro data analysis software (Molecular Devices, Sunnyvale, CA).

Co-immunoprecipitation of protein complexes and Western analysis

Whole cell or mucin granule lysates containing 500-1000μg/ml total protein were incubated overnight at 4°C with 3-10 μl (20-30μg) of a specific antibody. Twenty-five μl of Protein G dynal beads (Invitrogen, Carlsbad, CA) were added to bind the antibody-protein complex for 3 hours. Beads were washed three times with cold PBS, and proteins were eluted with 1X sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer and boiled 10 min before the proteins were resolved on SDS-PAGE gel. Resolved proteins were transferred to a 0.45 μM nitrocellulose membrane (BioRad, Hercules, CA), blocked with 5% skim milk, and either mouse anti-MARCKS (Millipore, Bedford, MA), rabbit anti-CSP, mouse anti-HSP70 (Abcam, Cambridge, UK), rabbit anti-Myosin Vc, rabbit anti-Cofilin, or rabbit anti-mCLCA3 antibody was used as the primary antibody to probe the membranes.

Visualization of the proteins occurred after probing with the secondary horseradish peroxidase-conjugated antibodies using an enhanced chemiluminescence kit (Chemicon, Buckinghamshire, UK) followed by exposure to film. The densitometry was analyzed by Labworks image acquisition and analysis software (UVP Inc, Upland, CA).

Ultrastructural Immunohistochemistry

Well differentiated cell cultures were washed with PBS then fixed on the transwell insert with 4% formaldehyde:1% glutaldehyde in phosphate buffer. In mucin granule membrane preparations, the granule membranes were fixed in the magnetic bead slurry. The tissue samples were embedded in Spurr resin, cut into ultrathin sections, and placed on stainless steel grids. Grids were blocked in 10% fetal bovine serum in PBS for 15 minutes at room temperature followed by a 5 minute wash in 0.5% bovine serum albumin in PBS. Primary antibody probing of the grids was done overnight at 4°C on the nutator, washed repeatedly for one hour in 0.5% BSA in PBS, and probed with gold labeled secondary antibody for 2 hours at room temperature. The appropriate whole molecule IgG was alternately used as the primary antibody negative control. The grids were washed in PBS only repeatedly over an hour, dried quickly, post-stained with uranyl acetate, and examined with a FEI/Philips EM 208S transmission electron microscope. Pan mucin antibody 17Q2 was used as a positive control to identify intact mucin granules.

Liquid chromatography tandem mass spectrometry (LC-MS/MS)

Protein bands separated on a 1-Dimensional sodium dodecyl sulfate-polyacrylamide

gel (SDS-PAGE) were excised, dried with a solvent, extracted, and treated with hydroxyethyl disulfide as a thiol blocking reagent under alkaline conditions at 60°C. The extracted peptides were reduced nearly to dryness under a stream of air prior to trypsin digestion in 50mM ammonium bicarbonate pH~7.8. Samples were then incubated overnight at 37°C before analysis by LC/MS.

Peptides were analyzed by reverse phase HPLC with electrospray ionization mass spectrometry. Separations were achieved with a C18 HPLC column (Phenomenex Jupiter Proteo: 150mm x 0.50mm I.D., 4um particle size, 90A pore size) and a mobile phase operated with a programmed gradient with 50mM acetic acid and acetonitrile. The instrument used for the analysis was a Thermo Surveyor HPLC coupled with a Thermo LTQ ion trap mass spectrometer. The mass spectrometer was operated in positive ion mode with an electrospray ionization (ESI) source. The mass spectrometer was operated in data dependent MS/MS scan mode scanning from m/z 420-2000 and collecting MS/MS spectra on the four most abundant ions in each scan.

Protein database searching

The acquired MS/MS spectra for each sample were searched using the BioWorks 3.1 SR1 SEQUEST algorithm (Thermo Electron, San Jose, CA) against the human nonredundant database. The nonredundant database was downloaded from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.ih.gov/blast/db/fasta). The nonredundant database was used for initial protein identification for tandem mass spectral data acquired in the ICR cell as well as the linear trap. Evaluation of total protein coverage

was done by creating a protein subset database consisting of *Homo sapiens* proteins only. Database searching parameters assumed proteolysis was performed using trypsin with the possibility of one internal cleavage residue. Searches were performed with trypsin specified as the enzyme with an allowance for up to two missed cleavage sites. Searches from replicates within an experiment were combined to generate a comprehensive list of peptides and proteins identified in a particular experiment. Acceptance levels for positive peptide identification were determined using cross-correlation scores (Xcorr). These scores aid in the determination of true positives, with higher scores increasing confidence in correct identifications. The minimum acceptable Xcorr for identified peptides was 2.0 (Vaughn et al., 2006, Peterman et al., 2005).

Statistical analysis

At least 5 replicate experiments were performed for each concentration of each reagent that was assayed. Controls such as medium alone or reagent vehicle were utilized. All reagents used in treating the cells were examined for cytotoxicity by measuring the total release of lactate dehydrogenase from the cells. All experiments were performed with reagents at non-cytotoxic concentrations.

Statistical analysis was performed on the data using Prism software (Graphpad, San Diego, CA) evaluating significance using one-way analysis of variance (ANOVA) with Bonferroni post-test corrections for multiple comparisons. Differences between treatments were considered significant at a p-value of < 0.05.

RESULTS

Specificity of mCLCA3 to hCLCA1 in airway epithelial cells

To determine whether or not there is hclca1 in airway epithelial cells, whole cell lysates from both NHBE and UNCN3T were analyzed by western blot analysis. Prior studies done in our lab evaluating hclca1 used a generously gifted rabbit anti-mclca3 antibody from Dr Achim Gruber, which was shown to associate with mucin granules in NHBE cells (Li et al., 2001). A new production of rabbit polyclonal anti-mclca3 antibody was generated as described above. While mclca3 is an ortholog of hclca1, this antibody to the mouse version had to be evaluated for its specificity to human clca1 in the NHBE air liquid interface system. Data has shown that in SDS-PAGE hclca1 appeared as a whole molecule (~125kDa) and two pieces (~83kDa; 50kDa) due to a cleavage site near the C-terminal end of the protein (Gibson et al., 2005). Western blot analysis with this new rabbit anti-mclca3 antibody detected hclca1 in whole cell lysates (Figure 1A). Furthermore, immunoprecipitation of cytosolic and plasma membrane fraction cell lysates with the anti-mclca3antibody followed by immunoblotting with a commercially available antibody synthesized to the peptide sequence in hclca1identified the same banding pattern (Figure 1B) in both fractions. Chromopure rabbit IgG (Jackson Immunoresearch, West Grove, PA) was used as the negative control. The levels of hCLCA1 did not appear affected by the 15 minute stimulation with PMA.

Transmission electron microscopy (TEM) of mucin granule membrane isolations

To further validate the cross reactivity of the rabbit anti-mCLCA3 antibody,

ultrastructural immunohistochemistry (ITEM) of well-differentiated NHBE cells examined the subcellular distribution of hCLCA1. Tissue sections were incubated with primary rabbit anti-mCLCA3 antibody (Figure 2A) followed by incubation with 12nm gold-labeled goat anti-rabbit secondary antibody. The micrographs shows that hCLCA1 appear to localize around the mucin granules membranes. There was little to no background seen in the negative controls (Figure 2B).

Validation of Immuno-isolation method of mucin granule preparation

In an attempt to visualize and verify that the mucin granule membrane isolations were indeed isolating granule membranes intact and fragmented, we used both standard TEM and ITEM. In Figure 3A, there is an intact mucin granule membrane. Figure 3B shows that the structures isolated are mucin granule membranes gold-labeled where hCLCA1 is associated. The positive control, primary pan mucin mouse anti-17Q2 antibody, further verifies that these structures are indeed mucin granule membranes (Figure 3C). The negative rabbit and mouse IgG controls are Figure 2D and Figure 2E, respectively.

Mucin granule membrane associated proteins

Mucin granule membranes isolated as previously described were eluted from the magnetic beads in SDS sample buffer, boiled 5 minutes, and separated by SDS-PAGE. Seven bands were excised from the gel and processed through LC-MS/MS. Table 1 represents the proteins identified. The majority of these proteins are cytoskeletal in nature (i.e. Myosin VC),

regulatory (i.e. Protein phosphatase 4, regulatory subunit 2) or function in docking and fusion (i.e. syntaxin 11).

Association of MARCKS, CSP, and HSP70 with the mucin granule membranes

MARCKS (Li et al., 2001), CSP, and HSP70 (Park et al., 2006) are reportedly linked to mucin secretion in airway epithelial cells, so we evaluated whether or not these proteins are associated with the membranes of mucin granules by Western blot analysis. Granule isolations from unstimulated and PMA stimulated well differentiated NHBE cells were separated from other whole cell organelles through differential centrifugation in an 86% Percoll gradient, 0.3M sucrose, then specifically targeted by an incubation with rabbit-anti-mCLCA3 antibody, the mucin granule membrane biomarker. Immuno-isolation blots were probed with anti-CSP, anti-HSP70, and anti-MARCKS antibodies. The results show (Figure 4A) that MARCKS, CSP, and HSP70 associate with mucin granule membranes. Densitometry analysis shows no significant difference in protein level of cells stimulated 15 minutes with 100 nM PMA and their media only control (Data not shown). Whole molecule rabbit IgG was the negative control. To validate the LC-MS/MS identification of Myosin Vc and Cofilin, we also evaluated mucin granule membrane isolations (Figure 4B). The results verify the associations.

CSP, HSP70, and hCLCA1 interact with MARCKS in NHBE cells

Since MARCKS, HSP70, CSP, and hCLCA1 all associate with the mucin granule membrane, we addressed whether or not they associate with each other. The co-immunoprecipitation of MARCKS from NHBE whole cell lysate followed by detection with anti-CSP, anti-HSP70, and anti-hCLCA1 antibodies in unstimulated and PMA stimulated well differentiated NHBE cells indicates that CSP, HSP70, and appear to all associate with MARCKS, thus directly or indirectly they appear bound to each other (Figure 5). Immunoblotting with anti-MARCKS antibody was the positive control for this analysis. The protein association with MARCKS did not appear affected by the 15 minute stimulation with PMA.

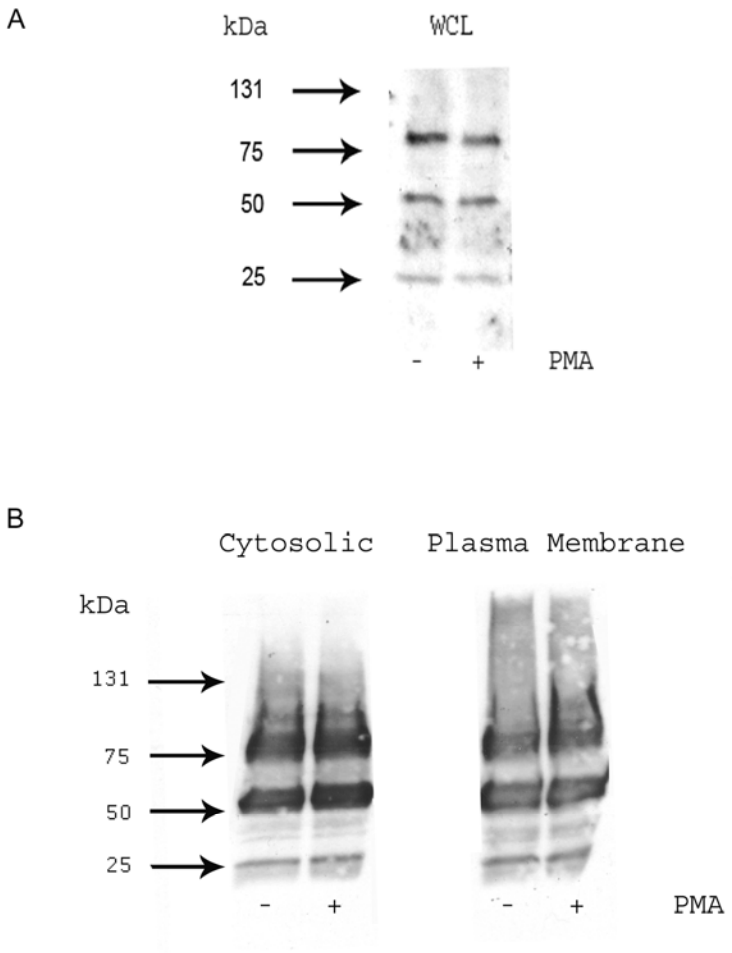
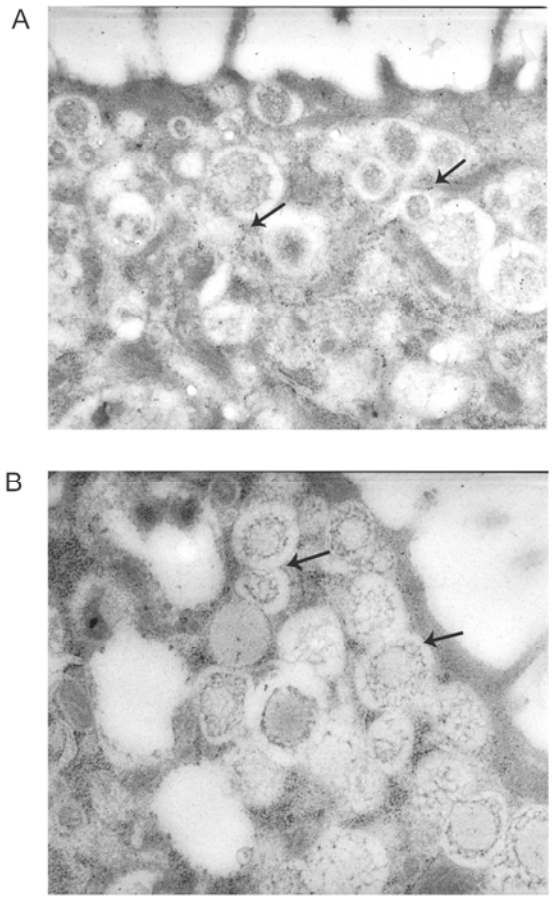
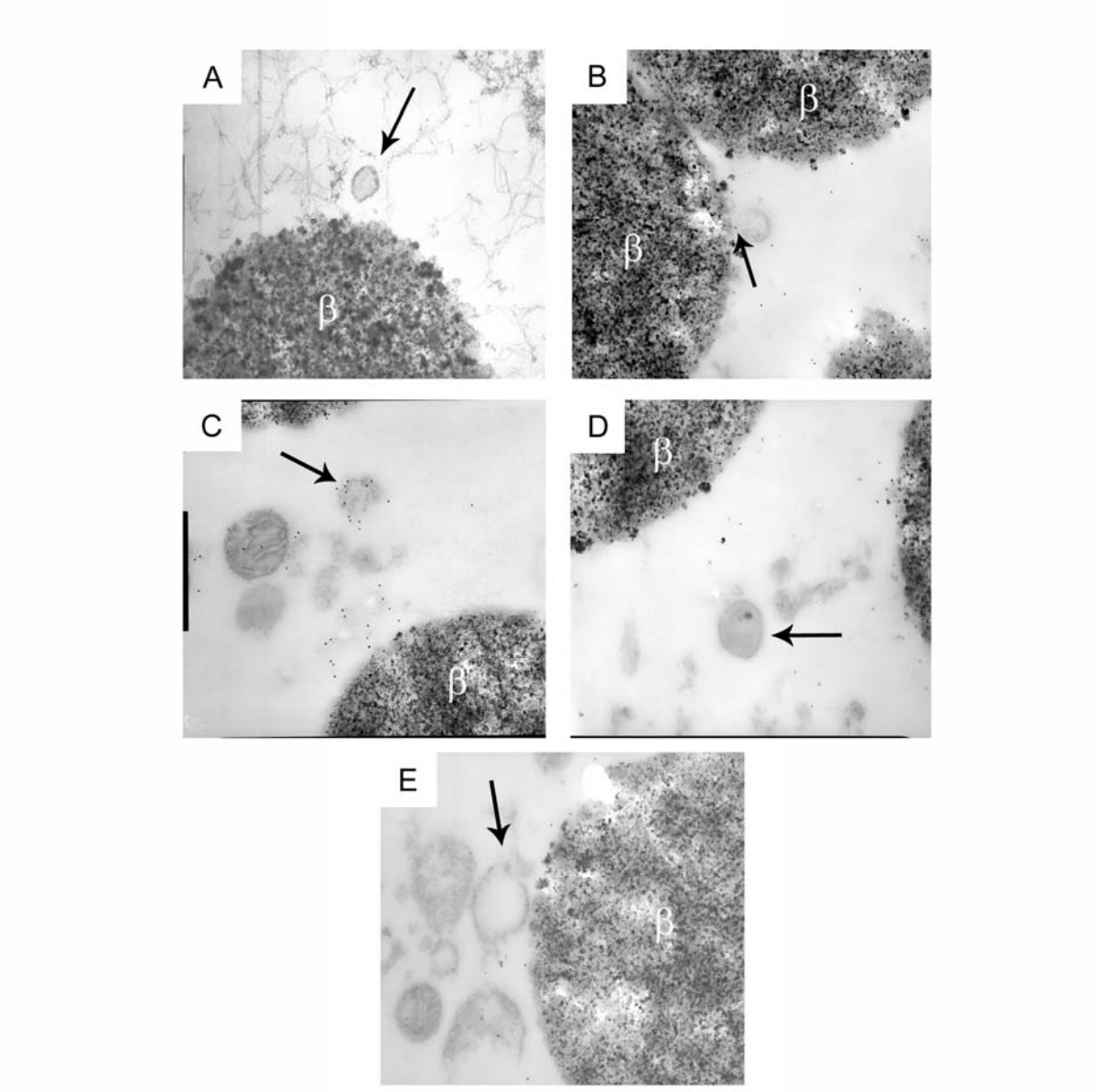


Figure 1:**hCLCA1 is found in airway epithelial cells**. A) Western blot analysis of whole cell lysates with rabbit anti-mCLCA3 antibody shows that hCLCA1 is found in NHBE cells. Stimulation with PMA for 15 minutes did not appear to affect the levels of hCLCA1. B) Immunoprecipitation of hCLCA1 from cytosolic and plasma membrane cell fractions with the anti-mCLCA3 antibody followed by incubation with a hCLCA1 specific antibody demonstrates that the anti-mCLCA3 does cross react with hCLCA1. It also appears that hCLCA1 is present in both cell fractions.



<u>Figure 2:</u> Associations of hCLCA1 with mucin granules from NHBE cells. Ultrastructural immunohistochemistry of well-differentiated NHBE cells examined the subcellular distribution of hCLCA1. After determining that the anti-mCLCA3 antibody does cross react with hCLCA1, ultra-thin sections of NHBE ALI culture on transwell inserts (A & B) were evaluated by ultrastructural immunohistochemistry as described in methods. The tissue sections were incubated with **A)** primary rabbit anti-mCLCA3 antibody followed by incubation with 12nm gold-labeled goat anti-rabbit secondary antibody. The negative rabbit IgG control is pictured in **B)**. The micrographs illustrate that hCLCA1 appears to localize around the mucin granules membranes (see arrows). There was little to no background seen in the negative controls.



<u>Figure 3:</u> TEM analysis of mucin granules isolated from the goblet cells of well-differentiated NHBE cells maintained in air/liquid interface culture system. Illustrated in panel A) is the structural view of mucin granule membranes (see arrows) near a magnetic dynal bead (β); B) hCLCA1 localized to mucin granule membrane; C) positive control pan mucin labeling of 17Q2 in mucin granule; D) Rabbit IgG negative control; E) Mouse IgG negative control. Primary antibody incubations were followed by 12nm gold-labeled secondary antibody. Gold appears as black dots indicating cross reactivity with the primary antibody.

Table 1. Additional mucin granule membrane associated proteins identified by liquid chromatography mass spectrometry (LC-MS/MS)

Protein/reference	Xcorr	Protein Accession Number	Charge	Peptide Sequence
MYH9 protein	5.665	29436380	2	K.LQVELDNVTGLLSQSDSK.S
Gelsolin (Amyloidosis, Finnish type)	5.283	4504165	2	-.AQPVQVAEGSEPDGFWEALGGK.-
keratin 1; Keratin-1; cytokeratin 1; hair alpha protein	4.684	17318569	2	-.LNDLEDALQQAK.-
heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa); BiP;	4.516	16507237	2	R.IEIESFYEGEDFSETLTR.A
mitochondrial malate dehydrogenase precursor	4.435	21735621	2	K.VAVLGASGGIGQPLSLLLK.N
bA255A11.8 (novel protein similar to annexin A2 (ANXA2)	4.318	12314197	2	K.SALSGHLETVILGLLK.T
Hypothetical protein DKFZp686P03159	4.318	34364597	2	-.SALSGHLETVILGLLK.-
Cathepsin B (Fragment)	4.3	4505863	2	-.NGPVEGAFSVYSDFLLYK.-
Crystal Structure Of Human Recombinant Procathepsin B At 3.2 Angstrom Resolution	4.3	2982114	2	K.NGPVEGAFSVYSDFLLYK.S
anterior gradient 2 homolog (Xenopus laevis) , agr2	4.116	30583839	2	-.LAEQFVLLNLVYETTDK.-
mutant beta-actin (beta'-actin)	4.084	28336	2	K.SYELPDGQVITIGNER.F
Annexin A2	3.904	16306978	2	R.RAEDGSVIDYELIDQDAR.D
ARC16-2	3.761	33150554	2	-.ALAVGGLGSIIR.-
Hypothetical protein DKFZp686E1899	3.632	57997483	2	-.QLETVLDDLDPENALLPAGFR.-
tropomodulin 3	3.632	6934244	2	K.QLETVLDDLDPENALLPAGFR.Q
PRO1708	3.611	7959791	3	-.RHPDYSVVLLLR.-
similar to beta-actin	3.502	37546764	2	-.SYKLPDGQVITIGNER.-
calmodulin 1 (phosphorylase kinase, delta)	3.458	30583815	2	R.VFDKDGNGYISAAELR.H
A Chain A, Human Serum Transferrin, Recombinant N-Terminal Lobe, Apo Form	3.41	4389230	2	-.DQYELLCLDNTR.-
Nuclease sensitive element binding protein-1	3.374	34098946	2	-.SVGDGETVEFDVVEGEK.-
Actin-like protein (Fragment)	3.294	62421184	2	-.VAPEEHPVLLTQAPLNPK.-
uracil DNA glycosylase	3.284	35053	2	-.LVINGNPITIFQER.-
Myosin regulatory light chain MRCL3 variant (Fragment)	3.259	62896697	2	-.ATSNVFAMFDQSQIQEFK.-
Keratin, type II cytoskeletal 3 (Cytokeratin 3) (K3) (CK3)	3.206	125098	2	-.FLEQQNKVLETK.-
unnamed protein product	3.19	28940	2	-.FTQAGSEVSALLGR.-
Unknown (protein for MGC:71545)	3.1	37747855	2	-.NLNEKDYELLCLDGTR.-
Hypothetical protein DKFZp451J0218	3.063	30268331	2	-.VSHLLGINVTDFTR.-
keratin 19; keratin, type I cytoskeletal 19; keratin, type I, 40-kd;	3.024	24234699	2	R.GQVGGQVSVEVDSAPGTDLAK.I
pyruvate dehydrogenase (lipoamide) beta; Pyruvate dehydrogenase, E1 beta	2.97	4505687	2	K.VFLLGEEVAQYDGAYK.V

Table 1 (continued)

