MARCKS Regulation of Mucin Secretion by Airway Epithelium *in Vitro*

Interaction with Chaperones

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We have reported previously that myristoylated alanine-rich C kinase substrate (MARCKS) is a key regulatory molecule controlling mucin secretion by airway epithelial cells *in vitro* and *in vivo*. The results of those studies supported a mechanism whereby MARCKS, upon phosphorylation by protein kinase C (PKC), translocates from plasma membrane to cytoplasm, where its binding to membranes of intracellular mucin granules is a key component of the secretory pathway. It remains unknown how MARCKS is targeted to and/or preferentially attaches to mucin granule membranes. We hypothesized that the chaperone cysteine string protein (CSP) may play an important role in this process. CSP was shown to associate with membranes of intracellular mucin granules in well-differentiated normal human bronchial epithelial (NHBE) cells *in vitro*, as determined by ultrastructural immunohistochemistry and Western blotting of isolated granule membranes. CSP in these cells complexed with MARCKS, as shown by co-immunoprecipitation. Given reported associations between CSP and a second chaperone, heat shock protein 70 (HSP70), a role for HSP70 in the MARCKS-dependent secretory mechanism also was investigated. HSP70 appeared to form a trimeric complex with MARCKS and CSP associated with mucin granule membranes within airway epithelial cells. Transfection of the HBE1 human bronchial epithelial cell line with siRNAs targeting sequences of MARCKS, CSP, or HSP70 resulted, in each case, in significant knockdown of expression of these proteins and subsequent attenuation of mucin secretion. The results provide the first evidence that CSP and HSP70, and their interactions with MARCKS, are involved in mucin secretion.

Keywords: MARCKS; cysteine string protein; heat shock protein 70; airway; mucin

Mucin (the glycoprotein component of mucus) is an important defense mechanism in airways, protecting lungs from microbes, particulates, and other deleterious inhaled substances. In diseases such as chronic bronchitis, asthma, bronchiectasis, or cystic fibrosis, excessive production and secretion of mucin contribute to airway obstruction and related complications. Intracellular mechanisms controlling the process of mucin secretion in airway epithelial cells have not been fully elucidated.

In goblet cells of the respiratory epithelium, mucin is synthesized and stored in cytoplasmic membrane-bound granules. Upon appropriate stimulation, the granules translocate to the cell periphery, where the granule membranes fuse with the plasma membrane and the contents of the granules (mucin) are released into the airway lumen via an exocytotic process (1). In previous studies, we demonstrated that the myristoylated

CLINICAL RELEVANCE

This research reveals an important role for the chaperone protein, cysteine string protein, in mucin secretion and suggests possible new therapeutic targets for treatment of disease characterized by excess mucus secretion.

alanine-rich C kinase substrate (MARCKS) protein is a central molecule regulating mucin secretion by airway epithelium *in vitro* (2) and *in vivo* (3). However, precise mechanism(s), signal transduction pathways, and other proteins involved in this process remain undetermined.

Under normal, constitutive conditions, cellular MARCKS is attached to the inner (cytoplasmic) face of the plasma membrane. When phosphorylated by activated protein kinase C (PKC), MARCKS translocates from the plasma membrane to the cytoplasm (4, 5). In previous studies in airway secretory cells, we demonstrated that MARCKS, upon phosphorylation, translocates to the cytoplasm, and there binds to membranes of intracellular mucin granules (2, 3). Binding of MARCKS to these granule membranes appears to be a critical component of the mucin secretory pathway, as inhibition of binding correlated with decreased mucin secretion (3). What is not known, however, is how MARCKS, after translocating to the cytoplasm upon phosphorylation by PKC, (1) is specifically targeted to mucin granule membranes, and (2) binds to these membranes.

Intracellular targeting and specific binding characteristics often are controlled by interactions between the protein of interest and a class of proteins collectively called "chaperones." In this study, initial attention was focused on the chaperone cysteine string protein (CSP). CSP, a member of the DnaJ family of highly conserved co-chaperones, is involved in intracellular movement of vesicles and exocytosis in several cell and tissue types (6–8), and recently has been shown to be present in airway epithelium (9).

A potential role for CSP in control of mucin secretion by human airway epithelial cells *in vitro* was investigated. The results showed that within well-differentiated normal human bronchial epithelial (NHBE) cells, CSP is localized to mucin granule membranes, and that MARCKS and CSP form a complex on these membranes. Since CSP is known to interact specifically with a second chaperone, heat shock protein 70 (HSP70) (10, 11), we then looked at possible involvement of HSP70 in the secretory process. HSP70 formed a trimeric complex with MARCKS and CSP within NHBE cells associated with mucin granule membranes. In addition, siRNAs targeting MARCKS, HSP70, or CSP knocked down expression of these respective proteins in the HBE1 human bronchial epithelial cell line, and also significantly attenuated mucin secretion when these cells were stimulated with the PKC-activating agent, phorbol 12-myristate 13-acetate (PMA). The results support an overall mechanism of airway mucin secretion, described previously (2, 3), whereby HSP70 binds to phosphorylated

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MARCKS in the cytoplasm and targets it to intracellularly stored mucin granules, where the MARCKS–HSP70 complex binds to CSP that is constitutively present on granule membranes. Thus, HSP70, CSP, and MARCKS appear to play integral and interconnected roles in the mucin secretion mechanism in airway epithelial cells.