Protein/reference	Xcorr	Protein Accession Number	Charge	Peptide Sequence
similar to hypothetical protein FLJ20420	2.903	37547262	2	-.RVAEELALEQAK.-
ASC-1 complex subunit P200	2.826	12061185	3	K.VKQCVHQIPSVMMEASIQPITR.T
keratin 4; Keratin-4; cytokeratin 4; keratin, type II cytoskeletal 4	2.815	17318574	2	-.NLDLDSIIAEVR.-
V_segment translation product	2.767	1552502	3	.MSTRLLCWMALCLLGAELSEAEVAQSPRYK.
pyruvate kinase M2	2.75	6018096	2	R.LAPITSDPTEATAVGAVEASFK.C
FLJ21945 protein	2.744	23272708	3	-.GICFLTDQLLLILVGKQK.-
Mitochondrial ornithine transporter 2	2.71	38372886	3	-.NEGIVALYSGLKATMIR.-
CLIP-associating protein CLASP1	2.67	13508645	3	-.SGNMIQSANDKNFDDEDSVDGNR.-
cytokeratin type II	2.631	4758618	2	-.LVDLEEALQKAK.-
E1A binding protein p400; p400 SWI2/SNF2-related protein; CAGH32 prote	2.63	15805014	3	-.RVLILSQMILMLDILEMFLNFHYLTYVR.-
TATA element modulatory factor 1	2.599	6005904	3	-.YQVELENLKDEYVRTLEETR.-
hypothetical protein FLJ14721	2.57	14249534	3	R.YPSPAELDAYAEKVANSPLSIKIFPTNIR.V
ras homolog gene family, member C; Aplysia RAS-related homolog 9	2.568	28395033	2	K.QVELALWDTAGQEDYDR.L
Integrin alpha-D precursor (Leukointegrin alpha D) (CD11d) (ADB2)	2.524	12643717	3	-.KPPQHSDFLTQISR.-
C11orf15 protein	2.523	31455206	3	-.RLFGHAQLIQSDDDIGDHQPFANAHDVLAR.-
protein kinase C-like 2	2.511	5453974	3	-.LEELHHKLQELNAHIVVSDPEDITDCPR.-
similar to cytoplasmic beta-actin	2.45	29736622	2	-.VAPDEHPILLTEAPLNPK.-
Hypothetical protein KIAA0143	2.449	2495710	3	SNGRHGAVGAPCAAPLSLGAAASAVEIAMPTI
inhibitory receptor IREM1	2.447	31790204	3	-.LSSAQVDQVEVEYVTMASLPK.-
fragile X mental retardation syndrome related protein 2; fragile X-ment	2.436	4758410	3	R.TDEDRTVMDGGLESDGPNMTENGLEDESR.I
unnamed protein product	2.436	34526743	2	R.SACATRQNSTSTKNTK.I
zinc finger protein 132 (clone pHZ-12)	2.436	4507979	3	-.PYSNLGQLPEVCTTQKLFECSNCGKAFLK.-
similar to nuclear pore complex interacting protein	2.434	37541436	3	-.KSAVQQLTPLLLR.-
CD1A protein	2.425	21594951	2	-.FILGLLDAGKAHLQR.-
Membrane alanine aminopeptidase precursor	2.415	37590640	3	-.YLSYTLNPDLIR.-
C59436 KIAA1391 protein [imported]	2.41	25535895	2	-.YINLEKEKDYPK.-
solute carrier family 24 (sodium/potassium/calcium exchanger), member 1	2.405	4759128	3	R.QKQAIYLFLLPIVFPLWLTVPDVR.R
low density lipoprotein-related protein 1B; low density lipoprotein rec	2.404	9055270	3	R.AWDTLYWTSSTTSSITRHTVDQTR.P
parkin isoform	2.403	20385800	3	-.EPQSLTRVDLSSSVLPGDSVGLAVILHTDSR.-
KIAA0713 protein	2.402	3882147	2	-.AQQKITEKDDQVK.-

Table 1 (continued)

Protein/reference	Xcorr	Protein Accession Number	Charge	Peptide Sequence
Unknown (protein for MGC:71515)	2.389	34785925	3	-.LLQQNVDICAEATCGMIAER.-
Thyroid hormone receptor interactor 6	2.385	12803689	3	-.EPGPGAKEEAAGISGPAGR.-
T00095 hypothetical protein KIAA0470	2.372	7512995	3	-.EDNKTDEGPDTPSYNR.-
SH3-domain binding protein 5 (BTK-associated); SH3 binding protein	2.368	4759058	3	-.TRSELVHKETAAR.-
hypothetical protein	2.366	12052938	3	K.GQAAPPAPPLPSSLDPPPPPAAVEVFQR.P
similar to Hypothetical protein KIAA0056	2.359	20481728	2	-.MVALRGLGSGLQPWCPLDLR.-
phosphodiesterase 10A	2.354	5729972	2	-.VIRGEETATWISSPSVAQK.-
unnamed protein product	2.354	30846	3	-.QAEGLSEDGAAMAVEPTQIQLSKR.-
unnamed protein product	2.336	34533353	3	.CLPVERRCDGLQDCGDGSDEAGCPDLACGR.
Similar to KIAA0052 protein	2.32	21619317	2	-.LYIPKDLRPVDNR.-
unnamed protein product	2.32	7022460	3	K.PLGLKDCIIVGGMDMVAQALELSR.K
unnamed protein product	2.318	10440008	3	-.MRPKVMWHLLR.-
similar to RIKEN cDNA 5031434M05	2.317	37540268	2	-.FCGLFMVLLSDHPSPDLGQK.-
RB1CC1 protein	2.314	17028471	3	-.VDSAMETSMMSVQENIHMLSEEKQR.-
unnamed protein product	2.305	14042913	2	R.FPLNGYCRLNSVQVLER.L
unnamed protein product	2.303	34531680	3	-.MPKGGCPKAPQQEELPLSSDMVEK.-
similar to DKFZP434P1750 protein	2.297	37541041	2	-.LALGTAEQR.-
similar to hypothetical protein FLJ31547	2.291	29746676	3	-.AIPSIIGVNNPECGFPLPMKEAPEILSGSNK.-
ubiquitin associated protein 2 isoform 2; AD-012 protein	2.29	22325366	3	-.SSYGLKGAWKNSVEEWTTEDWTEDLSETK.-
plastin 3; T isoform	2.283	7549809	2	-.PPYPKLGANMKK.-
breast carcinoma amplified sequence 1; Breast carcinoma amplified sequence	2.276	4502373	3	-.VDEVPGLSGQSDDVPAGKDIVDGKEK.-
stromal cell derived factor receptor 1 isoform b	2.272	6912646	3	K.NEGQDATMYCKSVGYPHPDWIWRK.K
Sec3-like isoform 1; homolog of yeast exocyst protein Sec3p; exocyst c	2.26	30410720	2	R.ELQVLDGANIQSIMASEK.Q
NACHT, LRR and PYD containing protein 10	2.258	28827807	3	-.KSQSQNLFSVKSSLSHGPK.-
transducer of regulated CREB protein 3	2.241	37693045	3	-.QQPPWKDEKHPGFR.-
chromosome 14 open reading frame 50	2.239	27312029	3	K.RLGGQTPYLMDQLGLRLGMWYWK.D
ubiquitin specific protease 32	2.233	28188773	3	-.NKDMSWPEEMSFIANSSKIDR.-
hypothetical protein	2.232	12053087	3	R.DVTFAQEFINLDGISLLTQMVESGTER.Y
unnamed protein product	2.232	34530583	3	.ALMNEKAQAALVEFVEDVNHAAIPREIPGK.I
unnamed protein product	2.228	10437181	3	-.SPDLYERQVCLLLLQLCSGLEHLK.-

Table 1 (continued)

Protein/reference	Xcorr	Protein Accession Number	Charge	Peptide Sequence
neuroblastoma-amplified protein	2.226	22164066	2	-.MLSVKFYSRQGQEQDGIFK.-
similar to Nck-associated protein 5 (NAP-5)	2.211	37547356	2	-.SGRLLFFARNYSVTTATR.-
RTKN protein	2.21	33871180	2	-.GRVCISDLRIPLMWK.-
dystrophin Dp116 isoform	2.208	5032295	3	-.TYHVKDLQGEIEAHTDVYHNLDENSQK.-
prostaglandin E receptor 4, subtype EP4; PGE receptor, EP4 subtype	2.204	4506259	3	-.NPDLQAIRIASVNPILDPWIYILLRK.-
A Chain A, Determination Cc-Chemokine Mcp-3, Nmr, 7 Structures	2.202	2624707	2	-.EAVIFKTKLDK.-
similar to soluble adenylyl cyclase	2.202	37552182	3	K.SSLCWFSREGLLATAQLMQALAYTK.L
similar to surface glycoprotein, Ig superfamily member	2.202	37541781	2	-.LVFQQLKRSQTK.-
MY9B_HUMAN Myosin IXb (Unconventional myosin-9b)	2.199	14548118	3	K.SPQVPRDIQEEELEVLLEEEAAGGDEDREK.E
KIAA0614 protein	2.192	34328016	3	K.ACNAHGGVFKDEIYIPLQEEDTK.K
R33083_1	2.191	3702295	3	-.PCGGKEFGLFEELSEGSFGWVTGIRR.-
LYST-interacting protein LIP8	2.19	29789293	3	-.PLQDLSPSSSAQALEELFPRYTSLR.-
SI:zC214P16.4 (novel protein similar to human protein phosphatase 1)	2.186	27884151	3	-.IMRPTDVPDTGLLCDLLWSDPDK.-
hypothetical protein	2.184	31873973	3	-.MVHSLPTAVPESPRIHPTRTPK.-
MTMR15 protein	2.181	28839601	3	-.EECIPEHMVRESKIMEAESQK.-
DKFZp434C0631 protein	2.179	28279320	2	K.NDHVLFYLENVFGR.A
Src substrate cortactin (Amplaxin) (Oncogene EMS1)	2.178	2498954	2	-.ELETGPKASHGYGGK.-
unnamed protein product	2.174	16549252	2	-.TCQVDQFSCGNGR.-
amylo-1,6-glucosidase, 4-alpha-glucanotransferase isoform 1; glycogen	2.172	4557275	3	-.WNPEALPSNTGEVNFQSGIIAARCAISK.-
hypothetical protein	2.172	30268329	2	-.EVMCQLGLHQK.-
hypothetical protein MGC46732	2.168	24308460	3	-.LKEESLSLFTILHDRILEIEK.-
similar to hypothetical protein	2.167	37541409	3	-.FYKPFNLMFYQMTGQDFQWEQEEGDK.-
dJ469A13.3 (continues in Em:AL031662 as dJ460J8.1)	2.162	13277305	2	-.PLLNDKNGTRNFQDFDCQ.-
syntaxin 11	2.157	3248918	3	-.WDVFSENLLADVKGR.-
cofilin 1 (non-muscle)	2.154	5031635	3	-.EILVGDVGQTVDDPYATFVKMLPDK.-
A-kinase anchor protein 3 (Protein kinase A anchoring protein 3)	2.153	14194457	2	-.SCDASLAELGDDKSGDASR.-
calreticulin precursor; Sicca syndrome antigen A	2.15	4757900	3	K.SGTIFDNFLITNDEAYAEEFGNETWGVTK.A
NDRG2 protein (Syld709613 protein)	2.148	20141615	3	-.TASLTSAASVDGNRSRSR.-
KIAA0550 protein	2.142	20521081	3	K.ELDESSVFVLGAVLYKNLDLILPTLR.N
transmembrane channel-like protein 7	2.141	33355703	3	R.IPSSKACGPFTNFNTTWEVIPK.T

Table 1 (continued)

Protein/reference	Xcorr	Protein Accession Number	Charge	Peptide Sequence
caspase 14 precursor; apoptosis-related cysteine protease	2.139	6912286	3	-.FQQAIDSREDPVSCAFVVLMAHGR.-
microtubule-associated protein 1B isoform 2	2.135	14165456	3	-.HPDVSMVDPEALAIEQNLGKALK.-
breast cancer antiestrogen resistance 3	2.133	4502371	3	-.NMPVNHQFPLASSMDLLSSRSPLAEHR.-
unnamed protein product	2.133	14042842	3	R.RASVKACIANVEPNQTVEINEQEALEEK.L
IKK-related kinase epsilon; IKK-related kinase epsilon; inducible Ikapp	2.131	7661946	2	R.DQVHEDRSIQQIQCCLDK.M
myosin VC; myosin 5C	2.127	9055284	2	-.SGSNAHVEDKVLASNPITEAVGNAK.-
neutral alpha glucosidase C type 2	2.126	25272050	3	-.STYQALLDSVTTDEDSTRFQIINEASK.-
hypothetical protein FLJ14871	2.123	14249582	3	R.RSQSASDAPLSQHTLETLLEEIKALR.E
Unknown (protein for IMAGE:3838106)	2.119	13325406	3	K.FNQCYGVLGVLDHLHGTDTMFKQTKAYER.I
hypothetical protein MGC39518	2.118	28376658	3	-.VIQDSNNELLEPVCHQLFELYRSSEVR.-
unnamed protein product	2.116	10434173	3	-.QHILPAEKEVTEFYVQNENSVDKWGK.-
unnamed protein product	2.115	28071062	3	-.MGIGTGHTSMNKGGKDVTLLELSVEK.R
FKSG16	2.112	16416764	2	-.VPFLFTIVPFSVLR.-
MGC4707 protein	2.109	15559265	3	-.NGDLLTMKEYHCLLQLLCPDFPLELTQK.-
ash1 (absent, small, or homeotic)-like	2.107	8922081	3	-.TLGKPDSLLVPAVASDSCNNSISLLSEK.-
ADCY7 protein	2.106	25058301	3	-.HQAILGMAFLVLAVFAALSVLMYVECLLR.-
KIAA1729 protein	2.104	12698003	3	K.SLTVDGNVGKLVGIDSFQPSVQK.Q
FLJ00056 protein	2.102	10440440	3	-.YGRLLEELLREAGPELSSECR.-
cytokine receptor-like factor 1	2.092	4758062	3	R.GPAAQSARRPPPLLPLLLLLCVLGAPR.A
ribosomal protein P0	2.092	4432757	2	R.VLALSVETDYTFPLAEK.V
RPLP0 protein	2.092	75771977|	2	-.VLALSVETDYTFPLAEK.-
ankyrin repeat and FYVE domain containing 1 isoform 2; ankyrin repeat	2.091	31317252	3	K.HSADVMSEMAQIAEALLQAGANPNMQDSK.(
AGLW2560	2.09	37182360	2	-.RSSWSATGSAAPFPPSDQPGTR.-
alpha-catenin-like protein	2.083	7019571	2	-.SNTLNIALDNMCKK.-
KPI-2 protein	2.083	27356940	3	-.KVFPLRWTAPELVTSFQDR.-
nebulin	2.079	4758794	3	-.INIPADMVSVLAAK.-
seven transmembrane helix receptor	2.075	21928245	3	-.SVTPPPFISPTSQLVITFSLTSLQESVTFR.-
KIAA0376	2.074	2280485	3	-.SFDSASQVPNPAAAAIPR.-
citron; rho-interacting, serine/threonine kinase 21	2.071	32698688	2	R.SDLYESELRESRLAAEEFK.R
hypermethylated in cancer 1	2.071	5729871	3	-.IHSGEKPYECQVCGGKFAQQR.-

Table 1 (continued)

Protein/reference	Xcorr	Protein Accession Number	Charge	Peptide Sequence
KIAA1200 protein	2.065	6330407	3	K.VGTAYEQLIGKLMDGEGDPDSPLWR.H
SH3-domain kinase binding protein 1; c-Cbl-interacting protein	2.058	13994242	3	-.PPSQSLTSSSLSSPDIFDSPSPEEDK.-
protein phosphatase 4, regulatory subunit 2	2.057	28372531	3	K.KEVCPVLDQFLCHVAKTGETMIQWSQFK.G
ubiquitin specific protease, proto-oncogene; Unph	2.057	4507853	3	-.PDAETQKSELGPLMR.-
MAGUK p55 subfamily member 6 (VELI-associated MAGUK 1) (VAM-1)	2.054	27764857	3	R.TSEFMPYVVFIAAPELETLRAMHK.A
KIAA1082 protein	2.052	14133233	2	K.GIPEHLMGKLGPNGER.S
A44256 zinc-finger protein ZNF76 - human	2.051	2136421	3	-.THTGERPFQCPFEGCGRSFTTSNIR.-
KIAA1950 protein	2.049	18916759	3	R.ALKDALVSTDAALQQLYVSAFSPAER.L
phosphatidylinositol 3-kinase	2.047	987948	2	K.AHRQGHMVKVDWLDR.L
unnamed protein product	2.046	21754782	2	K.HIDLLKIKVTSGVGDK.Q
similar to 16.7Kd protein	2.045	37549528	3	K.QFLECAENQGDIKLCEGFNEVLK.Q
T08775 hypothetical protein DKFZp586C1620.1	2.044	7512857	3	-.EDFFHCLKCNLCLAMNLQGRQVY.-
hypothetical protein MGC45962	2.042	22749313	3	-.MLVIAGGILAALLLLIVVVLCLYFKIHNALK.-
signal peptidase complex 18 kDa subunit	2.042	3641344	2	-.ARGFVPYIGIVTILMNDYPKFR.-
PIP3AP protein	2.041	34783714	3	R.EFYDSWHSKSTDYHGLLLPHIEGPEIK.V
FLJ00141 protein	2.035	18676488	3	-.LSTAITLLPVEEGR.-
XPA binding protein 2; XPA-binding protein 2	2.033	9910260	3	-.ALKLLPCSTKLWYR.-
KIAA0286	2.031	20521027	3	-.PVPPRLLTEEEYRIQGEVETR.-
capacitative calcium entry channel protein; identical to AJ006203	2.029	3264578	3	-.HWVVKLLTCMTIGFLFPMLSIAYLISPR.-
unnamed protein product	2.028	34528602	3	-.AQTHPAPPQTWAPFFTMHSSEQPCR.-
microtubule-based motor	2.027	4106715	3	R.SHAVFNIIFTQKRHDAETNITTEK.V
IGF-II mRNA-binding protein 1	2.026	21361352	3	-.LLVPTQYVGAIIGKEGATIRNITK.-
DEAD-box protein abstrakt [synthetic construct]	2.023	30584005	3	-.VPPVLQVLHCGDESMLDIGGER.-
KIAA1564 protein	2.018	34328020	2	-.WCSLPYEDSTWELKEDVDEGK.-
KIAA1196 protein	2.016	34013528	3	K.AENQALRDIPLSLMNDWKDEFK.A
ADAM 2 precursor (A disintegrin and metalloproteinase domain 2)	2.012	28202251	2	-.VCRNQRCVSSSYLGYDCTTDK.-
cyclin D-dependent kinases 4 and 6-binding protein/p16 product	2.012	861472	2	-.GSNHARIDAAEGPSAIPD.-
unnamed protein product	2.01	10435867	3	-.QWLSATKPPLSDR.-
hypothetical protein XP_352404	2.007	37560019	3	R.ALYTTLLMIPTRHANVDAVHDIANEDTV.-
very-long-chain acyl-CoA dehydrogenase	2.007	3273228	2	-.ALEQFATVVEAK.-

Table 1 (continued)

Protein/reference	Xcorr	Protein Accession Number	Charge	Peptide Sequence
TBX3-iso	2.003	7341107	3	K.EAFAPLTVQTDAARSSVHRHPFR.N
unnamed protein product	2.002	34536452	2	-.GPSLDIDTPDVNIEGPEGK.-
kinase-like protein	2	14041815	3	-.WSADMWRLGCLIWEVFNGPLPRAAALR.-
unnamed protein product	2	7023918	3	-.LSSSQGTIETSLQDIDSR.-

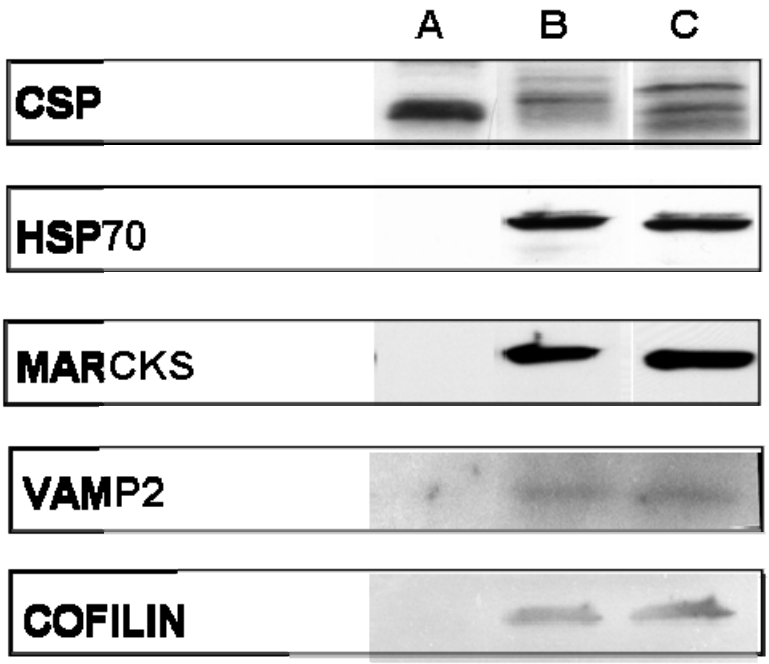


Figure 4. Western blot analysis of mucin granule immuno-isolations reveals that CSP, HSP70, and MARCKS are associated with the granule. Mucin granules were isolated as described. Granule isolations were separated from other whole cell organelles through differential centrifugation in a 86% Percoll gradient and more specifically targeted by incubation with the rabbit-anti-mCLCA3 antibody. Immuno-isolation blots were probed with anti-CSP, anti-HSP70, anti-MARCKS, anti-VAMP2, and anti-Cofilin antibodies in unstimulated (Lane C) and PMA stimulated (Lane B) well differentiated NHBE cells. Stimulated cells were treated for 15 minutes with 100 nM PMA. Whole molecule rabbit IgG was the negative control used for the immuno-isolations (Lane A).

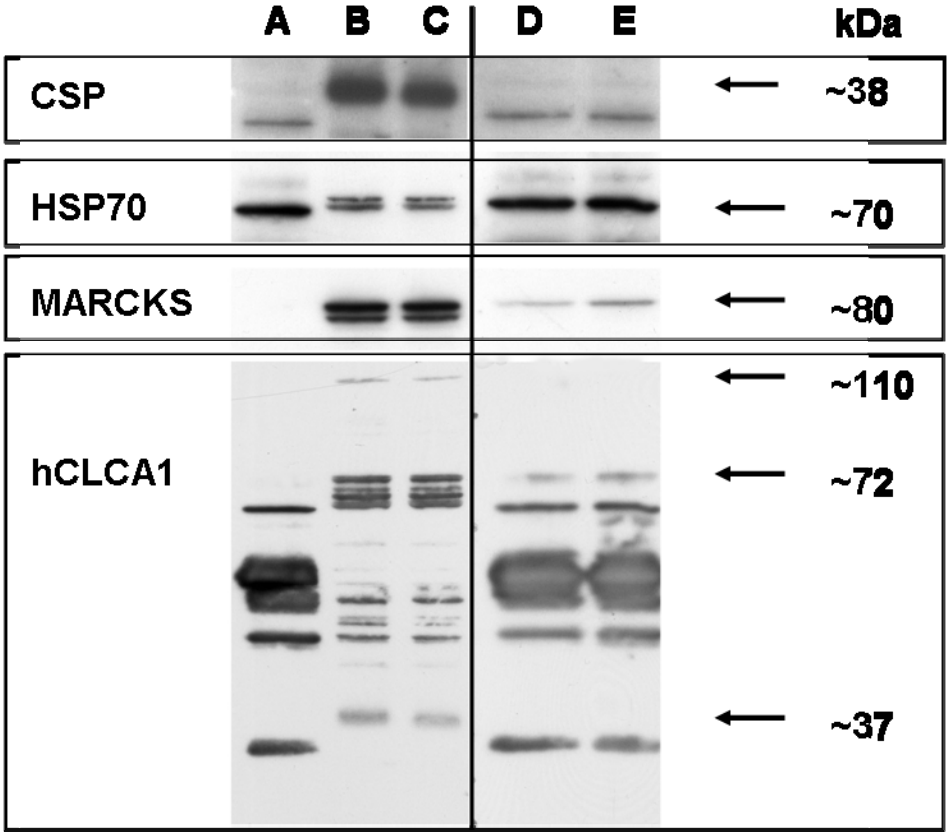


Figure 5. CSP, HSP70, MARCKS, and hCLCA1 are complexed in NHBE cells. Immunoprecipitation of MARCKS from whole cell lysate followed by immunoblotting for CSP, HSP70, and hCLCA1, in unstimulated (Lane D) and PMA stimulated (Lane E) NHBE cells indicates that these proteins appear associated with each other. Whole molecule rabbit IgG was the negative control used for the immuno-isolations (Lane A). Whole cell lysates of unstimulated (Lane C) and stimulated cells (Lane B) show the presence of these proteins. Immunoblotting with anti-MARCKS antibody was the positive control for this analysis. Stimulation with 100 nM PMA for 15 minutes did not appear to affect the association with MARCKS.

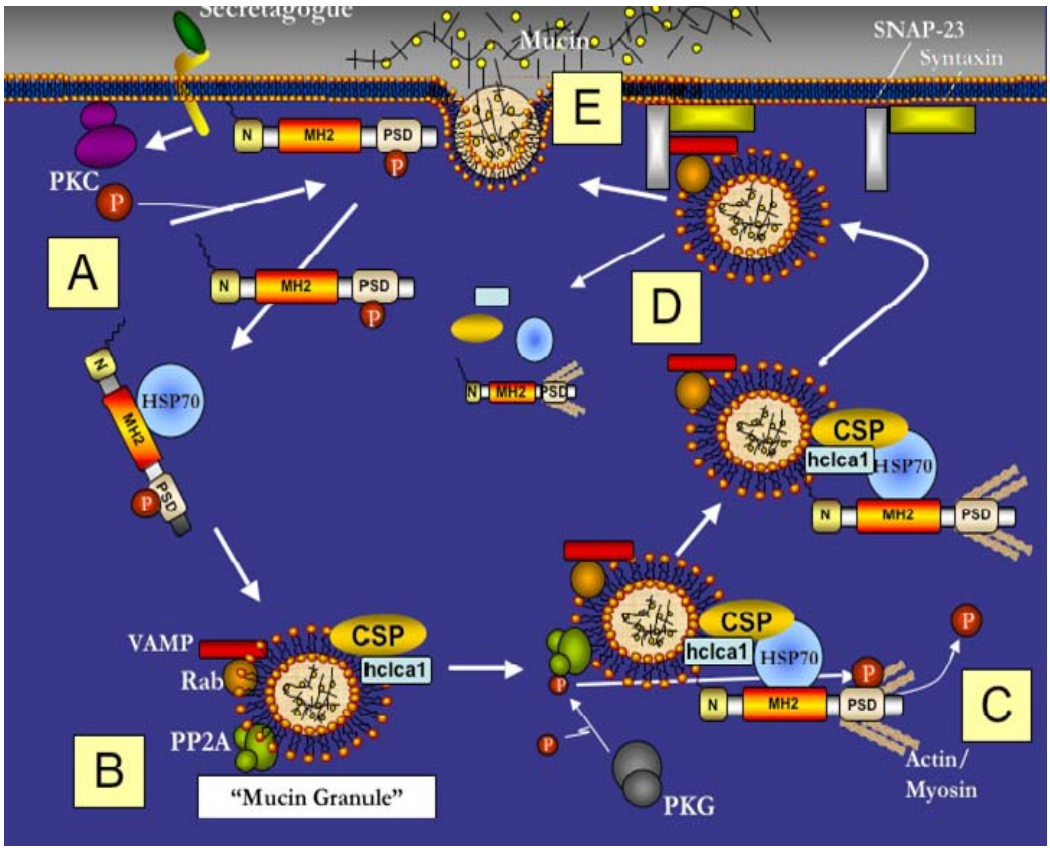


Figure 6: Hypothetical Pathway of Mucin Secretion *Event A:* The mucus secreting cell is stimulated by a secretagogue that activates PKC which in turn phosphorylates MARCKS on its PSD domain which causes it to detach its N-terminus from the plasma membrane and move into the cytoplasm. *Event B*: MARCKS is chaperoned to the preformed membrane-bound mucin granule by HSP70 that is bound to its MH2 domain. The MARCKS/HSP70 complex binds to the granule through an interaction with CSP and HSP70. *Event C:* PKG activates PROTEIN PHOSPHATASE 2, which dephosphorylates MARCKS allowing MARCKS to reinsert its N-terminus into the membrane granule. MARCKS then binds and uses ACTIN and MYOSIN (VC) to translocate to the plasma membrane. *Event D:* The docking protein Rab aligns the fusion protein vesicle soluble N-ethylmaleimide-sensitive factor attachment protein receptors (v-SNARE) vesicle-associated membrane protein (VAMP) on the granule to interact with its corresponding target SNARE (t-SNARE) on the plasma membrane. CSP is phosphorylated by PKA causing it to dissociate from the granule severing its interaction with HSP70 and MARCKS. *Event E:* The mucin contents are released into the airway lumen. A role for hCLCA1 has yet to be determined.

DISCUSSION

Secretory granule exocytosis studies have concentrated on certain cell types, which is probably due to their availability and ease of experimentation. Model systems of secretory granule exocytosis have grown (as reviewed in Burgoyne et al., 2003) to include catecholamine release from adrenal chrommafin cells (Evans et al., 2001), cortical granule secretion in oocytes (Smith et al.,2005), insulin secretion from pancreatic β-cells (Zhang et al., 2002), and mucin granules from intestinal and airway goblet cells (Li et al., 2001, Leverkoehne et al., 2002). Pioneering studies of secretory granule exocytosis were done in pancreatic exocrine cells (Burgoyne et al., 2003) and George Palade has been credited with delineating the pathway followed by secretory proteins. However, no one has taken a proteomic look at mucin secretion in airway epithelial cells specifically targeting the mucin granule.

The mechanisms of mucin secretion involve a cascade of events culminating in the transport of the mucin granule from the cytosol to the plasma membrane where it tethers and exudes its contents into the airway lumen. A logical extension to study this mechanism is to determine the proteins that are involved in physically translocating the mucin granule, i.e. membrane granule associated proteins. This study is novel because it is the first to specifically examine the mucin granule associated proteome.

The overall aim of this study was to delineate proteins associated with airway goblet cell mucin granules during regulated exocytosis. The method of subcellular fractionation is widely used in proteomic analysis of intracellular complexes and organelles, including endothelial membrane rafts (Guo Y et al.), neutrophil secretory vesicles (Jethwaney D et al.),

and insulin secretory granules (Brunner Y et al.). However, a technical complication of this method arises from the presence of contaminants, unwanted subcellular fragments that settle in the same density gradient as the target. Using a two tiered approach to subcellular fractionation, coined immuno-isolation, an antibody specific to the target organelle is used to further purify the isolation, thereby decreasing the amount of contaminants. This approach does, however, increase the amount of starting tissue needed.

In this communication, we report the immuno-isolation of mucin granules from airway epithelial cells using a self- forming Percoll density gradient followed by immuno-targeting of a known mucin granule membrane associated protein, mCLCA3. Specificity of the antibody generated to the mouse ortholog of hCLCA1 was first validated for use in *Homo sapiens* application. Immunoblotting well-differentiated normal bronchial epithelial cell whole cell lysates with the rabbit polyclonal anti-mCLCA3 antibody identified protein fragments sized at 110, 72, and 40 kDa. Furthermore, immunoprecipitation with the mCLCA3 antibody followed by analysis with the hCLCA1 specific antibody verified the previous results. These sizes are similar to what was seen in all other CLCA homologues thus far (Leverkoehne et al., 2002; Hauber et al., 2004; Robichaud et al., 2005). Therefore, the biochemical results are consistent with the proposed general model of CLCA protein structure and processing (reviewed in Pauli et al., 2000). Although we are still unsure of the function of hCLCA1, stimulation with PMA does not appear to effect hCLCA1 levels. This indicates that hCLCA1 does not seem to be downstream of PKC activation. The basis of this immuno-isolation procedure is that mCLCA3 is only a granule associated protein and can be used as a biomarker; however, we also see hCLCA1 in the plasma membrane fraction. This

can be explained since the method of dissociating the cells to extract proteins is, by nature of the process, physically disruptive, and mucin granule membranes may have also been disrupted during the process causing size shifts in the electrophoresis results.

Through ultrastructural analysis of the mucin granule membranes isolations it was revealed that both intact and fragmented membrane pieces were being isolated by our methods. A more targeted ITEM view with both the 17Q2 pan mucin and mCLCA3 antibody gold-labeling verified our findings. 17Q2 pan mucin antibody has been used extensively to measure mucins in ELISA and immunocytochemistry (Lin et al., 1989; Yang et al., 1992). Gold beads were found congregating around the Dynal beads identifying unassociated antibody bound thus proving the affinity of the Protein G dynal beads with the mCLCA3 antibody. Our studies did find disrupted membranes attached to mucins, so it is clear that our analysis was not exclusively of intact granules, even though that was initially our goal. IgG controls show little to no background, in fact, the misplaced gold-labeled beads were actually attached to chunks of dynal beads that were chipped off during the tissue sectioning preparation.

This is the first proteomic study of mucin granule associated proteins with the identification of > 100 proteins. One-dimensional gels coupled with liquid chromatography provide a good separation platform for soluble proteins (Vaughn et al., 2006). Here we excised seven bands and processed them for mass spectrometry analysis. An underlying aim of this study was to determine whether or not MARCKS, CSP, and HSP70, all key molecules previously published from our laboratory to be involved in mucin secretion (Li et al., 2001, Singer et al., 2004, Park et al., 2006), associate with the mucin granule membrane, thus

validating our hypothesis of mucin secretion (Figure 6). The band sizes chosen correlated to the expected migration sizes of those proteins. However, although HSP70 was seen, other proteins previously localized to the mucin granule such as MARCKS and hCLCA1 were not identified in the LC-MS/MS portion of this study. However, western analysis of mucin granule membrane fractions verified the association of MARCKS and hCLCA1 along with CSP. A limitation of these experiments, and subsequently the LC-MS/MS, is the recovery amount of mucin granules. A disadvantage of LC-MS/MS is that signals from proteins of low abundance can be masked by larger, more abundant proteins. Another complicating factor is that the excised bands were intermittent (~110kDA, 80kDa, 40kDa, etc) and not continuous serial sections, where the proteins was not identified ,possibly, because it was missed during this process. This could explain why we see MARCKS, CSP, and hCLCA1 in the Western blot analysis, but not in the LC-MS/MS data. There were a few contaminants of mitochondrial enzymes, keratins, and nuclear proteins we suspect were possibly, because the mCLCA3 antibody was polyclonal.

Many of the cytoskeletal related proteins identified are related to exocytosis. There are many published links between these proteins that relate back to a possible mechanism of mucin secretion. For example, Plastins are a class of Actin-binding proteins that cross-link Actin filaments into tight bundles (Ciborowski et al., 2007). Activation of cofilin, a major Actin depolymerizing protein, was shown in adrenal chromaffin cells to be necessary for exocytosis (Birkenfeld et al., 2001). Annexins, a family of calcium-dependent, membrane-associated proteins, are reported to function in endosome sorting, membrane-cytoskeletal linkage and control of fusion events in exocytosis (Burgoyne et al., 1989). Studies indicate an

essential role of Annexin A2 phosphorylation in regulating cofilin-dependent Actin cytoskeletal dynamics (de Graauw M et al., 2007). Gelsolins, Actin-binding proteins that regulate Actin-mediated movement by controlling assembly and disassembly of Actin by Acting-severing, are upregulated in the bronchial epithelium in asthma (Sun et al., 1999; Candiano et al., 2005). Myosin V is an Actin-based molecular motor that function as "molecular feet", transporting vesicles/organelles from one place to another along Actin tracts (Reck-Peterson et al., 2000; Langford et al., 2002). Further it is know that myosin V facilitates vesicle docking during exocytosis (Desnos et al., 2007). In the human genome there are three isoforms of Myosin V, myosin-Va, -Vb and –Vc. Recent studies in our lab have shown that Myosin Vc interacts with MARCKS and studies unraveling this interaction are ongoing (Lin, unpublished results). Here we also validated the LC-MS/MS findings that Cofilin and VAMP2 are mucin granule membrane associated proteins.

The regulatory proteins identified as associating with the mucin granule membrane probably did so while acting on other proteins (i.e. PKC, Protein phosphatase 1, Phosphodiesterase 10A). The interaction between Actin and myosin is primarily regulated by phosphorylation, and inhibition of PP2A inhibited secretion and led to increased phosphorylation of the myosin heavy and light chains at protein kinase C-specific sites in mast cell secretion (Holst et al., 2002). Protein phosphatase 1 and 2A were identified to dephosphorylate MARCKS in Swiss 3T3 cells and mouse fibroblasts (Clarke et al., 1993) as a result of studies of protein phosphatase inhibition with okadeic acid. Vesicle docking and fusion is regulated by SNAREs (soluble N-ethylmaleimide-sensitive fusion protein attachment protein) receptors of the transport vesicle and target membranes. Syntaxins are

SNARE proteins essential for exocytosis. It has been shown that Syntaxin 11 facilitates fusion in intracellular membrane trafficking events in lymphocyte-mediated secretion (Chen et al., 2001).

Another interesting finding of the study was that hCLCA1 binds MARCKS in a complex with CSP and HSP70. This is a novel finding that needs further analysis, but it supports the claim that hCLCA1/mCLCA3 is a soluble protein, likely a regulatory subunit, rather than a channel. Studies done by Gibson et al. determined that hCLCA1 and mCLCA3 proteins were secreted in bronchial alveolar lavage fluids from asthmatic patients and ovalbumin challenged mice (Gibson et al., 2005) as all fragment variants of these proteins. Furthermore, a CLCA family member, mCLCA1, was shown to directly interact with a large conductance potassium channel β subunit when co-transfected into HEK293 cells, which upregulated the calcium sensitivity and evoked a larger calcium activated chloride current than when it was transfected alone (Gibson et al., 2005). A capacitative activated entry channel protein subunit was also identified in this study to be granule membrane associated. It is tempting to speculate that the role of hCLCA1 in mucin granule exocytosis is regulation of the calcium influx that is well established in exocytosis events. This still does not provide an explanation for the hCLCA1 interaction, but it provides a possible mechanistic role for hCLCA1 in mucin secretion

In conclusion, the association of an abundance of cytoskeletal elements, chaperone and scaffolding proteins with mucin granules supports our overall hypothetical mechanism for mucin granule transport from the cytoplasm to the cell periphery. As we have argued, this process appears to occur as a series of highly cooperative and orchestrated events that

culminate with the release of mucin granule contents into the airway lumen. Through the application of proteomic tools we have been able to identify a plethora of potential therapeutic candidates which can be further investigated.

REFERENCES

Birkenfeld J, Kartmann B, Betz H, Roth D. Cofilin activation during Ca(2+)-triggered secretion from adrenal chromaffin cells. Biochem Biophys Res Commun. 2001 Aug 24;286(3):493-8.

Brunner Y, Couté Y, Iezzi M, Foti M, Fukuda M, Hochstrasser DF, Wollheim CB, Sanchez JC. Proteomics analysis of insulin secretory granules. Mol Cell Proteomics. 2007 Jun;6(6):1007-17.

Burgoyne RD, Geisow MJ. The annexin family of calcium-binding proteins. Cell Calcium. 1989 Jan;10(1):1-10.

Burgoyne RD, Morgan A. Secretory granule exocytosis. Physiol Rev. 2003 Apr;83(2):581-632. Review.

Candiano G, Bruschi M, Pedemonte N, Caci E, Liberatori S, Bini L, Pellegrini C, Viganò M, O'Connor BJ, Lee TH, Galietta LJ, Zegarra-Moran O. Gelsolin secretion in interleukin-4-treated bronchial epithelia and in asthmatic airways. Am J Respir Crit Care Med. 2005 Nov 1;172(9):1090-6.

Chamberlain LH, Burgoyne RD. Activation of the ATPase activity of heat-shock proteins Hsc70/Hsp70 by cysteine-string protein. Biochem J. 1997 Mar 15;322 (Pt 3):853-8.

Chamberlain LH, Burgoyne RD. The cysteine-string domain of the secretory vesicle cysteine-string protein is required for membrane targeting. Biochem J. 1998 Oct 15;335 (Pt 2):205-9.

Chen YA, Scheller RH. SNARE-mediated membrane fusion. Nat Rev Mol Cell Biol. 2001 Feb;2(2):98-106. Review.

Ciborowski P, Kadiu I, Rozek W, Smith L, Bernhardt K, Fladseth M, Ricardo-Dukelow M, Gendelman HE. Investigating the human immunodeficiency virus type 1-infected monocyte-derived macrophage secretome. Virology. 2007 Jun 20;363(1):198-209.

Clarke PR, Siddhanti SR, Cohen P, Blackshear PJ. Okadaic acid-sensitive protein phosphatases dephosphorylate MARCKS, a major protein kinase C substrate. FEBS Lett. 1993 Dec 20;336(1):37-42.

de Graauw M, Tijdens I, Smeets MB, Hensbergen PJ, Deelder AM, van de Water B. Annexin A2 phosphorylation mediates cell scattering and branching morphogenesis via cofilin activation. Mol Cell Biol. 2007 Dec 10.

Desnos C, Huet S, Fanget I, Chapuis C, Böttiger C, Racine V, Sibarita JB, Henry JP, Darchen F. Myosin Va mediates docking of secretory granules at the plasma membrane. J Neurosci. 2007 Sep 26;27(39):10636-45.

Eliyahu E, Shtraizent N, Tsaadon A, Shalgi R. Association between myristoylated alanin-rich C kinase substrate (MARCKS) translocation and cortical granule exocytosis in rat eggs. Reproduction. 2006 Feb;131(2):221-31.

Evans GJ, Morgan A, Burgoyne RD. Tying everything together: the multiple roles of cysteine string protein (CSP) in regulated exocytosis. Traffic. 2003 Oct;4(10):653-9. Review.

Evans GJ, Wilkinson MC, Graham ME, Turner KM, Chamberlain LH, Burgoyne RD, Morgan A. Phosphorylation of cysteine string protein by protein kinase A. Implications for the modulation of exocytosis. J Biol Chem. 2001 Dec 21;276(51):47877-85.

Evans MJ, Plopper CG. The role of basal cells in adhesion of columnar epithelium to airway basement membrane. Am. Rev. Respir. Dis. 1988; 138: 481–3.

Fahy JV. Remodeling of the airway epithelium in asthma. Am J Respir Crit Care Med. 2001 Nov 15;164(10 Pt 2):S46-51. Review.

Gibson A, Lewis AP, Affleck K, Aitken AJ, Meldrum E, Thompson N. hCLCA1 and mCLCA3 are secreted non-integral membrane proteins and therefore are not ion channels. J Biol Chem. 2005 Jul 22;280(29):27205-12.

Guo Y, Singleton PA, Rowshan A, Gucek M, Cole RN, Graham DR, Van Eyk JE, Garcia JG. Quantitative proteomics analysis of human endothelial cell membrane rafts: evidence of MARCKS and MRP regulation in the sphingosine 1-phosphate-induced barrier enhancement. Mol Cell Proteomics. 2007 Apr;6(4):689-96.

Hartwig JH, Thelen M, Rosen A, Janmey PA, Nairn AC, Aderem A. MARCKS is an actin filament crosslinking protein regulated by protein kinase C and calcium-calmodulin. Nature. 1992 Apr 16;356(6370):618-22.

Hauber HP, Tsicopoulos A, Wallaert B, Griffin S, McElvaney NG, Daigneault P, Mueller Z, Olivenstein R, Holroyd KJ, Levitt RC, Hamid Q. Expression of HCLCA1 in cystic fibrosis lungs is associated with mucus overproduction. Eur Respir J. 2004 Jun;23(6):846-50.

Holst J, Sim AT, Ludowyke RI. Protein phosphatases 1 and 2A transiently associate with myosin during the peak rate of secretion from mast cells. Mol Biol Cell. 2002 Mar;13(3):1083-98.

Jethwaney D, Islam MR, Leidal KG, de Bernabe DB, Campbell KP, Nauseef WM, Gibson BW. Proteomic analysis of plasma membrane and secretory vesicles from human neutrophils. Proteome Sci. 2007 Aug 10;5:12.

Kim KC, McCracken K, Lee BC, Shin CY, Jo MJ, Lee CJ, Ko KH.Airway goblet cell mucin: its structure and regulation of secretion. Eur Respir J. 1997 Nov;10(11):2644-9. Review.

Knight DA, Holgate ST. The airway epithelium: structural and functional properties in health and disease. Respirology. 2003 Dec;8(4):432-46. Review.

Krunkosky TM, Fischer BM, Martin LD, Jones N, Akley NJ, Adler KB. 2000 Effects of TNF-alpha on expression of ICAM-1 in human airway epithelial cells in vitro. Signaling pathways controlling surface and gene expression. Am. J. Respir. Cell Mol. Biol. 22: 685-692.

Kuperman DA, Huang X, Koth LL, Chang GH, Dolganov GM, Zhu Z, Elias JA, Sheppard D, Erle DJ. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. Nat Med. 2002 Aug;8(8):885-9.

Langford GM. Myosin-V, a versatile motor for short-range vesicle transport. Traffic. 2002 Dec;3(12):859-65.

Leverkoehne I, Gruber AD. The murine mCLCA3 (alias gob-5) protein is located in the mucin granule membranes of intestinal, respiratory, and uterine goblet cells. J Histochem Cytochem. 2002 Jun;50(6):829-38.

Li Y, Martin LD, Spizz G, Adler KB. MARCKS protein is a key molecule regulating mucin secretion by human airway epithelial cells in vitro. J Biol Chem. 2001 Nov 2;276(44):40982-90.

Lin H, Carlson DM, St George JA, Plopper CG, Wu R. An ELISA method for the quantitation of tracheal mucins from human and nonhuman primates. Am J Respir Cell Mol Biol. 1989 Jul;1(1):41-8.

Mundhenk L, Alfalah M, Elble RC, Pauli BU, Naim HY, Gruber AD. Both cleavage products of the mCLCA3 protein are secreted soluble proteins. J Biol Chem. 2006 Oct 6;281(40):30072-80.

Nakanishi A, Morita S, Iwashita H, Sagiya Y, Ashida Y, Shirafuji H, Fujisawa Y, Nishimura O, Fujino M. Role of gob-5 in mucus overproduction and airway hyperresponsiveness in asthma. Proc Natl Acad Sci U S A. 2001 Apr 24;98(9):5175-80.

Park J, Fang S, Adler KB. Regulation of airway mucin secretion by MARCKS protein involves the chaperones heat shock protein 70 and cysteine string protein. Proc Am Thorac Soc. 2006 Aug;3(6):493.

Park JA, Crews AL, Lampe WR, Fang S, Park J, Adler KB. Protein kinase C delta regulates airway mucin secretion via phosphorylation of MARCKS protein. Am J Pathol. 2007 Dec;171(6):1822-30.

Pauli BU, Abdel-Ghany M, Cheng HC, Gruber AD, Archibald HA, Elble RC. Molecular characteristics and functional diversity of CLCA family members. Clin Exp Pharmacol Physiol. 2000 Nov;27(11):901-5. Review.

Peterman SM, Dufresne CP, Horning S. The use of a hybrid linear trap/FT-ICR mass spectrometer for on-line high resolution/high mass accuracy bottom-up sequencing. J Biomol Tech. 2005 Jun;16(2):112-24.

Reck-Peterson SL, Provance DW Jr, Mooseker MS, Mercer JA. Class V myosins. Biochim Biophys Acta. 2000 Mar 17;1496(1):36-51.

Robichaud A, Tuck SA, Kargman S, Tam J, Wong E, Abramovitz M, Mortimer JR, Burston HE, Masson P, Hirota J, Slipetz D, Kennedy B, O'Neill G, Xanthoudakis S. Gob-5 is not

essential for mucus overproduction in preclinical murine models of allergic asthma. Am J Respir Cell Mol Biol. 2005 Sep;33(3):303-14.

Rogers DF. Physiology of airway mucus secretion and pathophysiology of hypersecretion. Respir Care. 2007 . Sep;52(9):1134-46; discussion 1146-9. Review.

Rossi AH, Salmon WC, Chua M, Davis CW. Calcium signaling in human airway goblet cells following purinergic activation. Am J Physiol Lung Cell Mol Physiol. 2007 Jan;292(1):L92-8.

Sakisaka T, Meerlo T, Matteson J, Plutner H, Balch WE. Rab-αGDI activity is regulated by a Hsp90 chaperone complex. EMBO J 2002;21: 6125–6135.

Seykora JT, Myat MM, Allen LA, Ravetch JV, Aderem A. Molecular determinants of the myristoyl-electrostatic switch of MARCKS. J Biol Chem. 1996 Aug 2;271(31):18797-802.

Singer M, Martin LD, Vargaftig BB, Park J, Gruber AD, Li Y, Adler KB. A MARCKS-related peptide blocks mucus hypersecretion in a mouse model of asthma. Nat Med. 2004 Feb;10(2):193-6.

Smith GB, Umbach JA, Hirano A, Gundersen CB.Interaction between constitutively expressed heat shock protein, Hsc 70, and cysteine string protein is important for cortical granule exocytosis in Xenopus oocytes. J Biol Chem. 2005 Sep 23;280(38):32669-75.

Sun HQ, Yamamoto M, Mejillano M, Yin HL. Gelsolin, a multifunctional Actin regulatory protein. J Biol Chem. 1999 Nov 19;274(47):33179-82. Review.

Thelen M, Rosen A, Nairn AC, Aderem A. Regulation by phosphorylation of reversible association of a myristoylated protein kinase C substrate with the plasma membrane. Nature. 1991 May 23;351(6324):320-2.

Vaughn CP, Crockett DK, Lim MS, Elenitoba-Johnson KS. Analytical characteristics of cleavable isotope-coded affinity tag-LC-tandem mass spectrometry for quantitative proteomic studies. J Mol Diagn. 2006 Sep;8(4):513-20.

Wright DT, Fischer BM, Li C, Rochelle LG, Akley NJ, Adler KB. Oxidant stress stimulates mucin secretion and PLC in airway epithelium via a nitric oxide-dependent mechanism. Am J Physiol. 1996 Nov;271(5 Pt 1):L854-61.

Wu TT, Castle JD. 1997. Evidence for colocalization and interaction between 37 and 39 kDa isoforms of secretory carrier membrane proteins (SCAMPs). J Cell Sci. 110: 1533-41.

Yang PC, Luh KT, Kuo SH, Wu CW. Immunocytochemistry and ELISA quantitation of mucin for diagnosis of malignant pleural effusions. Am Rev Respir Dis. 1992 Dec;146(6):1571-5.

Zhang H, Kelley WL, Chamberlain LH, Burgoyne RD, Lang J. Mutational analysis of cysteine-string protein function in insulin exocytosis. J Cell Sci. 1999 May;112 (Pt 9):1345-51.

CHAPTER III

A PROTEOMIC ANALYSIS OF PROTEINS ASSOCIATED WITH MUCIN GRANULE MEMBRANES OF AIRWAY SECRETORY CELLS

Authors: **Kimberly L Raiford and Kenneth B Adler.**

North Carolina State University, Raleigh, NC, United States.

Institution: **Department of Molecular Biomedical Sciences,**

College of Veterinary Medicine

North Carolina State University,

Raleigh, NC 27606.

ABSTRACT

Mucin hypersecretion can be highly deleterious when excess mucus leads to airway obstruction. Mucins, the highly glycosolated protein components of mucus, are stored in membrane-bound granules in goblet cells of the airway epithelium until their contents are secreted into the lumen during a process of regulated exocytosis. The precise mechanisms of mucin secretion have yet to be determined, but several mucin granule membrane - associated proteins have been identified in previous studies from this and other laboratories. Specifically, Myristoylated Alanine-Rich C Kinase Substrate (MARCKS), calcium-activated chloride channel 3 (mClca3, alias Gob-5), and the chaperones Cysteine String Protein (CSP) and Heat Shock Protein 70 (HSP70) are found in a complex associated with mucin granule membranes, and inhibition of any one of these proteins attenuates mucin secretion. Using Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) and proteomic analysis of mucin granule membranes isolated from 3 model systems; normal bronchial epithelial cells, the UNCN3T human airway epithelial cell line, and a colon adenocarcinoma (HT29) cell line, the above proteins, as well as several novel granule-associated proteins that could be involved in the mucin secretion process, were discovered. These novel proteins include: Calpain 1, MyosinVc, Capacitance Calcium Channel Entry Protein, Phosphodiesterase 10A, several protein phosphatases, RTKN (a scaffold protein), Syntaxin 11 (a docking protein), and Plastin 3. The roles of these proteins in the secretory process and how they interact with MARCKS and other chaperones may provide important insight into the conserved exocytosis machinery necessary for mucin secretion in the airway, and suggest potential new therapeutic targets to control excess mucus secretion.

INTRODUCTION

Chronic airway inflammation can result in morphologic and functional changes of the airway known as airway remodeling. Pathophysiological characteristics associated with this phenomenon are airway hyperresponsiveness, mucous cell hyperplasia, smooth muscle hypertrophy, and subepithelial fibrosis (Wills-Karp et al., 1998; Kuperman et al., 2002; Black JL et al., 2001). Chronic inflammatory diseases such as asthma, cystic fibrosis, and chronic bronchitis are associated with mucin hypersecretion resulting in excess mucus in the airways impairing mucociliary function, increased susceptibility to bacterial pathogens, vivification of inflammatory responses, and, in extreme cases, death (Rogers, 2007).

Secretory goblet cells produce and secrete a thin layer of mucus that coats the airway epithelium trapping inhaled irritants, pathogens, and potentially damaging particles. Mucus is a viscoelastic mixture of water, cell derived debris, mediators, and proteins; more specifically electrolytes, oxidants and antioxidants, and mucins (Rogers, 2007). Mucins, the highly glycosolated protein structural components of mucus, are stored in membrane-bound granules in the cytoplasm of respiratory epithelial goblet cells. During exocytosis, they are hydrated to form a gel, which is secreted into the airway lumen (Verdugo et al., 1990).

A defective cystic fibrosis transmembrane conductance regulator (CFTR) in airway epithelial cells contributes to the associated mucus hypersecretory phenotype, marked inflammation, infection, and obstruction responsible for the morbidity and mortality in patients with cystic fibrosis (Puchelle et al., 2002). CFTR defective epithelial cells have a decreased capacity for mucins packed inside the mucin granules to swell from the condensed phase inside the granules into an expanded gel phase (Puchelle et al., 2002). The abnormal

regulation of the flow of salts and water in concert with increased Actin content may also contribute to CF mucus hyperviscosity (Vasconcellos et al., 1994).

Mucin secretion is a process of regulated exocytosis involving membrane-bound mucin granules. An extracellular stimulus governs the translocation, docking, and fusion of the granule to the plasma membrane (Li et al., 2001). Even though the mechanism controlling mucin secretion has not been fully resolved, some progress has been made.

Most secretory granules have the same basic protein components that help facilitate docking and fusion with the target membrane: soluble N-ethylmaleimide-sensitive fusion attachment protein (SNAP) receptor (SNARE) (i.e. Syntaxin or SNAP-25), SNARE regulators (i.e. N-ethylmaleimide-sensitive fusion and α-SNAP), Rab proteins and effectors (i.e. Rab 3 and Noc2), calcium binding proteins (i.e. Calmodulin) (reviewed in Burgoyne et al., 2003), and Sec1/Munc18-like proteins (SM proteins). Despite the conservation of core exocytosis machinery, many variations occur in the control of secretory granule exocytosis that is related to the specialized physiological role of particular cell types.

Studies from our lab illustrated that Myristoylated alanine-rich C-kinase substrate (MARCKS), cysteine string protein (CSP), and heat shock protein 70 (HSP70) are involved in mucin secretion (Li et al., 2001; Singer et al., 2004, Park et al., 2006). A synthetic peptide corresponding to the first 24 sequences of the N-terminus of MARCKS inhibits constitutive and cholinergically stimulated mucin secretion in ovalbumin-sensitized mice (Singer et al., 2004). Short interfering ribonucleases (siRNAs) to the molecular chaperones HSP70 and CSP attenuate protein kinase C stimulated mucin secretion (Li et al., 2001; Park et al., 2006; unpublished data from Dr Adler's lab).

Human calcium-activated chloride channel and its murine ortholog mCLCA3 have been shown to play a role in goblet cell hyperplasia and mucus overproduction (Wills-Karp et al., 1998; Kuperman et al., 2002). Recently hCLCA1 has been identified as a secreted protein instead of the calcium activated chloride channel that its name implies (Gibson et al., 2005; Mundhenk et al., 2006). mCLCA3 was identified with transmission electron microscopy using gold-labeled secondary antibodies to be exclusively associated with mucin granule membranes of gastrointestinal, respiratory, and uterine goblet cells and other mucin-producing cells (Leverkoehne et al., 2002), so it has been used as a biomarker in mucin granule isolations (Singer et al., 2004) .

Knowledge of the machinery involved in mucin secretion would greatly contribute to our understanding of other regulated exocytosis processes. It is expected that there is conservation of key molecules across cell types and tissues. In this study, we evaluate the proteins that associate with the mucin granule membrane in normal bronchial epithelial cells and a diseased epithelial cell line, cystic fibrosis UNCN3T. We also evaluate mucin secretion and evaluate the levels of MARCKS, CSP, HSP70, and hCLCA1 in primary normal bronchial epithelial cells, the UNCN3T cell line, and the intestinal cell line HT29-18N2, are stimulated with the PKC agonist, phorbol-myristate-acetate (PMA). As expected, the results show conservation of several proteins which include plastin 3, annexin 2, and myosin V, in addition to CSP, MARCKS, HSP70, and hCLCA1.

MATERIALS AND METHODS

Cell Culture

Three different model systems were used: primary normal bronchial epithelial (NHBE) and UNCN3T (cystic fibrosis bronchial epithelial cell line) cells grown at air/liquid interface (Li et al., 2001), and HT29-18N2 (colon adenocarcinoma HT29 cell line) (Phillips et al. 1995). HT29-18N2 cells were utilized, because they can differentiate into a columnar monolayer consisting predominantly of cells filled with mucin secretory granules (Phillips et al. 1995).

Primary culture of NHBE cells in air/liquid interface culture was described previously (Krunkosky et al., 2000). Briefly, commercially available normal human bronchial epithelial (NHBE) cells (Lonza, Cambridge, MA) were seeded into vented T75 tissue culture flasks at a density of 500 cells/cm^2. The cells were expanded in growth medium at 5% CO_2 at 37ºC to a confluence of 85-90%, dissociated from the flasks using 0.25% trypsin/EDTA, and frozen in liquid nitrogen as passage-2 cells (2 X 10^6 cells/ml).

Air/liquid interface cultures of NHBE cells were established on Transwell®-Clear culture 0.4μm pore polyester inserts (Costar, Cambridge, MA) thinly coated with rat-tail collagen type I (Collaborative Biomedical, Bedford, MA). Frozen NHBE cells were recovered and seeded at a density of 2 x 10^4 cells/cm^2 onto the apical surface of the inserts with complete media alone in the basolateral compartment. The complete medium was composed a 50:50 mixture of bronchial epithelial growth medium and high glucose (4.5 g/L) Dulbecco's modified Eagle's medium containing a final concentration of 50 μg/ml gentamicin, 5 μg/ml insulin, 10 μg/ml transferrin, 0.5 μg/ml epinephrine, 6.5 ng/ml

triiodothyronine, 0.5 ng/ml human recombinant epidermal growth factor, 0.5 μg/ml hydrocortisone, 50 ng/ml amphotericin-B, 0.13 mg/ml bovine pituitary extract, 5 x 10–8 mol/L all-trans retinoic acid, 1.5 μg/ml bovine serum albumin, and 20 U/ml nystatin. Cells were grown submerged in a 5% CO_2 atmosphere at 37°C, and media was changed the next day, then every other day until the cells reached 90% confluence. At this point, air/liquid interface (ALI) was established by removing the apical media (Day 0 ALI), whereas basolateral media was changed daily for 14 days. Mucin was observed at 14 days in culture (Day 7 ALI) and cilia were apparent by 18 days in culture (Day 11 ALI), however, experiments were conducted on cells at 21 days in culture (Day 14 ALI) ensuring that the culture was well differentiated. An immortalized cystic fibrosis cell line, UNCN3T, grown in the same manner as the NHBE cells was also utilized.

Primary cells of intestinal HT29-18N2 cells were grown under submerged conditions in complete media composed of high glucose (4.5 g/L) Dulbecco's modified Eagle's medium containing a final concentration of 10% fetal bovine serum, and 10 U/μg/ml penicillin/streptomycin (Fisher) in a 5% CO_2 atmosphere at 37°C, and media was changed the next day then every other day until the cells reached confluence.

Double sandwich enzyme-linked immunosorbent assay (ELISA) of mucin secretion

The apical surface of the cells was washed in phosphate buffer saline (PBS), pH 7 using gentle agitation for 5 minutes prior to baseline and experimental mucin samples collections to remove the accumulated mucus. Both "baseline" and "experimental" collections of secreted mucin from each well were collected in order to minimize variability

by allowing each well to serve as its own control. Baseline secreted mucin samples were collected in the apical medium after the cells were incubated with medium alone for the desired time period and stored at -20^{o}C. The cells were rested for ~18-24 hours then exposed to the protein kinase C (PKC) agonist phorbol-12-myristate-13-acetate (PMA)- containing medium or medium alone for the same amount of time used in the baseline collection.

Experimental secreted mucin samples were collected from the apical medium samples and pooled with two subsequent apical surface washes using 1mM dithiothreitol (Sigma) in PBS. The addition of 50mM β-mercaptaethanol denatured mucins in medium to aid in antibody binding. Samples were centrifuged at 8000 rpm for 5 minutes to remove dead cells and heavy debris then 50µl was coated into a 96 well Costar (#3590, EIA/RIA with high binding) plate in triplicate. A serial dilution of known mucin standard stock was also coated onto the plate in triplicate. The amount of mucin secreted was then quantified with the double sandwich ELISA method using the pan mucin 1:1000 dilution of 17Q2 antibody (Covance, Berkley, CA) that cross reacts with a carbohydrate epitope on human mucins as described previously (Li et al., 2001).

Absorbance values were determined by the microplate reader system, and the linear regression and protein concentrations calculated by SoftMax Pro data analysis software (Molecular Devices, Sunnyvale, CA). Mucin secretion was reported as fold change from the control.

Mucin granule immuno-isolations

These immuno-isolations were performed using a modification of the protocol from

Wu et al (1997). After treatments, cells were washed in PBS and then collected in isolation buffer (PBS, 1 mM phenylmethyl sulfonyl fluoride, protease inhibitor cocktail 1, phosphatase inhibitor cocktail (Sigma, St. Louis, MO) using a rubber policemen. The collected cells were lysed by brief sonication, and the lysates were spun at 600 x g for 10 minutes at 4°C. The supernatants were added to 1.9 volumes of 86% Percoll, 0.3M sucrose, 5mM MOPS (4-Morpholinepropanesulfonic acid), 1mM EDTA, and 0.2µg/ml DPPD (N,N'-diphenyl-4-phenylenediamine) ph 6.8, (Sigma), and centrifuged for 30 minutes at 17,000 x g in a Sorvall Discovery 100S ultracentrifuge (Sorvall, Inc. Newtown, CT). The crude granules were transferred from the bottom of the self-formed gradient to a new tube, diluted with 3 volumes of 0.3M sucrose containing 2mM MOPS, 1mM EDTA, and 0.2µg/ml DPPD, and centrifuged for 15 minutes at 2000 x g. The pellet was reconstituted in PBS, incubated with an antibody to gob-5/mclca3 (ortholog to human clca1) overnight at 4°C on a nutator. The rabbit polyclonal gob-5 antibody was generated to the mclca3 peptide epitope ESWKAKPEYTRPKLE (Covance, Denver, PA). After incubation, the antibody-granule complex was applied to protein G coated Dynal beads. The beads were washed thoroughly and the complex was eluted with Na-citrate pH 2.5 or loading dye.

Protein subcellular fractionation

After treatments, cells were washed with ice-cold PBS containing a phosphatase inhibitor (Active Motif Inc, Carlsbad, CA) and then scraped into lysis buffer (50 mM Tris, pH 7.5, 1 mM ethylenediamine tetraacetic acid, 100 mM NaCl, 1 mM phenylmethyl sulfonyl fluoride) using rubber policemen. The collected cellular mixture was lysed by brief

sonication. Lysates were spun at 14,000 x g at 4°C in an Eppendorf 5417R centrifuge (Eppendorf Corp., Hamburg, Germany) for 30 minutes, to separate the cytosolic and membrane fractions. The supernatant was kept as the cytosolic sample while the pellet was resuspended in lysis buffer containing 0.01% Triton-100, dissolved by sonication, and incubated on ice for 30 minutes. Following incubation, the samples were centrifuged again at 14,000 x g at 4°C for 30 minutes, and the supernatant separated from the pellet mixture was kept as the membrane fraction.

For preparation of whole cell crude lysates, the disrupted cellular mixture was centrifuged at 15,000 rpm in an Eppendorf 5417R centrifuge (Eppendorf Corp., Hamburg, Germany) for 15minutes at 4°C. The supernatant was collected as the whole crude cell lysate.

The protein concentration of all cell lysate samples was quantified by Bradford assay (BioRad Laboratories, Hercules, CA). Bovine serum albumin was used as the standard and serial dilutions were made from the initial stock concentration of 400 ng/ml. Absorbance values were determined by the microplate reader system, and linear regression and protein concentrations calculated by SoftMax Pro data analysis software (Molecular Devices, Sunnyvale, CA).

Immunoprecipitation and Western analysis

Whole cell or mucin granule lysates containing 500-1000μg/ml total protein were incubated overnight at 4°C with 3-10 μl (20-30μg) of a specific antibody overnight at 4°C with gentle agitation. Twenty-five microliters of Protein G dynal beads (Invitrogen, Carlsbad, CA) were added to bind the antibody-protein complex for 3 hours at 4°C with gentle

agitation. Beads were washed three times with cold PBS, and proteins were eluted with 1X sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer and boiled 10 min before the proteins were resolved on SDS-PAGE gel. Resolved proteins were transferred to a polyvinylidene difluoride membrane (Micron Separation Inc., Westborough, MA), blocked with 5% skim milk, and membrane were probed with either anti-MARCKS (Upstate), anti-CSP (Chemicon), anti-HSP70, anti-hCLCA1(IMGENEX) or anti-gob5.

Visualization of the proteins occurred after probing with the secondary horseradish peroxidase-conjugated antibodies using the enhanced chemiluminescence method. The densitometry was analyzed by Quantity One image acquisition and analysis software (BioRad Laboratories, Hercules, CA).

Liquid chromatography tandem mass spectrometry (LC-MS/MS)

Protein bands were excised from bands on a 1-Dimensional gel, dried with a solvent, extracted, and treated with hydroxyethyl disulfide as a thiol blocking reagent under alkaline conditions at 60°C. The extracted peptides were reduced nearly to dryness under a stream of air prior to trypsin digestion in 50mM ammonium bicarbonate pH~7.8. Samples were then incubated overnight at 37°C before analysis by LC/MS.

Peptides were analyzed by reverse phase HPLC with electrospray ionization mass spectrometry. Separations were achieved with a C18 HPLC column (Phenomenex Jupiter Proteo: 150mm x 0.50mm I.D., 4um particle size, 90A pore size) and a mobile phase operated with a programmed gradient with 50mM acetic acid and acetonitrile. The instrument used for the analysis was a Thermo Surveyor HPLC coupled with a Thermo LTQ

ion trap mass spectrometer. The mass spectrometer was operated in positive ion mode with an electrospray ionization (ESI) source. The mass spectrometer was operated in data dependent MS/MS scan mode scanning from m/z 420-2000 and collecting MS/MS spectra on the four most abundant ions in each scan.

Protein database searching

The acquired MS/MS spectra for each sample were searched using the BioWorks 3.1 SR1 SEQUEST algorithm (Thermo Electron, San Jose, CA) against the human nonredundant database. The nonredundant database was downloaded from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.ih.gov/blast/db/fasta). The nonredundant database was used for initial protein identification for tandem mass spectral data acquired in the ICR cell as well as the linear trap. Evaluation of total protein coverage was done by creating a protein subset database consisting of *Homo sapiens* proteins only. Database searching parameters assumed proteolysis was performed using trypsin with the possibility of one internal cleavage residue. Searches were performed with trypsin specified as the enzyme with an allowance for up to two missed cleavage sites. Searches from replicates within an experiment were combined to generate a comprehensive list of peptides and proteins identified in a particular experiment. Acceptance levels for positive peptide identification were determined using cross-correlation scores (Xcorr). These scores aid in the determination of true positives, with higher scores increasing confidence in correct identifications. The minimum acceptable Xcorr for identified peptides was 2.0 (Vaughn et al., 2006; Peterman et al., 2005)

Statistical analysis

At least 5 replicate experiments were performed for each concentration of each reagent that was assayed. Controls such as medium alone or reagent vehicle were utilized. All reagents used in treating the cells were examined for cytotoxicity by measuring the total release of lactate dehydrogenase from the cells. All experiments were performed with reagents at non-cytotoxic concentrations.

Statistical analysis was performed on the data using Prism software (Graphpad, San Diego, CA) evaluating significance using one-way analysis of variance (ANOVA) with Bonferroni post-test corrections for multiple comparisons. Differences between treatments were considered significant at a p-value of < 0.05.

RESULTS

Effects of PMA on mucin secretion

The amount of mucin secreted was quantified with the double sandwich ELISA method using the pan mucin 17Q2 antibody that cross reacts with a carbohydrate epitope on human mucins as described previously (Li et al., 2001). As illustrated in Figure 1, PMA stimulated mucin secretion by UNCN3T and NHBE cells is maximal after 15 minutes exposure to PMA (Figure 1A and C, respectively) so this time point was chosen for additional experiments. HT29-18N2 cells stimulated with 500nM PMA elicited a significant increase at 120 minutes.

Western Analysis of UNCN3T, NHBE, and HT29-18N2 cells

MARCKS, phosphorylated-MARCKS (Li et al., 2001), CSP, and HSP70 (Park et al., 2006; Park JA et al., 2005) all have been reported to have a role in mucin secretion. Here we evaluate UNCN3T and HT29-18N2 cells for their levels of MARCKS, HSP70, CSP, pMARCKS, and Actin following PMA stimulation 15 minutes. The results show (Figure 2) that all proteins are present in each cell similarly to NHBE cells. Overall, the concentration of these proteins appears more robust in the cystic fibrosis samples. Densitometry analysis shows an increase in pMARCKS and MARCKS in NHBE cells stimulated 15 minutes with PMA versus their media only control after normalizing to ACTIN (Figure 2B). There is a ~1.9 fold increase in pMARCKS in NHBE cells and a ~2 fold decrease in HT29-18N2 cells. The pMARCKS identified different banding patterns for the cell lines versus NHBE cells. MARCKS and pMARCKS resolves at 80kDa, but have also show a band at 110 kDa.

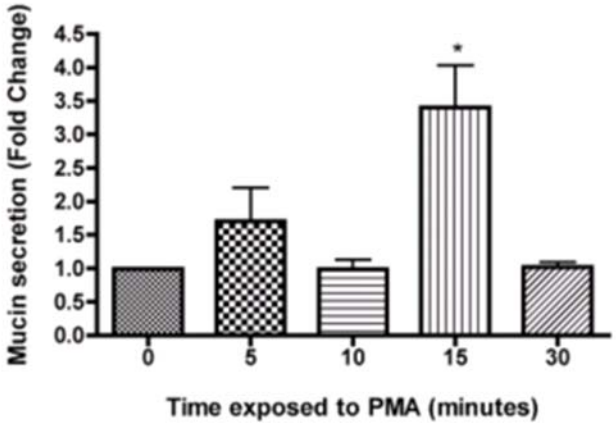
UNCN3T cells and HT29-18N2 cells only identify the 110kDa band. We are unsure of this result.

The CSP bands show a ~1.9 fold increase in UNCN3T cells and a ~2.9 fold decrease in HT29-18N2 cells, while the levels in NHBE cells are relatively the same (~1.43). These results indicate that bronchial epithelial cell types have more similar regulation of exocytotic key core machinery than the intestinal cells. These data could provide an explanation as to why HT29-18N2 cells take longer to respond to PMA and increase mucin secretion.

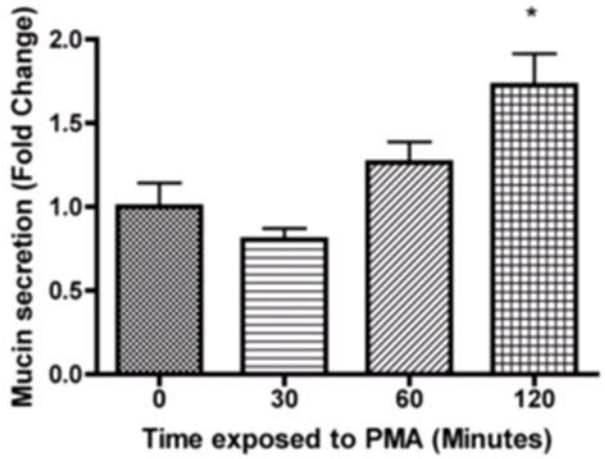
Mucin granule membrane associated proteins

Mucin granule membranes isolated as previously described were eluted from the magnetic beads in SDS sample buffer, boiled 5 minutes, and separated by SDS-PAGE. Seven bands were excised from the gel and processed through LC-MS/MS. Table 1 represents the proteins identified in both NHBE and CF UNCN3T cells. The proteins in Table 2 are a subset of the 249 proteins identified that have an Xcorr value greater than 4, which ensures a high confidence that these proteins are mucin granule membrane associated. Table 3 lists the proteins that were only identified in isolations of UNCN3T cells.

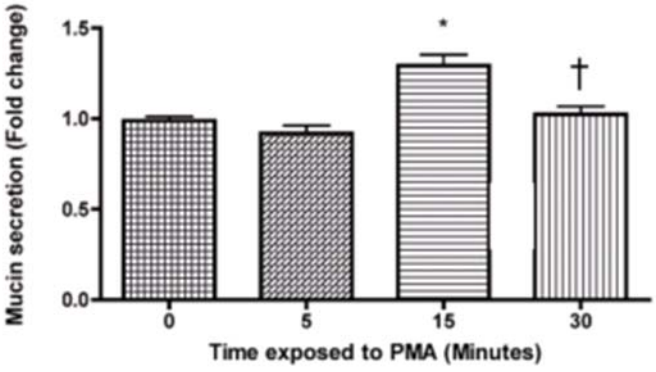
Figure 1. **ELISA measuring mucin secretion in 3 cell types**. UNCN3T, HT29-18N2, and NHBE cells were exposed to PMA throughout a range of times and concentrations. The NHBE and UNCN3T cells were exposed to PMA following 9days in ALI, while HT29-18N2 cells were exposed on Day 6. A) UNCN3T CF cells were exposed to 100 nM PMA or vehicle control for 0, 5, 10, 15, or 30 minutes, and mucin secretion was quantified by ELISA as described in the text using the pan mucin 17Q2 antibody. Mucin secretion was significantly increased after exposure to PMA at 15 minutes. B) HT29-18N2 cells were exposed to 500 nM PMA or vehicle control for 0, 30, 60, 120 minutes and there was significant increase in mucin secretion at 120 minutes. C) NHBE cells mucin secretion was significantly increased after exposure to PMA at 15 minutes. *, Significantly different from vehicle control ($P < 0.01$). †, Significantly different from vehicle control ($P < 0.01$). Data are presented as mean ± SEM (n = 12 at each point.)



UNCN3T



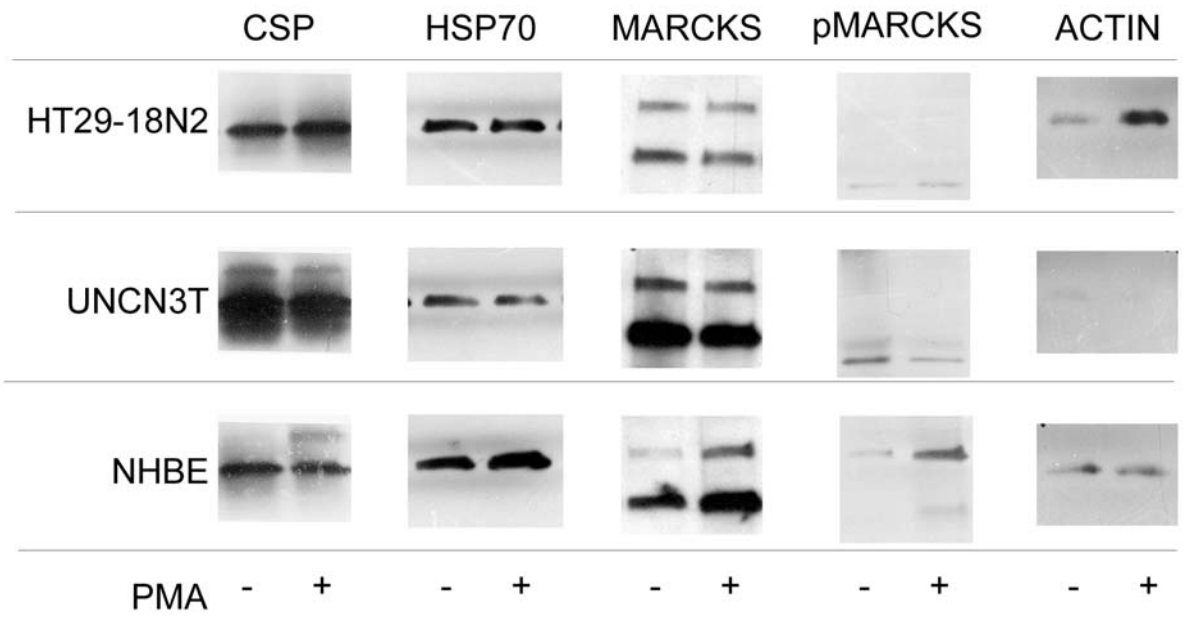
HT29-18N2



NHBE

Figure 2: Western Analysis of CSP, HSP70, ACTIN, MARCKS, and pMARCKS show that they are present in unstimulated and Protein Kinase C stimulated HT29-18N2 and UNCN3T cells. Cells were exposed to PMA for 15 minutes. Protein extractions from whole cell lysates were quantitated by a Bradford assay and equal amounts of protein were loaded into a 4-15% polyacrylamide gel. A) Immunoblot of the key proteins was analyzed by stripping and reprobing the same membrane (3 times). Phospho-MARCKS was analyzed first followed by MARCKS, CSP and HSP70, and Actin. B) Densitometry analysis normalized to Actin shows an increase in pMARCKS (~1.9) and MARCKS (~1.7) in NHBE cells following treatment with PMA, while the opposite trend is seen in HT29-18N2 cells decreasing in similar ratios. The CSP bands show a ~1.9 fold increase in UNCN3T cells and a ~2.9 fold decrease in HT29-18N2 cells. Trends in the regulation of the bronchial epithelial cells are similar and differ greatly from the intestinal cells.

A


CSP
HSP70
MARCKS
pMARCKS
ACTIN
HT29-18N2
UNCN3T
NHBE
PMA - + - + - + - + - +

B

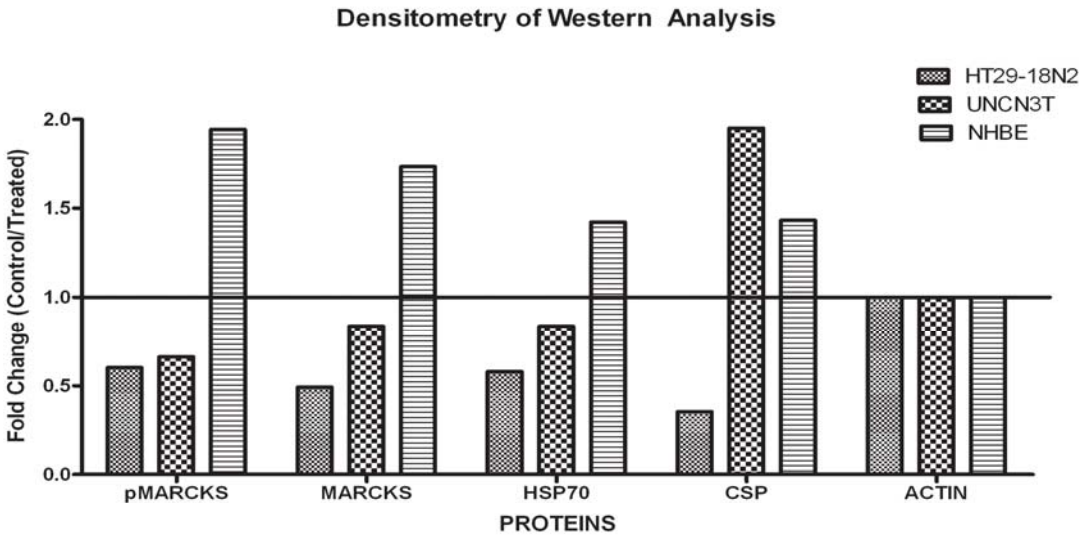

Densitometry of Western Analysis
HT29-18N2
UNCN3T
NHBE
Fold Change (Control/Treated)
2.0
1.5
1.0
0.5
0.0
pMARCKS
MARCKS
HSP70
CSP
ACTIN
PROTEINS

Table 1: Comprehensive list of 249 mucin granule membrane associated proteins found in both NHBE and UNCN3T cells

Protein/reference	Xcorr	Protein Accession Number	Charge	Peptide Sequence
HSPA8 protein	5.99	13938297	2	-.SINPDEAVAYGAAVQAAILSGDK.-
MYH9 protein	5.665	29436380	2	-K.LQVELDNVTGLLSQSDSK.S-
similar to Heat shock cognate 71 kDa protein	5.62	37550676	2	-.IINEPTAAAIAYGLDK.-
NADH-ubiquinone oxidoreductase 75 kDa subunit,	5.61	128826	2	-.YDDIEGANYFQQANELSK.-
gelsolin (amyloidosis, Finnish type); Gelsolin	5.283	4504165	2	-R.AQPVQVAEGSEPDGFWEALGGK.A-
keratin 10, type I, epidermal	5.10	88041	2	-.GSLGGGFSSGGFSGGSFSR.-
mitochondrial trifunctional protein, alpha subunit precursor	4.89	20127408	2	-.DSIFSNLTGQLDYQGFEK.-
NADPH-cytochrome P-450 reductase	4.85	11414998	2	-.NIIVFYGSQTGTAEEFANR.-
cytokeratin 9	4.698	435476	2	-.GGGGSFGYSYGGGSGGGFSASSLGGGFGGGSR.-
keratin 1; Keratin-1; cytokeratin 1; hair alpha protein	4.684	17318569	2	-.LNDLEDALQQAK.-
heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	4.516	16507237	2	-R.IEIESFYEGEDFSETLTR.A-
protein disulfide isomerase related protein	4.50	4758304	2	-.GESDPAYQQYQDAANNLR.-
transmembrane protein (63kD), endoplasmic reticulum	4.49	19920317	2	-.VQSLQATFGTFESILR.-
SDHA protein	4.44	26996830	2	-.LGANSLLDLVVFGR.-
myosin heavy chain nonmuscle form A	4.44	625305	2	-.IAQLEEQLDNETK.-
mitochondrial malate dehydrogenase precursor	4.435	21735621	2	-K.VAVLGASGGIGQPLSLLLK.N-
bA255A11.8 (novel protein similar to annexin A2 (ANXA2)	4.318	12314197	2	-K.SALSGHLETVILGLLK.T-
Cathepsin B (Fragment)	4.3	2982114	2	-.NGPVEGAFSVYSDFLLYK.-
dnaK-type molecular chaperone HSPA6	4.26	87626	2	-.IINEPTAAAIAYGLDR.-
similar to 78 kDa glucose-regulated protein prec	4.21	37547096	2	-.NQLTSNPKNTVFDAK.-
AGR2 (Anterior gradient 2)	4.116	68012756	2	-.LAEQFVLLNLVYETTDK.-
mutant beta-actin (beta'-actin)	4.084	28336	2	-K.SYELPDGQVITIGNER.F-
hydroxysteroid (17-beta) dehydrogenase 4	4.04	4504505	2	-.GALVVVNDLGGDFK.-
glycerol-3-phosphate dehydrogenase (EC 1.1.99.5)	3.99	7446012	2	-.LAFLNVQAAEEALPR.-
heat shock 70kDa protein 9B precursor	3.98	24234688	2	-.SQVFSTAADGQTQVEIK.-
dnaK-type molecular chaperone HSPA1L	3.93	2119712	2	-.AQIHDLVLVGGSTR.-
heat shock 70kDa protein 1-like; heat shock 70kD	3.93	27436929	2	-.AKIHDIVLVGGSTR.-
Annexin A2	3.904	16306978	2	-R.RAEDGSVIDYELIDQDAR.D-
smooth muscle myosin heavy chain 11 isoform S	3.90	13124879	2	-.TQLEELEDELQATEDAK.-
ABCD3 protein	3.81	16307246	2	-.IANPDQLLTQDVEK.-

Table 1 (continued)

Protein/reference	Xcorr	Protein Accession Number	Charge	Peptide Sequence
Pyruvate kinase, M2 isozyme	3.80	125604	2	-.FGVEQDVDMVFASFIR.-
junction plakoglobin; gamma-catenin	3.79	4504811	2	-.LLNDEDPVVVTK.-
heat shock 70kDa protein 8 isoform 2	3.78	24234686	2	-.GPAVGIDLGTTYSCVGVFQHGK.-
A Chain A, Human Acid-Beta-Glucosidase	3.77	33357737	2	-.SYFSEEGIGYNIIR.-
ARC16-2	3.761	33150554	2	-.ALAVGGLGSIIR.-
carcinoembryonic antigen-related cell adhesio	3.68	19923221	2	-.SDPVTLNVLYGPDVPTISPSK.-
ribophorin I	3.65	4506675	2	-.SEDLLDYGPFR.-
tropomodulin 3	3.632	6934244	2	-K.QLETVLDDLDPENALLPAGFR.Q-
alpha enolase	3.61	2661039	2	-.FGANAILGVSLAVCK.-
keratin 6A [synthetic construct]	3.61	30584049	2	-.AIGGGLSSVGGGSSTIK.-
PRO1708	3.611	7959791	3	-.RHPDYSVVLLLR.-
similar to beta-actin	3.502	37546764	2	-.SYKLPDGQVITIGNER.-
heat shock protein 90-beta	3.49	72222	2	-.GVVDSEDLPLNISR.-
unnamed protein product	3.49	21752190	2	-.GVVDSEDIPLNLSR.-
monoamine oxidase A	3.49	30584981	2	-.DVPAVEITHTFWER.-
long-chain fatty-acid-coenzyme A ligase 1	3.46	4503651	2	-.IIVVMDSYGSELVER.-
calmodulin 1 (phosphorylase kinase, delta)	3.458	30583815	2	-R.VFDKDGNGYISAAELR.H-
ACTN4 protein	3.42	33874637	2	-.ETTDTDTADQVIASFK.-
A Chain A, Human Serum Transferrin, Recombinant N-Terminal Lobe, Apo Form	3.41	4389230	2	-.DQYELLCLDNTR.-
hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), alpha subunit, isoform CRA_b	3.388	119621109	2	-.DSIFSNLTGQLDYQGFEK.-
Nuclease sensitive element binding protein-1	3.374	34098946	2	-.SVGDGETVEFDVVEGEK.-
N-ethylmaleimide-sensitive factor	3.37	11079228	2	-.NFSGAELEGLVR.-
transketolase	3.31	4507521	2	-.ILATPPQEDAPSVDIANIR.-
Actin-like protein (Fragment)	3.294	62421162	2	-.VAPEEHPVLLTQAPLNPK.-
heat shock protein	3.29	4204880	2	-.FEELNADLFR.-
uracil DNA glycosylase	3.284	35053	2	-.LVINGNPITIFQER.-
myosin regulatory light chain MRCL2	3.259	15809016	2	-R.ATSNVFAMFDQSQIQEFK.E-
Myosin regulatory light chain MRCL3 variant (Fragment)	3.259	62896697	2	-.ATSNVFAMFDQSQIQEFK.-
lamin A/C	3.23	30584609	2	-.NSNLVGAAHEELQQSR.-
keratin 6 irs	3.206	15618995	2	-.FLEQQNQVLETK.-
Keratin, type II cytoskeletal 3 (Cytokeratin 3) (K3) (CK3)	3.206	125098	2	-.FLEQQNKVLETK.-

Table 1 (continued)

Protein/reference	Xcorr	Protein Accession Number	Charge	Peptide Sequence
mitochondrial ATP synthase, H+ transporting F1 complex beta subunit	3.19	89574029	2	-.FTQAGSEVSALLGR.-
acid alpha-glucosidase preproprotein;	3.18	4503849	2	-.STGGILDVYIFLGPEPK.-
keratin 6A [synthetic construct]	3.094	30584049	2	-.ISIGGGSCAISGGYGSR.-
Hypothetical protein DKFZp451J0218	3.063	Q86T83_HUMAN	2	-.VSHLLGINVTDFTR.-
lamin A/C isoform 1 precursor; 70 kDa lamin	3.06	27436946	2	-.SVGGSGGGSFGDNLVTR.-
NSAP1 protein	3.05	26454828	2	-.NLANTVTEEILEK.-
keratin 19; keratin, type I cytoskeletal 19; keratin, type I, 40-kd	3.024	24234699	2	-R.GQVGGQVSVEVDSAPGTDLAK.I-
calnexin	3.02	7709904	2	-.APVPTGEVYFADSFDR.-
complement component 3 precursor; acylation	3.01	4557385	2	-.VPVAVQGEDTVQSLTQGDGVAK.-
nucleolin	2.978	4885511	2	-.GYAFIEFASFEDAK.-
pyruvate dehydrogenase (lipoamide) beta; Pyruvate dehydrogenase, E1 beta	2.97	4505687	2	-K.VFLLGEEVAQYDGAYK.V-
solute carrier family 25 (mitochondrial carrier)	2.93	21361103	2	-.YLGLYNDPNSNPK.-
Phosphoenolpyruvate carboxykinase, mitochondrial precursor	2.91	3287892	2	-.LGTPVLQALGDGDFVK.-
LIM domain and actin binding 1 isoform a	2.90	165905589	2	-.SQDVELWEGEVVK.-
similar to hypothetical protein FLJ20420	2.903	37547262	2	-.RVAEELALEQAK.-
carnitine palmitoyltransferase II	2.86	4503023	2	-.LNFELTDALK.-
A Chain A, Crystal Structure Of Human P32, A Doughnut-Shaped Acidic Mitochondria	2.837	4930073	2	-K.VEEQEPELTSTPNFVVEVIK.N-
ASC-1 complex subunit P200	2.826	12061185	3	-K.VKQCVHQIPSVMMEASIQPITR.T-
keratin 7; keratin, simple epithelial type I, K7	2.83	30089956	2	-.VDALNDEINFLR.-
keratin 4; Keratin-4; cytokeratin 4; keratin, type II cytoskeletal 4	2.815	17318574	2	-.NLDLDSIIAEVR.-
V_segment translation product	2.767	1552502	3	-.MSTRLLCWMALCLLGAELSEAEVAQSPRYK.-
pyruvate kinase M2	2.75	6018096	2	-R.LAPITSDPTEATAVGAVEASFK.C-
FLJ21945 protein	2.744	23272708	3	-.GICFLTDQLLLILVGKQK.-
Mitochondrial ornithine transporter 2	2.71	38372886	3	-.NEGIVALYSGLKATMIR.-
solute carrier family 25, member 13 (citrin)	2.70	7657581	2	-.IAPLEEGTLPFNLAEAQR.-
CLIP-associating protein CLASP1	2.67	13508645	3	-.SGNMIQSANDKNFDDEDSVDGNR.-
cytokeratin type II	2.631	4758618	2	-.LVDLEEALQKAK.-
E1A binding protein p400; p400 SWI2/SNF2-related protein; CAGH32 prote	2.63	15805014	3	-.RVLILSQMILMLDILEMFLNFHYLTYVR.-
TATA element modulatory factor 1	2.599	6005904	3	-.YQVELENLKDEYVRTLEETR.-
keratin protein K6irs	2.596	28372503	2	-.DLDLDSIIAEVR.-
Calpain 1, large subunit	2.58	14250593	2	-.KAPSDLYQIILK.-

Table 1 (continued)

Protein/reference	Xcorr	Protein Accession Number	Charge	Peptide Sequence
hypothetical protein FLJ14721	2.57	14249534	3	-R.YPSPAELDAYAEKVANSPLSIKIFPTNIR.V-
ras homolog gene family, member C; Aplysia RAS-related homolog 9	2.568	28395033	2	-K.QVELALWDTAGQEDYDR.L-
Integrin alpha-D precursor (Leukointegrin alpha D) (CD11d) (ADB2)	2.524	12643717	3	-.KPPQHSDFLTQISR.-
C11orf15 protein	2.523	31455206	3	-.RLFGHAQLIQSDDDIGDHQPFANAHDVLAR.-
calcium channel, voltage-dependent, N type, alpha 1B subunit	2.495	4502523	3	-.TDTGEQVDEFPCGLESPAWTCENGTECR.-
similar to cytoplasmic beta-actin	2.45	29736622	2	-.VAPDEHPILLTEAPLNPK.-
Hypothetical protein KIAA0143	2.449	2495710	3	-R.PSNGRHGAVGAPCAAPLSLGAAASAVEIAMPTR.V-
inhibitory receptor IREM1	2.447	31790204	3	-.LSSAQVDQVEVEYVTMASLPK.-
fragile X mental retardation syndrome related protein 2; fragile X-ment	2.436	4758410	3	-R.TDEDRTVMDGGLESDGPNMTENGLEDESR.P-
unnamed protein product	2.436	34526743	2	-R.SACATRQNSTSTKNTK.I-
zinc finger protein 132 (clone pHZ-12)	2.436	4507979	3	-.PYSNLGQLPEVCTTQKLFECSNCGKAFLK.-
similar to nuclear pore complex interacting protein	2.434	37541436	3	-.KSAVQQLTPLLLR.-
CD1A protein	2.425	21594951	2	-.FILGLLDAGKAHLQR.-
Membrane alanine aminopeptidase precursor	2.415	37590640	3	-.YLSYTLNPDLIR.-
C59436 KIAA1391 protein [imported]	2.41	25535895	2	-.YINLEKEKDYPK.-
solute carrier family 24 (sodium/potassium/calcium exchanger), member 1	2.405	4759128	3	-R.QKQAIYLFLLPIVFPLWLTVPDVR.R-
low density lipoprotein-related protein 1B	2.404	9055270	3	-R.AWDTLYWTSSTTSSITRHTVDQTR.P-
parkin isoform	2.403	20385800	3	-.EPQSLTRVDLSSSVLPGDSVGLAVILHTDSR.-
KIAA0713 protein	2.402	3882147	2	-.AQQKITEKDDQVK.-
SSB3 protein	2.4	28958160	3	-.YRHTFCSLLGRDEDSWGLSYTGLLHHK.-
Unknown (protein for MGC:71515)	2.389	34785925	3	-.LLQQNVDICAEATCGMIAER.-
Thyroid hormone receptor interactor 6	2.385	12803689	3	-.EPGPGAKEEAAGISGPAGR.-
hypothetical protein KIAA0470	2.372	7512995	3	-.EDNKTDEGPDTPSYNR.-
SH3-domain binding protein 5 (BTK-associated); SH3 binding protein	2.368	4759058	3	-.TRSELVHKETAAR.-
hypothetical protein	2.366	12052938	3	-K.GQAAPPAPPLPSSLDPPPPPAAVEVFQR.P-
similar to Hypothetical protein KIAA0056	2.359	20481728	2	-.MVALRGLGSGLQPWCPLDLR.-
dystrophin	2.354	119619470	3	-.QAEGLSEDGAAMAVEPTQIQLSKR.-
phosphodiesterase 10A	2.354	5729972	2	-.VIRGEETATWISSPSVAQK.-
Low-density lipoprotein receptor-related protein 3 precursor	2.336	84028221	3	-.CLPVERRCDGLQDCGDGSDEAGCPDLACGR.-
DEAD (Asp-Glu-Ala-Asp) box polypeptide 49	2.32	31542656	3	-.PLGLKDCIIVGGMDMVAQALELSR.-
Similar to KIAA0052 protein	2.32	21619317	2	-.LYIPKDLRPVDNR.-

Table 1 (continued)

Protein/reference	Xcorr	Protein Accession Number	Charge	Peptide Sequence
unnamed protein product	2.32	7022460	3	-K.PLGLKDCIIVGGMDMVAQALELSR.K-
unnamed protein product	2.318	10440008	3	-.MRPKVMWHLLR.-
similar to RIKEN cDNA 5031434M05	2.317	37540268	2	-.FCGLFMVLLSDHPSPDLGQK.-
RB1CC1 protein	2.314	17028471	3	-.VDSAMETSMMSVQENIHMLSEEKQR.-
PERIPENTONAL HEXON-ASSOCIATED PROTEIN (PROTEIN IIIA)	2.313	123064	3	-R.ANPTHEKVLAIVNALAENRAIR.P-
unnamed protein product	2.305	14042913	2	-R.FPLNGYCRLNSVQVLER.L-
unnamed protein product	2.303	34531680	3	-.MPKGGCPKAPQQEELPLSSDMVEK.-
unnamed protein product	2.303	34531680	3	-.MPKGGCPKAPQQEELPLSSDMVEK.-
similar to DKFZP434P1750 protein	2.297	37541041	2	-.LALGTAEQR.-
ubiquitin associated protein 2 isoform 2; AD-012 protein	2.29	22325366	3	-.SSYGLKGAWKNSVEEWTTEDWTEDLSETK.-
plastin 3; T isoform	2.283	7549809	2	-.PPYPKLGANMKK.-
breast carcinoma amplified sequence 1; Breast carcinoma amplified seque	2.276	4502373	3	-.VDEVPGLSGQSDDVPAGKDIVDGKEK.-
neuroplastin isoform a precursor	2.272	9257240	3	-.NEGQDATMYCKSVGYPHPDWIWRK.-
stromal cell derived factor receptor 1 isoform b	2.272	6912646	3	-K.NEGQDATMYCKSVGYPHPDWIWRK.K-
Sec3-like isoform 1; homolog of yeast exocyst protein Sec3p; exocyst	2.26	30410720	2	-R.ELQVLDGANIQSIMASEK.Q-
NACHT, LRR and PYD containing protein 10	2.258	28827807	3	-.KSQSQNLFSVKSSLSHGPK.-
SI:dZ146N9.1 (novel protein similar to human general control of amino-a	2.252	33284844	2	-.KLDAGSQLSLIDDLHR.-
transducer of regulated CREB protein 3	2.241	37693045	3	-.QQPPWKDEKHPGFR.-
chromosome 14 open reading frame 50	2.239	27312029	3	-K.RLGGQTPYLMDQLGLRLGMWYWK.D-
ubiquitin specific protease 32	2.233	28188773	3	-.NKDMSWPEEMSFIANSSKIDR.-
hypothetical protein	2.232	12053087	3	-R.DVTFAQEFINLDGISLLTQMVESGTER.Y-
similar to paraneoplastic antigen MA2	2.232	146219841	3	-.ALMNEKAQAALVEFVEDVNHAAIPREIPGK.-
unnamed protein product	2.232	34530583	3	-K.ALMNEKAQAALVEFVEDVNHAAIPREIPGK.D-
NKF3 kinase family member	2.228	148368962	3	-.SPDLYERQVCLLLLQLCSGLEHLK.-
KIAA2004 protein	2.227	24899174	2	-.EILTEQDVNGAVLK.-
erythrocyte membrane protein 4.1N	2.212	16356663	3	-.DSEGIDIMLGVCANGLLIYRDRLR.-
similar to Nck-associated protein 5 (NAP-5)	2.211	37547356	2	-.SGRLLFFARNYSVTTATR.-
RTKN protein	2.21	33871180	2	-.GRVCISDLRIPLMWK.-
dystrophin Dp116 isoform	2.208	5032295	3	-.TYHVKDLQGEIEAHTDVYHNLDENSQK.-
prostaglandin E receptor 4, subtype EP4; PGE receptor, EP4 subtype	2.204	4506259	3	-.NPDLQAIRIASVNPILDPWIYILLRK.-
A Chain A, Determination Cc-Chemokine Mcp-3, Nmr, 7 Structures	2.202	2624707	2	-.EAVIFKTKLDK.-

Table 1 (continued)

Protein/reference	Xcorr	Protein Accession Number	Charge	Peptide Sequence
similar to soluble adenylyl cyclase	2.202	37552182	3	-K.SSLCWFSREGLLATAQLMQALAYTK.L-
similar to surface glycoprotein, Ig superfamily member	2.202	37541781	2	-.LVFQQLKRSQTK.-
keratin 2a	2.198	4557703	2	-.WELLQQMNVGTR.-
GR AF-1 specific protein phosphatase	2.192	7108919	3	-.ACNAHGGVFKDEIYIPLQEEDTK.-
KIAA0614 protein	2.192	34328016	3	-K.ACNAHGGVFKDEIYIPLQEEDTK.K-
dipeptidyl-peptidase 9, isoform CRA_b	2.191	119589606	3	-.PCGGKEFGLFEELSEGSFGWVTGIRR.-
LYST-interacting protein LIP8	2.19	29789293	3	-.PLQDLSPSSSAQALEELFPRYTSLR.-
SI:zC214P16.4 (novel protein similar to human protein phosphatase 1)	2.186	27884151	3	-.IMRPTDVPDTGLLCDLLWSDPDK.-
hypothetical protein	2.184	31873973	3	-.MVHSLPTAVPESPRIHPTRTPK.-
myotubularin related protein (MTMR15)	2.181	28839601	3	-.EECIPEHMVRESKIMEAESQK.-
hypothetical protein	2.179	28279320	2	-K.NDHVLFYLENVFGR.A-
Src substrate cortactin (Amplaxin) (Oncogene EMS1)	2.178	2498954	2	-.ELETGPKASHGYGGK.-
similar to methyltransferase-like protein 1 isoform a; D1075-like gene	2.177	37546365	3	-K.PGTGIMAAETWNVAEAEAPQPQKR.Y-
unnamed protein product	2.174	16549252	2	-.TCQVDQFSCGNGR.-
amylo-1,6-glucosidase, 4-alpha-glucanotransferase isoform 1; glycogen	2.172	4557275	3	-.WNPEALPSNTGEVNFQSGIIAARCAISK.-
hypothetical protein	2.172	30268329	2	-.EVMCQLGLHQK.-
hypothetical protein MGC46732	2.168	24308460	3	-.LKEESLSLFTILHDRILEIEK.-
NDRG family member 3	2.162	56203225	2	-.PLLNDKNGTRNFQDFDCQ.-
syntaxin 11	2.157	3248918	3	-.WDVFSENLLADVKGR.-
cofilin 1 (non-muscle)	2.154	5031635	3	-.EILVGDVGQTVDDPYATFVKMLPDK.-
A-kinase anchor protein 3 (Protein kinase A anchoring protein 3)	2.153	14194457	2	-.SCDASLAELGDDKSGDASR.-
calreticulin precursor	2.15	4757900	3	K.SGTIFDNFLITNDEAYAEEFGNETWGVTK.A
NDRG2 protein (Syld709613 protein)	2.148	20141615	3	-.TASLTSAASVDGNRSRSR.-
KIAA0550 protein	2.142	20521081	3	-K.ELDESSVFVLGAVLYKNLDLILPTLR.N-
transmembrane channel-like protein 7	2.141	33355703	3	-R.IPSSKACGPFTNFNTTWEVIPK.T-
CSMD2 protein	2.14	34190748	3	-.EGSRFHHGDTLKFECQPAFELVGQK.-
similar to thymine-DNA glycosylase	2.14	37543803	3	-.SEYHAVLQRPAAR.-
caspase 14 precursor; apoptosis-related cysteine protease	2.139	6912286	3	-.FQQAIDSREDPVSCAFVVLMAHGR.-
microtubule-associated protein 1B isoform 2	2.135	14165456	3	-.HPDVSMVDPEALAIEQNLGKALK.-
breast cancer antiestrogen resistance 3	2.133	4502371	3	-.NMPVNHQFPLASSMDLLSSRSPLAEHR.-
unnamed protein product	2.133	14042842	3	-R.RASVKACIANVEPNQTVEINEQEALEEK.L-

Table 1 (continued)

Protein/reference	Xcorr	Protein Accession Number	Charge	Peptide Sequence
IKK-related kinase epsilon; IKK-related kinase epsilon; inducible Ikapp	2.131	7661946	2	-R.DQVHEDRSIQQIQCCLDK.M-
myosin VC; myosin 5C	2.127	9055284	2	-.SGSNAHVEDKVLASNPITEAVGNAK.-
neutral alpha glucosidase C type 2	2.126	25272050	3	-.STYQALLDSVTTDEDSTRFQIINEASK.-
hypothetical protein FLJ14871	2.123	14249582	3	-R.RSQSASDAPLSQHTLETLLEEIKALR.E-
Similar to dactylaplasia	2.122	13938471	3	-R.VWSIAISPLLSSFVTGTACCGHFSPLR.I-
chromosome 5 open reading frame 4	2.119	39644469	3	-K.FNQCYGVLGVLDHLHGTDTMFKQTKAYER.H-
Acyl-CoA dehydrogenase family member 11	2.116	74750393	3	-.QHILPAEKEVTEFYVQNENSVDKWGK.-
chromosome 14 open reading frame 70	2.115	28071062	3	-.MGIGTGHTSMNKGGKDVTLLELSVEK.R
Solute carrier family 46 member 3	2.112	38511860	2	-.VPFLFTIVPFSVLR.-
hypothetical protein LOC79096 isoform 4	2.109	15559265	3	-.NGDLLTMKEYHCLLQLLCPDFPLELTQK.-
ash1 (absent, small, or homeotic)-like	2.107	8922081	3	-.TLGKPDSLLVPAVASDSCNNSISLLSEK.-
adenylate cyclase 7 (ADCY7)	2.106	25058301	3	-.HQAILGMAFLVLAVFAALSVLMYVECLLR.-
zinc finger (KIAA1729) protein	2.104	58761535	3	-K.SLTVDGNVGKLVGIDSFQPSVQK.Q-
FLJ00056 protein	2.102	10440440	3	-.YGRLLEELLREAGPELSSECR.-
cytokine receptor-like factor 1	2.092	4758062	3	-R.GPAAQSARRPPPLLPLLLLLCVLGAPR.A-
ribosomal protein P0	2.092	4432757	2	-R.VLALSVETDYTFPLAEK.V-
ankyrin repeat and FYVE domain containing 1 isoform 2; ankyrin repeat	2.091	31317252	3	-K.HSADVMSEMAQIAEALLQAGANPNMQDSK.G-
AGLW2560	2.09	37182360	2	-.RSSWSATGSAAPFPPSDQPGTR.-
SKI-interacting protein; nuclear receptor coactivator, 62-kD;	2.09	6912676	3	-.NLDKDMYGDDLEAR.-
alpha-catenin-like protein	2.083	7019571	2	-.SNTLNIALDNMCKK.-
KPI-2 protein	2.083	27356940	3	-.KVFPLRWTAPELVTSFQDR.-
KIAA0039	2.079	37541272	3	-.TMADQLYLENIDEFVTDQNK.-
nebulin	2.079	4758794	3	-.INIPADMVSVLAAK.-
seven transmembrane helix receptor	2.075	21928245	3	-.SVTPPPFISPTSQLVITFSLTSLQESVTFR.-
KIAA0376	2.074	2280485	3	-.SFDSASQVPNPAAAAIPR.-
citron; rho-interacting, serine/threonine kinase 21	2.071	32698688	2	-R.SDLYESELRESRLAAEEFK.R-
hypermethylated in cancer 1	2.071	5729871	3	-.IHSGEKPYECQVCGGKFAQQR.-
similar to Probable ubiquitin carboxyl-terminal hydrolase FAF-X	2.07	37547080	2	-.RSYIIKCIEDIK.-
KIAA1200 protein	2.065	6330407	3	-K.VGTAYEQLIGKLMDGEGDPDSPLWR.H-
Pleckstrin homology domain-containing family H member 1	2.065	55741447	3	-.VGTAYEQLIGKLMDGEGDPDSPLWR.-
par-3 partitioning defective 3 homolog B isoform a	2.061	119120907	3	-K.IHHLEYTDGGILDPDDVLADVVEDK.D-

Table 1 (continued)

Protein/reference	Xcorr	Protein Accession Number	Charge	Peptide Sequence
ribonuclease L; ribonuclease 4; interferon-induced 2-5A-dependent RNas	2.059	10863929		-.WTTKINECVMKK.-
SH3-domain kinase binding protein 1; c-Cbl-interacting protein	2.058	13994242	3	-.PPSQSLTSSSLSSPDIFDSPSPEEDK.-
protein phosphatase 4, regulatory subunit 2	2.057	28372531	3	-K.KEVCPVLDQFLCHVAKTGETMIQWSQFK.G-
ubiquitin specific protease, proto-oncogene; Unph	2.057	4507853	3	-.PDAETQKSELGPLMR.-
membrane protein, MAGUK p55 subfamily member 6 (VELI-associated MAGUK 1) (VAM-1)	2.054	27764857	3	-R.TSEFMPYVVFIAAPELETLRAMHK.A-
KIAA1082 protein	2.052	14133233	2	-K.GIPEHLMGKLGPNGER.S-
zinc-finger protein ZNF76	2.051	2136421	3	-.THTGERPFQCPFEGCGRSFTTSNIR.-
KIAA1950 protein	2.049	18916759	3	-R.ALKDALVSTDAALQQLYVSAFSPAER.L-
phosphatidylinositol 3-kinase	2.047	987948	2	-K.AHRQGHMVKVDWLDR.L-
unnamed protein product	2.046	21754782	2	-K.HIDLLKIKVTSGVGDK.Q-
similar to Coiled-coil-helix-coiled-coil-helix domain-containing protein 2 isoform 3;similar to 16.7Kd protein	2.045	37549528	3	-K.QFLECAENQGDIKLCEGFNEVLK.Q-
hypothetical protein DKFZp586C1620.1	2.044	7512857	3	-.EDFFHCLKCNLCLAMNLQGRQVY.-
hypothetical protein MGC45962	2.042	22749313	3	-.MLVIAGGILAALLLLIVVVLCLYFKIHNALK.-
signal peptidase complex 18 kDa subunit	2.042	3641344	2	-.ARGFVPYIGIVTILMNDYPKFR.-
A Chain A, Apo Form Of Human Mitochondrial Aldehyde Dehydrogenase	2.04	28949044	2	-.ELGEYGLQAYTEVK.-
histidyl-tRNA synthetase-like; HARS-related; histidine-tRNA ligase hom	2.039	15029520	3	-.NNPKLLTQLHYCESTGIPLVVIIGEQELK.-
XPA binding protein 2; XPA-binding protein 2	2.033	9910260	3	-.ALKLLPCSTKLWYR.-
KIAA0286	2.031	20521027	3	-.PVPPRLLTEEEYRIQGEVETR.-
capacitative calcium entry channel protein; identical to AJ006203	2.029	3264578	3	-.HWVVKLLTCMTIGFLFPMLSIAYLISPR.-
unnamed protein product	2.028	34528602	3	-.AQTHPAPPQTWAPFFTMHSSEQPCR.-
microtubule-based motor	2.027	4106715	3	-R.SHAVFNIIFTQKRHDAETNITTEK.V-
IGF-II mRNA-binding protein 1	2.026	21361352	3	-.LLVPTQYVGAIIGKEGATIRNITK.-
bA11M20.1 (TATA box binding protein (TBP)-associated factor, RNA polyme	2.025	13559031	3	-.LRPPPEGSAGSCAPVPAAAAVAAGPEPAPAGPAK.-
DEAD-box protein abstrakt [synthetic construct]	2.023	30584005	3	-.VPPVLQVLHCGDESMLDIGGER.-
similar to FLJ00251 protein	2.023	37541591	3	-.RCFPHVHAVSFRSCPTGEK.-
KIAA1564 protein	2.018	34328020	2	-.WCSLPYEDSTWELKEDVDEGK.-
KIAA1196 protein	2.016	34013528	3	-K.AENQALRDIPLSLMNDWKDEFK.A-
unknown	2.013	10441986	3	-.MRRELLAGILLR.-
ADAM 2 precursor (A disintegrin and metalloproteinase domain 2)	2.012	28202251	2	-.VCRNQRCVSSSYLGYDCTTDK.-
cyclin D-dependent kinases 4 and 6-binding protein/p16 product	2.012	861472	2	-.GSNHARIDAAEGPSAIPD.-
unnamed protein product	2.01	10435867	3	-.QWLSATKPPLSDR.-

Table 1 (continued)

Protein/reference	Xcorr	Protein Accession Number	Charge	Peptide Sequence
unnamed protein product	2.01	10435867	3	-.QWLSATKPPLSDR.-
hypothetical protein XP_352404	2.007	37560019	3	-R.ALYTTLLMIPTRHANVDAVHDIANEDTV.-
very-long-chain acyl-CoA dehydrogenase	2.007	3273228	2	-.ALEQFATVVEAK.-
protein phosphatase 1, regulatory (inhibitor) subunit 12A; myosin phosphatase	2.004	4505317	3	-K.SLLSSTSTTTKITTGSSSAGTQSSTSNR.L-
TBX3-isoform	2.003	7341107	3	-K.EAFAPLTVQTDAARSSVHRHPFR.N-
PIP3AP protein	2.002	34783714	3	-K.EVTEKEVTLHLLPGEQLLCEASTVLK.Y-
skeletal muscle specific actinin, alpha 3	2.002	4557241	2	-.LVPSRDQTLQEELARQQVNER.-
unnamed protein product	2.002	34536452	2	-.GPSLDIDTPDVNIEGPEGK.-
unnamed protein product	2.002	34536452	2	-.GPSLDIDTPDVNIEGPEGK.-
TANK-binding kinase 1	2	7019547	3	-.LSSSQGTIETSLQDIDSR.-

Table 2: Mucin granule associated proteins with an Xcorr value > 4

Protein/reference	Xcorr	Protein Accession Number	Charge	Peptide Sequence
HSPA8 protein	5.99	13938297	2	-.SINPDEAVAYGAAVQAAILSGDK.-
MYH9 protein	5.665	29436380	2	-K.LQVELDNVTGLLSQSDSK.S-
similar to Heat shock cognate 71 kDa protein	5.62	37550676	2	-.IINEPTAAAIAYGLDK.-
NADH-ubiquinone oxidoreductase 75 kDa subunit,	5.61	128826	2	-.YDDIEGANYFQQANELSK.-
gelsolin (amyloidosis, Finnish type); Gelsolin	5.283	4504165	2	-R.AQPVQVAEGSEPDGFWEALGGK.A-
keratin 10, type I, epidermal	5.10	88041	2	-.GSLGGGFSSGGFSGGSFSR.-
mitochondrial trifunctional protein, alpha subunit precursor	4.89	20127408	2	-.DSIFSNLTGQLDYQGFEK.-
NADPH-cytochrome P-450 reductase	4.85	11414998	2	-.NIIVFYGSQTGTAEEFANR.-
cytokeratin 9	4.698	435476	2	-.GGGGSFGYSYGGGSGGGFSASSLGGGFGGGSR.-
keratin 1; Keratin-1; cytokeratin 1; hair alpha protein	4.684	17318569	2	-.LNDLEDALQQAK.-
heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	4.516	16507237	2	-R.IEIESFYEGEDFSETLTR.A-
protein disulfide isomerase related protein	4.50	4758304	2	-.GESDPAYQQYQDAANNLR.-
transmembrane protein (63kD), endoplasmic reticulum	4.49	19920317	2	-.VQSLQATFGTFESILR.-
SDHA protein	4.44	26996830	2	-.LGANSLLDLVVFGR.-
myosin heavy chain nonmuscle form A	4.44	625305	2	-.IAQLEEQLDNETK.-
mitochondrial malate dehydrogenase precursor	4.435	21735621	2	-K.VAVLGASGGIGQPLSLLLK.N-
bA255A11.8 (novel protein similar to annexin A2 (ANXA2)	4.318	12314197	2	-K.SALSGHLETVILGLLK.T-
Cathepsin B (Fragment)	4.3	2982114	2	-.NGPVEGAFSVYSDFLLYK.-
dnaK-type molecular chaperone HSPA6	4.26	87626	2	-.IINEPTAAAIAYGLDR.-
similar to 78 kDa glucose-regulated protein prec	4.21	37547096	2	-.NQLTSNPKNTVFDAK.-
AGR2 (Anterior gradient 2)	4.116	68012756	2	-.LAEQFVLLNLVYETTDK.-
mutant beta-actin (beta'-actin)	4.084	28336	2	-K.SYELPDGQVITIGNER.F-
hydroxysteroid (17-beta) dehydrogenase 4	4.04	4504505	2	-.GALVVVNDLGGDFK.-

Table 3. Mucin granule associated proteins exclusively seen in UNCN3T cells

Protein/reference	Xcorr	Protein Accession Number	Charge	Peptide Sequence
HSPA8 protein	5.99	13938297	2	-.SINPDEAVAYGAAVQAAILSGDK.-
similar to Heat shock cognate 71 kDa protein	5.62	37550676	2	-.IINEPTAAAIAYGLDK.-
NADH-ubiquinone oxidoreductase 75 kDa subunit,	5.61	128826	2	-.YDDIEGANYFQQANELSK.-
keratin 10, type I, epidermal	5.10	88041	2	-.GSLGGGFSSGGFSGGSFSR.-
uracil DNA glycosylase	5.10	35053	2	-.WGDAGAEYVVESTGVFTTMEK.-
mitochondrial trifunctional protein, alpha subunit precursor	4.89	20127408	2	-.DSIFSNLTGQLDYQGFEK.-
NADPH-cytochrome P-450 reductase	4.85	11414998	2	-.NIIVFYGSQTGTAEEFANR.-
cytokeratin 9	4.64	435476	2	-.VQALEEANNDLENK.-
protein disulfide isomerase related protein	4.50	4758304	2	-.GESDPAYQQYQDAANNLR.-
transmembrane protein (63kD), endoplasmic reticulum	4.49	19920317	2	-.VQSLQATFGTFESILR.-
SDHA protein	4.44	26996830	2	-.LGANSLLDLVVFGR.-
myosin heavy chain nonmuscle form A	4.44	625305	2	-.IAQLEEQLDNETK.-
dnaK-type molecular chaperone HSPA6	4.26	87626	2	-.IINEPTAAAIAYGLDR.-
similar to 78 kDa glucose-regulated protein prec	4.21	37547096	2	-.NQLTSNPKNTVFDAK.-
hydroxysteroid (17-beta) dehydrogenase 4	4.04	4504505	2	-.GALVVVNDLGGDFK.-
glycerol-3-phosphate dehydrogenase (EC 1.1.99.5)	3.99	7446012	2	-.LAFLNVQAAEEALPR.-
heat shock 70kDa protein 9B precursor	3.98	24234688	2	-.SQVFSTAADGQTQVEIK.-
dnaK-type molecular chaperone HSPA1L	3.93	2119712	2	-.AQIHDLVLVGGSTR.-
heat shock 70kDa protein 1-like; heat shock 70kD	3.93	27436929	2	-.AKIHDIVLVGGSTR.-
smooth muscle myosin heavy chain 11 isoform S	3.90	13124879	2	-.TQLEELEDELQATEDAK.-
ABCD3 protein	3.81	16307246	2	-.IANPDQLLTQDVEK.-
Pyruvate kinase, M2 isozyme	3.80	125604	2	-.FGVEQDVDMVFASFIR.-
junction plakoglobin; gamma-catenin	3.79	4504811	2	-.LLNDEDPVVVTK.-
heat shock 70kDa protein 8 isoform 2	3.78	24234686	2	-.GPAVGIDLGTTYSCVGVFQHGK.-
A Chain A, Human Acid-Beta-Glucosidase	3.77	33357737	2	-.SYFSEEGIGYNIIR.-
carcinoembryonic antigen-related cell adhesio	3.68	19923221	2	-.SDPVTLNVLYGPDVPTISPSK.-
ribophorin I	3.65	4506675	2	-.SEDLLDYGPFR.-
alpha enolase	3.61	2661039	2	-.FGANAILGVSLAVCK.-
keratin 6A [synthetic construct]	3.61	30584049	2	-.AIGGGLSSVGGGSSTIK.-

Table 3 (continued)

Protein/reference	Xcorr	Protein Accession Number	Charge	Peptide Sequence
unnamed protein product	3.49	21752190	2	-.GVVDSEDIPLNLSR.-
long-chain fatty-acid-coenzyme A ligase 1	3.46	4503651	2	-.IIVVMDSYGSELVER.-
ACTN4 protein	3.42	33874637	2	-.ETTDTDTADQVIASFK.-
N-ethylmaleimide-sensitive factor	3.37	11079228	2	-.NFSGAELEGLVR.-
transketolase	3.31	4507521	2	-.ILATPPQEDAPSVDIANIR.-
heat shock protein	3.29	4204880	2	-.FEELNADLFR.-
lamin A/C	3.23	30584609	2	-.NSNLVGAAHEELQQSR.-
acid alpha-glucosidase preproprotein;	3.18	4503849	2	-.STGGILDVYIFLGPEPK.-
S100 calcium binding protein A11	3.15	30584939	2	-.TEFLSFMNTELAAFTK.-
lamin A/C isoform 1 precursor; 70 kDa lamin	3.06	27436946	2	-.SVGGSGGGSFGDNLVTR.-
NSAP1 protein	3.05	26454828	2	-.NLANTVTEEILEK.-
calnexin	3.02	7709904	2	-.APVPTGEVYFADSFDR.-
complement component 3 precursor; acylation-st	3.01	4557385	2	-.VPVAVQGEDTVQSLTQGDGVAK.-
solute carrier family 25 (mitochondrial carrier)	2.93	21361103	2	-.YLGLYNDPNSNPK.-
PPCM_HUMAN Phosphoenolpyruvate carboxykinase, mitochondrial precursor	2.91	3287892	2	-.LGTPVLQALGDGDFVK.-
LIM domain and actin binding 1 isoform a	2.90	165905589	2	-.SQDVELWEGEVVK.-
carnitine palmitoyltransferase II	2.86	4503023	2	-.LNFELTDALK.-
keratin 7; keratin, simple epithelial type I, K7	2.83	30089956	2	-.VDALNDEINFLR.-
solute carrier family 25, member 13 (citrin)	2.70	7657581	2	-.IAPLEEGTLPFNLAEAQR.-

DISCUSSION

We report here a comparative proteomic analysis of mucin granule membranes from normal bronchial epithelial cells and a cystic fibrosis cell line, UNCN3T. We are attempting to elucidate the core molecules associated with mucin secretion. Our findings support the presence of key molecules already published to be implicated in mucin secretion, i.e. MARCKS, CSP, and HSP70 in UNCN3T cells and an intestinal cell line, HT29-18N2 . We also report that UNCN3T cells respond to PKC activation by PMA stimulation in a manner similar to NHBE cells, however, intestinal HT29-18N2 cells are delayed in their response requiring 2 hours to achieve a significant increase. NHBE and UNCN3T cells respond significantly by 15 minutes. This finding was unexpected, because cystic fibrosis cells are chronic inflammatory cells and presumptively are hyperresponsive. It would have been expected that these cells expressed an increase in mucin secretion faster that the normal bronchial epithelial cells. HT29-18N2 cells are grown in a submerged condition differing from the airway epithelial cells, therefore, the delayed response to PMA as compared to the NHBE cells may be due to this effect.

It is interesting to note overall concentration levels from all the analyzed proteins were highest in the UNCN3T cells. This could be attributed to disease since we suspect that the proteins involved in the exocytosis of mucin granules would be more concentrated in these cells that are chronically stimulated to secrete mucus. These cells also respond more robustly with the amount of mucin secreted following exposure to PMA at 15 minutes, but the activation of MARCKS is not echoed. We suspect that PKC phosphorylation has already peaked prior to when it was measured by Western analysis in these cells, possibly by 5

minutes. We also see that the levels of CSP are increased in these cells, but this could be an artifact of overexposure in the densitometry analysis since the signals for CSP were so intense.

In the mass spectrometry results, we have identified 249 mucin granule membrane (Table 1) associated proteins that appeared in at least two experiments with an Xcorr value of 2 or more. The majority of these proteins are cytoskeletal or regulators of cytoskeletal components. However, the identified proteins revealed the following functional categories: proteases, metabolism, protein folding, redox regulation, ATPases, calcium regulation, signaling components, exocytotic mechanisms, and related functions. The data generated was from three separate experiments. Twelve KIAA proteins were identified in this screen. KIAA proteins are products of large cDNAs identified by the Kazusa cDNA project. This Kazusa project was the first to extensively sequence more than 2000 human cDNAs in their entirety focusing particularly on large cDNAs encoding large proteins (Nagase et al., 2006). Fifteen are identified as hypothetical proteins. By definition, hypothetical proteins are predicted, but there is no experimental evidence that it is expressed in vivo. Fourteen proteins are unnamed, and 2 proteins are annotated as gene products whose process are not known or cannot be inferred. Follow up analysis of these proteins are warranted since they have been identified in multiple experiments.

When comparing the cystic fibrosis cells with the NHBE cells, the results significantly overlapped with the exception of a few proteins that were seen in the UNCN3T cells only (Table 3), (i.e. Calpain, Calnexin, and N-ethylmaleimide-sensitive factor). The results from this method of LC-MS/MS are not quantitative, therefore, I expect that these

differences are due more probably to technical aspects such as the time mucin granule membranes were isolated. This factor dictates the proteins associated with the granule at that point of exocytosis. A more quantitative method of analysis is required and would be an appropriate future study.

In summary of the proteins identified in these analysis restrictive to the UNCN3T cell mucin granule isolations, calpains are Ca(2+)-dependent cysteine proteases found to be involved in many cellular responses relating to exocytosis, including cytoskeletal rearrangements and vesicular trafficking. When calpains are inhibited by calpeptin, a calpain inhibitor, it blocks pancreatic beta-cell spreading and insulin secretion (Parnaud et al., 2005). In a study of mitochondrial release of cytochrome c and Smac in neutrophils from patients with cystic fibrosis that exhibited delayed apoptosis and markedly increased calpastatin, calpain-1 protein levels were decreased compared to neutrophils from control individuals (Altznauer et al., 2004).

N-ethylmaleimide-sensitive factor (NSF) is an ATPase involved in disassembling SNARE complexes (Burgoyne et al., 2003). The molecular chaperone calnexin colocalizes with CSP in the endoplasmic reticulum and with CFTR at the apical membrane domain in Calu-3 airway cells (Zhang et al., 2002). CSP has been well documented for its role in regulated exocytosis. CSP has also been shown to interact with CFTR and is coexpressed with CFTR at the apical membrane domain of epithelial cells. Identifying Calnexin and CSP (in the previous chapter) to be granule membrane associated proteins also suggests a possible relationship with CFTR and the mucin granule membrane. Identification of Calnexin is also

interesting, because calnexin mRNA expression is decreased in studies of HT29 cancer cells (Yeates et al., 1997).

Proteins such as cathespin B, a protease, were only seen in NHBE isolations. Another protein, S100 calcium binding protein A11, interacts with a variety of intracellular target proteins that are cell specific, and S100 proteins regulate effector proteins, specific steps of signaling pathways, or cellular functions, which possibly could makes it a good therapeutic target (Kanamori et al., 2004).

The proteins that were identified with an Xcorr score greater than 4 were cytoskeleton or cytoskeletal related proteins and mitochondrial metabolism proteins. These results were expected and are similar to what was described in Chapter 2. MARCKS, CSP, and hCLCA1 were not identified by mass spectrometry analysis in this study. We suspect that other proteins that are separating at the same size as our "target proteins" are so abundant that they are masking detection of the targets. This occurs when the signals from the more abundant proteins overshadow the less abundant proteins so much that the signals from these less abundant proteins appear at levels similar to background noise. We will be exploring different methods of mass spectrometry analysis to try an elucidate them.

In conclusion, the proteins identified provide numerous avenues for future studies as these proteins should be analyzed more thoroughly for their role in mucin secretion. Here we clearly demonstrate that our strategy for mucin granule membrane analysis has determined a set of mucin granule membrane associated proteins. These data may help to unravel the mechanisms of mucin secretion as well as providing candidates for future therapeutic approaches to treat mucin hypersecretion.

REFERENCES

Altznauer F, Conus S, Cavalli A, Folkers G, Simon HU. Calpain-1 regulates Bax and subsequent Smac-dependent caspase-3 activation in neutrophil apoptosis. J Biol Chem. 2004 Feb 13;279(7):5947-57.

Burgoyne RD, Geisow MJ. The annexin family of calcium-binding proteins. Cell Calcium. 1989 Jan;10(1):1-10.

Burgoyne RD, Morgan A. Secretory granule exocytosis. Physiol Rev. 2003 Apr;83(2):581-632. Review.

Gibson A, Lewis AP, Affleck K, Aitken AJ, Meldrum E, Thompson N. hCLCA1 and mCLCA3 are secreted non-integral membrane proteins and therefore are not ion channels. J Biol Chem. 2005 Jul 22;280(29):27205-12.

Kanamori T, Takakura K, Mandai M, Kariya M, Fukuhara K, Sakaguchi M, Huh NH, Saito K, Sakurai T, Fujita J, Fujii S. Increased expression of calcium-binding protein S100 in human uterine smooth muscle tumours. Mol Hum Reprod. 2004 Oct;10(10):735-42.

Krunkosky TM, Fischer BM, Martin LD, Jones N, Akley NJ, Adler KB. 2000 Effects of TNF-alpha on expression of ICAM-1 in human airway epithelial cells in vitro. Signaling pathways controlling surface gene expression. Am. J. Respir. Cell Mol. Biol. 22: 685-692.

Kuperman DA, Huang X, Koth LL, Chang GH, Dolganov GM, Zhu Z, Elias JA, Sheppard D, Erle DJ. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. Nat Med. 2002 Aug;8(8):885-9.

Leverkoehne I, Gruber AD. The murine mCLCA3 (alias gob-5) protein is located in the mucin granule membranes of intestinal, respiratory, and uterine goblet cells. J Histochem Cytochem. 2002 Jun;50(6):829-38.

Li Y, Martin LD, Spizz G, Adler KB. MARCKS protein is a key molecule regulating mucin secretion by human airway epithelial cells in vitro. J Biol Chem. 2001 Nov 2;276(44):40982-90.

Mundhenk L, Alfalah M, Elble RC, Pauli BU, Naim HY, Gruber AD. Both cleavage products of the mCLCA3 protein are secreted soluble proteins. J Biol Chem. 2006 Oct 6;281(40):30072-80.

Nagase T, Koga H, Ohara O. Kazusa mammalian cDNA resources: towards functional characterization of KIAA gene products. Brief Funct Genomic Proteomic. 2006 Mar;5(1):4-7.

Park J, Fang S, Adler KB. Regulation of airway mucin secretion by MARCKS protein involves the chaperones heat shock protein 70 and cysteine string protein. Proc Am Thorac

Soc. 2006 Aug;3(6):493.

Park JA, Crews AL, Lampe WR, Fang S, Park J, Adler KB. Protein kinase C delta regulates airway mucin secretion via phosphorylation of MARCKS protein. Am J Pathol. 2007 Dec;171(6):1822-30.

Park JA, Crews AL, Lampe WR, Fang S, Park J, Adler KB. Protein Kinase C{delta} Regulates Airway Mucin Secretion via Phosphorylation of MARCKS Protein. Am J Pathol. 2007 Nov 30.

Park JA, He F, Martin LD, Li Y, Chorley BN, Adler KB. Human neutrophil elastase induces hypersecretion of mucin from well-differentiated human bronchial epithelial cells in vitro via a protein kinase C{delta}-mediated mechanism. Am J Pathol. 2005 Sep;167(3):651-61.

Parnaud G, Hammar E, Rouiller DG, Bosco D. Inhibition of calpain blocks pancreatic beta-cell spreading and insulin secretion. Am J Physiol Endocrinol Metab. 2005 Aug;289(2):E313-21.

Peterman SM, Dufresne CP, Horning S. The use of a hybrid linear trap/FT-ICR mass spectrometer for on-line high resolution/high mass accuracy bottom-up sequencing. J Biomol Tech. 2005 Jun;16(2):112-24.

Phillips TE, Ramos R, Duncan SL.1995. HT29-18N2 differentiation in a protein-free medium. In Vitro Cell Dev Biol Anim. 31:421-3.

Puchelle E, Bajolet O, Abély M. Airway mucus in cystic fibrosis. Paediatr Respir Rev. 2002 Jun;3(2):115-9.

Rogers DF. Physiology of airway mucus secretion and pathophysiology of hypersecretion. Respir Care. 2007 . Sep;52(9):1134-46; discussion 1146-9. Review.

Singer M, Martin LD, Vargaftig BB, Park J, Gruber AD, Li Y, Adler KB. A MARCKS-related peptide blocks mucus hypersecretion in a mouse model of asthma. Nat Med. 2004 Feb;10(2):193-6.

Vasconcellos CA, Allen PG, Whol ME, Drazen JM, Janmeuy PA, Stossel TP. Reduction in viscosity of cystic fibrosis sputum in vitro by gelsolin. Science 1994(263):969–971.

Vaughn CP, Crockett DK, Lim MS, Elenitoba-Johnson KS. Analytical characteristics of cleavable isotope-coded affinity tag-LC-tandem mass spectrometry for quantitative proteomic studies. J Mol Diagn. 2006 Sep;8(4):513-20.

Verdugo P. Goblet cells secretion and mucogenesis. Annu Rev Physiol. 1990;52:157-76. Review.

Wu TT, Castle JD. 1997. Evidence for colocalization and interaction between 37 and 39 kDa isoforms of secretory carrier membrane proteins (SCAMPs). J Cell Sci. 110: 1533-41.

Yeates LC, Powis G. The expression of the molecular chaperone calnexin is decreased in cancer cells grown as colonies compared to monolayer. Biochem Biophys Res Commun. 1997 Sep 8;238(1):66-70

Zhang H, Peters KW, Sun F, Marino CR, Lang J, Burgoyne RD, Frizzell RA. Cysteine string protein interacts with and modulates the maturation of the cystic fibrosis transmembrane conductance regulator. J Biol Chem. 2002 Aug 9;277(32):28948-58.

SUMMARY AND FUTURE DIRECTIONS

The studies presented in this dissertation investigated mucin granule membrane associated proteins involved in mucin secretion using well-differentiated normal human bronchial epithelial (NHBE) cells, a cystic fibrosis cell line (UNCN3T), and an intestinal colon cancer cell line (HT29-18N2) *in vitro.* The air liquid interface (ALI) culture system of airway epithelial cells provides an *in vitro* model system that differentiates into mucus and ciliated cells that mimic *in vivo* conditions (Li et al., 2001). The HT29-18N2 and UNCN3T cells were utilized in these studies because they were able to provide an enriched population of epithelial goblet cells containing mucin granules for subsequent mucin granule isolations.

The aim of this project was to provide novel insight into mechanisms of mucin secretion. Mucin secretion is a process of regulated exocytosis where membrane bound mucin granules stored in the cytoplasm of goblet cells are transported to the plasma membrane where they dock, fuse, and release their mucin contents into the airway lumen. The niche of the study was related to mucin granule associated protein identification. An antibody generated to a previously identified mucin granule membrane associated protein, mCLCA3, an ortholog of hCLCA1, was produced and used as a biomarker for mucin granule membranes (Leverkoehne et al., 2002).

Calcium activated chloride channel 1 (hCLCA1), the first identified human member of calcium activated chloride channel family, was cloned and assigned molecular and functional characterization in 1998 by Gruber et al. (1998). The hCLCA1 gene and promoter region are located on chromosome 1 at p22-p31 and span 31,902 bp (Gruber et al., 1998). The hCLCA1 gene is translated into a 914-amino acid protein with a signal sequence, 13

consensus sites for protein kinase C (PKC) phosphorylation, 3 consensus sites for phosphorylation by Ca2+/calmodulin-dependent kinase II, and four disputed putative transmembrane domains that were based on hydrophobicity plots.

An alternative model for hCLCA1 structure has been predicted using SMART predictions based on sequence homology to the α2-integrin collagen receptor domain, suggesting that all the CLCA family members have a von Willebrand factor domain A (VWA domain) that covers the area previously thought to have 3 transmembrane domains (Gibson et al., 2005; Pawlowski et al., 2006).

The calculated mass for hCLCA1 is 100.9 kDa, but *in vitro* translation of hCLCA1 resulted in a 100-kDa product that increased in size to 125 kDa after glycosylation (Gruber et al. ,1998). CLCA family members are cleaved by a hydrolase near the carboxyl-terminal, and in hCLCA1 it yields a ~75kDa and ~35kDa product (Pawloski et al., 2006) that is fully secreted into the extracellular environment (Mundhenk et al, 2006).

hCLCA1 protein is expressed in the digestive tract including the small intestine, colon and appendix, and it plays an important role in fluid and electrolyte transport and in mucus secretion (Gruber et al.,1998). hCLCA1 has been extensively analyzed as a candidate modulator in the pathogenesis of cystic fibrosis (Hauber et al., 2004). In cystic fibrosis patients, several electrolyte trafficking pathways are deficient including sodium and chloride channels other than CFTR. The combination of these deficiencies contribute to a variety of complex pathological changes in epithelial tissues, mainly in the lung, intestine, and pancreas of these patients(Hauber et al., 2004). The expression of hCLCA1 is increased in the epithelial cells of patients with asthma and COPD (Nakano et al., 2006; Hauber et al., 2005).

A gene transfection study showed that mucus production and murine Muc5ac and Gob-5 expression are induced by interleukin 13 (IL-13) in Stat6+/- mice (Kuperman et al., 2005). Kim et al. (2007) investigated whether or not histamine induces MUC5AC production in NCI-H292 cells in an attempt to evaluate which pathway is involved in this mucin production and found that histamine-induced MUC5AC production occurs via the upregulation of hCLCA1.

Gob-5, the murine ortholog of hCLCA1, is expressed and strictly localizes in airway epithelium, especially in mucus-producing cells in mice sensitized with ovalbumin (Nakanishi et al., 2001). Adenoviral gene transfer with an antisense Gob-5 construct suppresses the development of airway hyperreactivity and mucus overproduction in ovalbumin-sensitized mice. Contrarily, the overexpression of gob-5 in airway epithelium transfected with a sense Gob-5 construct via an adenoviral vector results in mucus overproduction and airway hyperreactivity (Nakanishi et al., 2001).

The hCLCA1 protein strongly associates with membranes requiring strong pH or detergent for phase separation (Gibson et al., 2005), and gold labeling of mucin granules isolated using Percoll density gradient indicates that hCLCA1 protein associates with the granule membrane (Park et al., 2006; Leverkoehne et al., 2002). Subcellular fractionation of hCLCA1 transfected HEK cells find it in the membrane fractions or phases of stringent washes, and using high or low pH solutions as stripping agents have been shown to remove membrane associated proteins (Gibson et al., 2005). Immunofluorescent detection of hCLCA1 following these washes demonstrated that hCLCA1 was removed, but not totally indicating that it interacts with other proteins (Gibson et al., 2005).

The subcellular distribution of hCLCA1 protein was evaluated in the well differentiated NHBE ALI culture system. A method of immuno-isolation of mucin granules was optimized and verified for its efficacy in mucin granule membrane isolations. This was a novel study where proteomic analysis elucidated mucin granule membrane associated proteins during the process of regulated exocytosis stimulated by the PKC antagonist, PMA.

The results of these studies elucidated numerous novel proteins associated with the mucin granule during the regulated process of mucin secretion, which were discussed at the conclusion of each chapter. Proteins identified are categorized as cytoskeletal, regulatory, and accessory proteins that function as proteases, in metabolism, protein folding, redox regulation, ATPases, calcium regulation, signaling components, and exocytotic mechanisms. The general mechanism of secretion is known to involve cytoskeletal rearrangement via phosphorylation and dephosphorylation of Actin and myosin subunits, release of calcium stores, and SNARE mediated docking and fusion (Burgoyne et al., 2003.). Cytoskeletal and scaffold proteins identified such as Myosin Vc, ACTIN-like proteins, Microtubule motor proteins, Annexins, and Plastin3 proteins all have published roles in exocytosis. A host of regulatory proteases, phosphatases, and kinases associations were also identified in these studies.

The majority of the proteins identified with an Xcorr value of at least 4 were chaperone proteins from the Heat shock family or related proteins such as glucose-regulated protein 78 (GRP78). As previously discussed, Heat shock proteins are a family activated to respond to a wide range of stressors. HSP70 is specifically involved in maintenance of

cellular integrity via protein translocation folding (reviewed in Pilon M et al., 1999). HSP70, the inducible form of HSC70, is a molecular chaperone that can be activated by CSP (Evans et al., 2003). GRP78 is in the normal pathway leading to secretion. Reduction of constitutive GRP78 levels results in increased secretion of proteins that are normally retained in the ER (Dorner et al., 1990).

The other proteins can be classified as enzymes or cytoskeletal components such as myosin and beta actin. Cathespin B is a protease found to have a unique role in secretory vesicles for the production of biologically active peptides (Hook, 2005). Protein-disulfide isomerase (PDI) is a multi-functional protein that plays a key role in the co- and post-translational modification of secretory and cell surface proteins in all eukaryote cells (Freedman et al., 1994). As an enzyme, PDI catalyzes the rearrangement of pre-existing disulfide bonds as well as formation or breakage of disulfide bonds, depending on the reduction potential of the environment (Freedman et al., 1994).

Gelsolins are actin-binding proteins that regulate actin-mediated movement by controlling assembly and disassembly of actin by severing them (Sun HQ et al., 1999). Gelsolins are upregulated in the bronchial epithelium in asthma (Candiano G et al., 2005). Annexins are calcium-dependent membrane-associated proteins that function in endosome sorting, membrane-cytoskeletal linkage and control of fusion events in exocytosis (Burgoyne RD et al., 1989). Studies indicate an essential role of Annexin A2 phosphorylation in regulating cofilin-dependent Actin cytoskeletal dynamics (de Graauw M et al., 2007).

There are a few mitochondrial proteins that have been identified as mucin granule associated with an Xcorr score greater than four, i.e. mitochondrial malate dehydrogenase,

succinate dehydrogenase (SDHA) and NADH-ubiquinone oxidoreductase proteins play a role in mitochondrial metabolism and respiration (Isaac et al., 2007; Easlon et al. 2008). Adler et al. (1982) evaluate porcine tracheal goblet cell ultrastructure and identified that microtubules and microfilaments are observed in close association with both mucin granules and coiled filamentous mitochondria, suggesting a role in the intracellular movement of these organelles (Adler et al., 1982).

It was expected that our mass spectrometry analysis would have identified SNARE proteins such as VAMP2, Rab GTPases, molecular chaperones CSP, MARCKS, and hCLCA1, but it did not. Verification of mucin granule membrane associations for these proteins was provided by immunocytochemistry and Western blot analysis. The low yield of the mucin granule membranes along with the extensive granule immunoisolation procedure introduced complications in the subsequent mass spectrometry analysis. A disadvantage of LC-MS/MS is that signals from proteins of low abundance can be masked by larger, more abundant proteins, so we had to verify by Western blot analysis.

This investigation has also provided a novel interaction between MARCKS and hCLCA1 on the mucin granule membrane. The function of this interaction is still elusive.

These data provide a generous amount of potential targets for therapeutics for mucin hypersecretion in the respiratory and intestinal mucosa.

Future studies should focus on:

1) validating the associations of these proteins via a secondary analysis such as Western blot.
2) whether or not proteins identified are necessary for mucin secretion. One

can evaluate the effects of inhibitors, siRNA, and dominant negative constructs specific to these proteins for attenuation of mucin secretion.

3) whether or not the protein spots increased or decreased on the 2D gels in each cell type correlated to the proteins associated with the mucin granule membranes. Mass spectrometry followed by Western analysis verification may be assayed for these proteins.

4) Determining the function of the MARCKS/hCLCA1 interaction by creating blocking peptides to specific domains of both proteins.

The overall contribution of this study was generating a list of possible hypotheses to be evaluated. This study has provided novel findings for protein-protein interaction.

COMBINED REFERENCES

Altznauer F, Conus S, Cavalli A, Folkers G, Simon HU. Calpain-1 regulates Bax and subsequent Smac-dependent caspase-3 activation in neutrophil apoptosis. J Biol Chem. 2004 Feb 13;279(7):5947-57.

Arbuzova A, Murray D, McLaughlin S. MARCKS, membranes, and calmodulin: kinetics of their interaction. Biochim Biophys Acta. 1998 Nov 10;1376(3):369-79. Review.

Arbuzova A, Schmitz AA, Vergères G. Cross-talk unfolded: MARCKS proteins. Biochem J. 2002 Feb 15;362(Pt 1):1-12. Review.

Barnes PJ. Cytokine modulators as novel therapies for asthma. Annu Rev Pharmacol Toxicol. 2002; 42:81-98. Review.

Birkenfeld J, Kartmann B, Betz H, Roth D. Cofilin activation during Ca(2+)-triggered secretion from adrenal chromaffin cells. Biochem Biophys Res Commun. 2001 Aug 24;286(3):493-8.

Black JL, Roth M, Lee J, Carlin S, Johnson PR.Mechanisms of airway remodeling. Airway smooth muscle. Am J Respir Crit Care Med. 2001 Nov 15;164(10 Pt 2):S63-6.

Boal F, Le Pevelen S, Cziepluch C, Scotti P, Lang J. Cysteine-string protein isoform beta (Cspbeta) is targeted to the trans-Golgi network as a non-palmitoylated CSP in clonal beta-cells. Biochim Biophys Acta. 2007 Feb;1773(2):109-19.

Brunner Y, Couté Y, Iezzi M, Foti M, Fukuda M, Hochstrasser DF, Wollheim CB, Sanchez JC. Proteomics analysis of insulin secretory granules. Mol Cell Proteomics. 2007 Jun;6(6):1007-17.

Burgoyne RD, Geisow MJ. The annexin family of calcium-binding proteins. Cell Calcium. 1989 Jan;10(1):1-10.

Burgoyne RD, Morgan A. Secretory granule exocytosis. Physiol Rev. 2003 Apr;83(2):581-632. Review.

Candiano G, Bruschi M, Pedemonte N, Caci E, Liberatori S, Bini L, Pellegrini C, Viganò M, O'Connor BJ, Lee TH, Galietta LJ, Zegarra-Moran O. Gelsolin secretion in interleukin-4-treated bronchial epithelia and in asthmatic airways. Am J Respir Crit Care Med. 2005 Nov 1;172(9):1090-6.

Chamberlain LH, Burgoyne RD. Activation of the ATPase activity of heat-shock proteins Hsc70/Hsp70 by cysteine-string protein. Biochem J. 1997 Mar 15;322 (Pt 3):853-8.

Chamberlain LH, Burgoyne RD. The cysteine-string domain of the secretory vesicle cysteine-string protein is required for membrane targeting. Biochem J. 1998 Oct 15;335 (Pt 2):205-9.

Chen YA, Scheller RH. SNARE-mediated membrane fusion. Nat Rev Mol Cell Biol. 2001 Feb;2(2):98-106. Review.

Ciborowski P, Kadiu I, Rozek W, Smith L, Bernhardt K, Fladseth M, Ricardo-Dukelow M, Gendelman HE. Investigating the human immunodeficiency virus type 1-infected monocyte-derived macrophage secretome. Virology. 2007 Jun 20;363(1):198-209.

Clarke PR, Siddhanti SR, Cohen P, Blackshear PJ. Okadaic acid-sensitive protein phosphatases dephosphorylate MARCKS, a major protein kinase C substrate. FEBS Lett. 1993 Dec 20;336(1):37-42.

Danks K, Wade JA, Batten TF, Walker JH, Ball SG, Vaughan PF. Redistribution of F-actin and large dense-cored vesicles in the human neuroblastoma SH-SY5Y in response to secretagogues and protein kinase Calpha activation. Brain Res Mol Brain Res. 1999 Feb 5;64(2):236-45.

de Graauw M, Tijdens I, Smeets MB, Hensbergen PJ, Deelder AM, van de Water B. Annexin A2 phosphorylation mediates cell scattering and branching morphogenesis via cofilin activation. Mol Cell Biol. 2007 Dec 10.

Desnos C, Huet S, Fanget I, Chapuis C, Böttiger C, Racine V, Sibarita JB, Henry JP, Darchen F. Myosin Va mediates docking of secretory granules at the plasma membrane. J Neurosci. 2007 Sep 26;27(39):10636-45.

Eliyahu E, Shtraizent N, Tsaadon A, Shalgi R. Association between myristoylated alanin-rich C kinase substrate (MARCKS) translocation and cortical granule exocytosis in rat eggs. Reproduction. 2006 Feb;131(2):221-31.

Evans GJ, Morgan A, Burgoyne RD. Tying everything together: the multiple roles of cysteine string protein (CSP) in regulated exocytosis. Traffic. 2003 Oct;4(10):653-9. Review.

Evans GJ, Wilkinson MC, Graham ME, Turner KM, Chamberlain LH, Burgoyne RD, Morgan A. Phosphorylation of cysteine string protein by protein kinase A. Implications for the modulation of exocytosis. J Biol Chem. 2001 Dec 21;276(51):47877-85.

Evans MJ, Plopper CG. The role of basal cells in adhesion of columnar epithelium to airway basement membrane. Am. Rev. Respir. Dis. 1988; 138: 481–3.

Fahy JV. Remodeling of the airway epithelium in asthma. Am J Respir Crit Care Med. 2001 Nov 15;164(10 Pt 2):S46-51. Review.

Gibson A, Lewis AP, Affleck K, Aitken AJ, Meldrum E, Thompson N. hCLCA1 and mCLCA3 are secreted non-integral membrane proteins and therefore are not ion channels. J Biol Chem. 2005 Jul 22;280(29):27205-12.

Groneberg DA, Eynott PR, Oates T, Lim S, Wu R, Carlstedt I, Nicholson AG, Chung KF. Expression of MUC5AC and MUC5B mucins in normal and cystic fibrosis lung. Respir Med. 2002 Feb;96(2):81-6.

Guo Y, Singleton PA, Rowshan A, Gucek M, Cole RN, Graham DR, Van Eyk JE, Garcia JG. Quantitative proteomics analysis of human endothelial cell membrane rafts: evidence of MARCKS and MRP regulation in the sphingosine 1-phosphate-induced barrier enhancement. Mol Cell Proteomics. 2007 Apr;6(4):689-96.

Hartwig JH, Thelen M, Rosen A, Janmey PA, Nairn AC, Aderem A. MARCKS is an actin filament crosslinking protein regulated by protein kinase C and calcium-calmodulin. Nature. 1992 Apr 16;356(6370):618-22.

Hauber HP, Tsicopoulos A, Wallaert B, Griffin S, McElvaney NG, Daigneault P, Mueller Z, Olivenstein R, Holroyd KJ, Levitt RC, Hamid Q. Expression of HCLCA1 in cystic fibrosis lungs is associated with mucus overproduction. Eur Respir J. 2004 Jun;23(6):846-50.

Hegab AE, Sakamoto T, Nomura A, Ishii Y, Morishima Y, Iizuka T, Kiwamoto T, Matsuno Y, Homma S, Sekizawa K. Niflumic acid and AG-1478 reduce cigarette smoke-induced mucin synthesis: the role of hCLCA1. Chest. 2007 Apr;131(4):1149-5.

Holst J, Sim AT, Ludowyke RI. Protein phosphatases 1 and 2A transiently associate with myosin during the peak rate of secretion from mast cells. Mol Biol Cell. 2002 Mar;13(3):1083-98.

Houston JC, De Navasquez S, Trounce JR. A clinical and pathological study of fatal cases of status asthmaticus. Thorax 1953; 8: 207-213.

Jethwaney D, Islam MR, Leidal KG, de Bernabe DB, Campbell KP, Nauseef WM, Gibson BW. Proteomic analysis of plasma membrane and secretory vesicles from human neutrophils. Proteome Sci. 2007 Aug 10;5:12.

Kanamori T, Takakura K, Mandai M, Kariya M, Fukuhara K, Sakaguchi M, Huh NH, Saito K, Sakurai T, Fujita J, Fujii S. Increased expression of calcium-binding protein S100 in human uterine smooth muscle tumours. Mol Hum Reprod. 2004 Oct;10(10):735-42.

Khvotchev M, Dulubova I, Sun J, Dai H, Rizo J, Südhof TC. Dual modes of Munc18-1/SNARE interactions are coupled by functionally critical binding to syntaxin-1 N terminus. J Neurosci. 2007 Nov 7;27(45):12147-55.

Kim KC, McCracken K, Lee BC, Shin CY, Jo MJ, Lee CJ, Ko KH.Airway goblet cell mucin: its structure and regulation of secretion. Eur Respir J. 1997 Nov;10(11):2644-9. Review.

Knight DA, Holgate ST. The airway epithelium: structural and functional properties in health and disease. Respirology. 2003 Dec;8(4):432-46. Review.

Krunkosky TM, Fischer BM, Martin LD, Jones N, Akley NJ, Adler KB. 2000 Effects of TNF-alpha on expression of ICAM-1 in human airway epithelial cells in vitro. Signaling pathways controlling surface and gene expression. Am. J. Respir. Cell Mol. Biol. 22: 685-692.

Kuperman DA, Huang X, Koth LL, Chang GH, Dolganov GM, Zhu Z, Elias JA, Sheppard D, Erle DJ. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. Nat Med. 2002 Aug;8(8):885-9.

Langford GM. Myosin-V, a versatile motor for short-range vesicle transport. Traffic. 2002 Dec;3(12):859-65.

Lapierre L, Kumar R, Hales C, Navarre J, Bhartur S, Burnette J, Provance D, Mercer J, Bahler M, Goldenring J. Myosin vb is associated with plasma membrane recycling systems. Mol. Biol. Cell. 2001;12:1843–1857.

Leikauf GD, Borchers MT, Prows DR, Simpson LG. Mucin apoprotein expression in COPD. Chest. 2002 May;121(5 Suppl):166S-182S. Review.

Leverkoehne I, Gruber AD. The murine mCLCA3 (alias gob-5) protein is located in the mucin granule membranes of intestinal, respiratory, and uterine goblet cells. J Histochem Cytochem. 2002 Jun;50(6):829-38.

Li Y, Martin LD, Spizz G, Adler KB. MARCKS protein is a key molecule regulating mucin secretion by human airway epithelial cells in vitro. J Biol Chem. 2001 Nov 2;276(44):40982-90.

Lin H, Carlson DM, St George JA, Plopper CG, Wu R. An ELISA method for the quantitation of tracheal mucins from human and nonhuman primates. Am J Respir Cell Mol Biol. 1989 Jul;1(1):41-8.

Mundhenk L, Alfalah M, Elble RC, Pauli BU, Naim HY, Gruber AD. Both cleavage products of the mCLCA3 protein are secreted soluble proteins. J Biol Chem. 2006 Oct 6;281(40):30072-80.

Nagase T, Koga H, Ohara O. Kazusa mammalian cDNA resources: towards functional characterization of KIAA gene products. Brief Funct Genomic Proteomic. 2006 Mar;5(1):4-7.

Nakanishi A, Morita S, Iwashita H, Sagiya Y, Ashida Y, Shirafuji H, Fujisawa Y, Nishimura O, Fujino M. Role of gob-5 in mucus overproduction and airway hyperresponsiveness in asthma. Proc Natl Acad Sci U S A. 2001 Apr 24;98(9):5175-80.

Oppenshaw PJM, Turner-Warwick M. Observations on sputum production in patients with variable airflow obstruction; implications for the diagnosis of asthma and chronic bronchitis. Respir Med 1989; 83: 25-31.

Park J, Fang S, Adler KB. Regulation of airway mucin secretion by MARCKS protein involves the chaperones heat shock protein 70 and cysteine string protein. Proc Am Thorac Soc. 2006 Aug;3(6):493.

Park JA, Crews AL, Lampe WR, Fang S, Park J, Adler KB. Protein kinase C delta regulates airway mucin secretion via phosphorylation of MARCKS protein. Am J Pathol. 2007 Dec;171(6):1822-30.

Park JA, Crews AL, Lampe WR, Fang S, Park J, Adler KB. Protein Kinase C{delta} Regulates Airway Mucin Secretion via Phosphorylation of MARCKS Protein. Am J Pathol. 2007 Nov 30.

Park JA, He F, Martin LD, Li Y, Chorley BN, Adler KB. Human neutrophil elastase induces hypersecretion of mucin from well-differentiated human bronchial epithelial cells in vitro via a protein kinase C{delta}-mediated mechanism. Am J Pathol. 2005 Sep;167(3):651-61.

Park KS, Wells JM, Zorn AM, Wert SE, Laubach VE, Fernandez LG, Whitsett JA. Transdifferentiation of ciliated cells during repair of the respiratory epithelium. Am J Respir Cell Mol Biol. 2006 Feb;34(2):151-7.

Parnaud G, Hammar E, Rouiller DG, Bosco D. Inhibition of calpain blocks pancreatic beta-cell spreading and insulin secretion. Am J Physiol Endocrinol Metab. 2005 Aug;289(2):E313-21.

Pauli BU, Abdel-Ghany M, Cheng HC, Gruber AD, Archibald HA, Elble RC. Molecular characteristics and functional diversity of CLCA family members. Clin Exp Pharmacol Physiol. 2000 Nov;27(11):901-5. Review.

Peterman SM, Dufresne CP, Horning S. The use of a hybrid linear trap/FT-ICR mass spectrometer for on-line high resolution/high mass accuracy bottom-up sequencing. J Biomol Tech. 2005 Jun;16(2):112-24.

Phillips TE, Ramos R, Duncan SL.1995. HT29-18N2 differentiation in a protein-free medium. In Vitro Cell Dev Biol Anim. 31:421-3.

Pilon M, Schekman R. Protein translocation: how Hsp70 pulls it off. Cell. 1999 Jun 11;97(6):679-82. Review.

Porumb T, Crivici A, Blackshear PJ, Ikura M. Calcium binding and conformational properties of calmodulin complexed with peptides derived from myristoylated alanine-rich C kinase substrate (MARCKS) and MARCKS-related protein (MRP). Eur Biophys J. 1997;25(4):239-47.

Puchelle E, Bajolet O, Abély M. Airway mucus in cystic fibrosis. Paediatr Respir Rev. 2002 Jun;3(2):115-9.

Puchelle E, Zahm JM, Tournier JM, Coraux C. Airway epithelial repair, regeneration, and remodeling after injury in chronic obstructive pulmonary disease. Proc Am Thorac Soc. 2006 Nov;3(8):726-33. Review.

Reck-Peterson SL, Provance DW Jr, Mooseker MS, Mercer JA. Class V myosins. Biochim Biophys Acta. 2000 Mar 17;1496(1):36-51.

Ren Q, Barber HK, Crawford GL, Karim ZA, Zhao C, Choi W, Wang CC, Hong W, Whiteheart SW. Endobrevin/VAMP-8 is the primary v-SNARE for the platelet release reaction. Mol Biol Cell. 2007 Jan;18(1):24-33.

Robichaud A, Tuck SA, Kargman S, Tam J, Wong E, Abramovitz M, Mortimer JR, Burston HE, Masson P, Hirota J, Slipetz D, Kennedy B, O'Neill G, Xanthoudakis S. Gob-5 is not essential for mucus overproduction in preclinical murine models of allergic asthma. Am J Respir Cell Mol Biol. 2005 Sep;33(3):303-14.

Rogers DF. Physiology of airway mucus secretion and pathophysiology of hypersecretion. Respir Care. 2007 . Sep;52(9):1134-46; discussion 1146-9. Review.

Rose MC, Voynow JA. Respiratory tract mucin genes and mucin glycoproteins in health and disease. Physiol Rev. 2006 Jan;86(1):245-78. Review.

Rose MC. Mucins: structure, function, and role in pulmonary diseases. Am J Physiol. 1992 Oct;263(4 Pt 1):L413-29. Review.

Rossi AH, Salmon WC, Chua M, Davis CW. Calcium signaling in human airway goblet cells following purinergic activation. Am J Physiol Lung Cell Mol Physiol. 2007 Jan;292(1):L92-8.

Sakisaka T, Meerlo T, Matteson J, Plutner H, Balch WE. Rab-αGDI activity is regulated by a Hsp90 chaperone complex. EMBO J 2002;21: 6125–6135.

Seykora JT, Myat MM, Allen LA, Ravetch JV, Aderem A. Molecular determinants of the myristoyl-electrostatic switch of MARCKS. J Biol Chem. 1996 Aug 2;271(31):18797-802.

Singer M, Martin LD, Vargaftig BB, Park J, Gruber AD, Li Y, Adler KB. A MARCKS-related peptide blocks mucus hypersecretion in a mouse model of asthma. Nat Med. 2004 Feb;10(2):193-6.

Smith GB, Umbach JA, Hirano A, Gundersen CB.Interaction between constitutively expressed heat shock protein, Hsc 70, and cysteine string protein is important for cortical granule exocytosis in Xenopus oocytes. J Biol Chem. 2005 Sep 23;280(38):32669-75.

Söllner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, Rothman JE. SNAP receptors implicated in vesicle targeting and fusion. Nature. 1993 Mar 25;362(6418):318-24.

Spina D. Epithelium smooth muscle regulation and interactions. Am. J. Respir. Crit. Care Med. 1998; 158: S141–5.

Stahl B, Tobaben S, Südhof TC. Two distinct domains in hsc70 are essential for the interaction with the synaptic vesicle cysteine string protein. Eur J Cell Biol. 1999 Jun;78(6):375-81.

Sun HQ, Yamamoto M, Mejillano M, Yin HL. Gelsolin, a multifunctional Actin regulatory protein. J Biol Chem. 1999 Nov 19;274(47):33179-82. Review.

Thelen M, Rosen A, Nairn AC, Aderem A. Regulation by phosphorylation of reversible association of a myristoylated protein kinase C substrate with the plasma membrane. Nature. 1991 May 23;351(6324):320-2.

Vääräniemi J, Palovuori R, Lehto VP, Eskelinen S. Translocation of MARCKS and reorganization of the cytoskeleton by PMA correlates with the ion selectivity, the confluence, and transformation state of kidney epithelial cell lines. J Cell Physiol. 1999 Oct;181(1):83-95.

Vasconcellos CA, Allen PG, Whol ME, Drazen JM, Janmeuy PA, Stossel TP. Reduction in viscosity of cystic fibrosis sputum in vitro by gelsolin. Science 1994(263):969–971.

Vaughn CP, Crockett DK, Lim MS, Elenitoba-Johnson KS. Analytical characteristics of cleavable isotope-coded affinity tag-LC-tandem mass spectrometry for quantitative proteomic studies. J Mol Diagn. 2006 Sep;8(4):513-20.

Verdugo P. Goblet cells secretion and mucogenesis. Annu Rev Physiol. 1990;52:157-76. Review.

Wang K, Wen FQ, Feng YL, Ou XM, Xu D, Yang J, Deng ZP. Increased expression of human calcium-activated chloride channel 1 gene is correlated with mucus overproduction in Chinese asthmatic airway. Cell Biol Int. 2007 Nov;31(11):1388-95.

Williams OW, Sharafkhaneh A, Kim V, Dickey BF, Evans CM. Airway mucus: From production to secretion. Am J Respir Cell Mol Biol. 2006 May;34(5):527-36. Review.

Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, Donaldson DD. Interleukin-13: central mediator of allergic asthma. Science. 1998 Dec 18;282(5397):2258-61.

Wohnsland F, Schmitz AA, Steinmetz MO, Aebi U, Vergéres G. Interaction between actin and the effector peptide of MARCKS-related protein. Identification of functional amino acid segments. J Biol Chem. 2000 Jul 7;275(27):20873-9.

Wright DT, Fischer BM, Li C, Rochelle LG, Akley NJ, Adler KB. Oxidant stress stimulates mucin secretion and PLC in airway epithelium via a nitric oxide-dependent mechanism. Am J Physiol. 1996 Nov;271(5 Pt 1):L854-61.

Wu DY, Wu R, Chen Y, Tarasova N, Chang MM. PMA stimulates MUC5B gene expression through an Sp1-based mechanism in airway epithelial cells. Am J Respir Cell Mol Biol. 2007 Nov;37(5):589-97.

Wu TT, Castle JD. 1997. Evidence for colocalization and interaction between 37 and 39 kDa isoforms of secretory carrier membrane proteins (SCAMPs). J Cell Sci. 110: 1533-41.

Yang PC, Luh KT, Kuo SH, Wu CW. Immunocytochemistry and ELISA quantitation of mucin for diagnosis of malignant pleural effusions. Am Rev Respir Dis. 1992 Dec;146(6):1571-5.

Yeates LC, Powis G. The expression of the molecular chaperone calnexin is decreased in cancer cells grown as colonies compared to monolayer. Biochem Biophys Res Commun. 1997 Sep 8;238(1):66-70.

Zhang H, Kelley WL, Chamberlain LH, Burgoyne RD, Lang J. Mutational analysis of cysteine-string protein function in insulin exocytosis. J Cell Sci. 1999 May;112 (Pt 9):1345-51.

Zhang H, Peters KW, Sun F, Marino CR, Lang J, Burgoyne RD, Frizzell RA. Cysteine string protein interacts with and modulates the maturation of the cystic fibrosis transmembrane conductance regulator. J Biol Chem. 2002 Aug 9;277(32):28948-58.