MATERIALS AND METHODS

Cell Culture

NHBE cells. Expansion, cryopreservation, and culture of NHBE cells in air–liquid interface were performed as described previously (12). Briefly, NHBE cells (Cambrex, San Diego, CA) were seeded in vented T75 flasks (500 cells/cm²) and cultured until cells reached 85 to 90% confluence. Cells then were dissociated by trypsin/EDTA and frozen as passage-2. Air–liquid interface culture was initiated by seeding passage-2 cells (2×10^4 cells/cm²) in Transwell clear culture inserts (Costar, Cambridge, MA) thinly coated with rat tail collagen, type I (Collaborative Biomedical, Bedford, MA). Cells were cultured submerged in medium at 37°C in an atmosphere of 5% CO₂ for 5 to 7 days until nearly confluent. At that time, an air–liquid interface was created by removing the apical medium and feeding cells basolaterally. Medium was changed daily thereafter. Cells were cultured for an additional 14 days to allow full differentiation.

HBE1 cells. For studies involving transfection of cells with siRNAs, HBE 1 cells, a papilloma virus–transformed human bronchial epithelial cell line (13) capable of mucin secretion when cultured in air–liquid interface (2, 14) were used. Briefly, cells were grown submerged in serum-free Ham's F-12/DMEM medium (supplemented with 5 µg/ml insulin, 10 ng/ml epidermal growth factor, 0.1 µM dexamethasone, 5 µg/ml transferrin, 20 ng/ml cholera toxin, 50 ng/ml amphotericin B, 130 µg/ml bovine pituitary extract, 50 µg/ml gentamicin, and 22 U/ml nystatin). After the cells reached approximately 80% confluence, air–liquid interface culture was initiated as described above for NHBE cells. A day before transfection, cells were dissociated with Versene (Invitrogen, Carlsbad, CA) and re-seeded in 12-well culture plates at 1×10^5 cells/cm².

Measurement of Mucin Secretion by Enzyme-Linked Immunosorbent Assay

Before collection of “baseline” and “test” mucin samples, the accumulated mucus at the apical surface of the cells was removed by washing with phosphate-buffered saline (PBS), pH 7.2, containing 1 mM dithiothreitol (DTT). To collect the *baseline* secretion, cells were incubated with medium alone for 30 minutes, and secreted mucin in the apical medium was collected and reserved. Cells were rested for 24 hours and then exposed to medium containing the selected stimulatory and/or inhibitory reagents (or appropriate controls) for a 15- or 30-minute period, after which secreted mucin was collected and reserved as the *test* sample. Both baseline and test secretions were analyzed by double sandwich enzyme-linked immunosorbent assay (ELISA) using the pan-mucin antibody 17Q2 (1:1,000 dilution; Covance, Berkeley, CA) as the primary antibody (15). The ratio of test/baseline was used to quantify mucin secretion, allowing each culture dish to serve as its own control and thus minimizing deviation caused by variability among culture wells. Levels of mucin secretion were reported as percentage of the medium or solvent control as reported previously (2, 16).

Western Blotting

Total proteins were extracted from cells using a general lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; 1% NP-40; and protease inhibitors) and centrifuged at 14,000 rpm ($16,000 \times g$) for 10 minutes at 4°C. The supernatants were transferred to new tubes and mixed with 2× sample buffer and boiled for 5 minutes. After flash spinning, the samples were loaded into wells and proteins transferred to a nitrocellulose membrane (0.45 µM; Bio-Rad, Hercules, CA) using a semi-dry electrophoretic transfer cell according to the manufacturer's instructions. After transfer, the membrane was rinsed with PBS and blocked in 3% nonfat dry milk for 1 hour at room temperature. The membrane was then incubated with the primary antibody diluted in PBS/0.05% Tween 20 (PBST) with 1% bovine serum albumin (BSA)

overnight at 4°C. After washing with PBST, the membrane was incubated with secondary antibody diluted in PBST with 1% BSA for 1 hour at room temperature. After washing three times with PBST, proteins on the membrane were detected using an Amersham ECL kit followed by exposure to Hyperfilm (Amersham Bioscience, Buckinghamshire, UK). The optical density of protein bands from Western blotting was analyzed by Lab Works software (Upland, CA).

Co-Immunoprecipitation

Immunoprecipitation was performed using Dynal beads coated with protein A according to the manufacturer's instructions (Dynal, Great Neck, NY). Total protein was extracted from cells using an immunoprecipitation lysis buffer specifically designed to maintain protein–protein interactions (20 mM sodium phosphate, pH 7.5; 500 mM NaCl; 0.1% SDS; 1% NP-40; and protease inhibitors). Proteins were diluted to approximately 1 mg/ml using PBS, and 5 to 10 µl of antibody added to approximately 1 ml cell lysate. The sample was incubated overnight at 4°C with gentle shaking, and an appropriate amount of Dynal beads coated with protein A added to the antigen–antibody complex (~50 µl of gel per 5 µg of antibody). The sample was incubated with gentle mixing for 2 hours at room temperature, and the immobilized protein A–bound complexes washed three times with 0.5 ml of the lysis buffer. Fifty microliters of 2× Laemmli sample buffer was added before boiling for 5 minutes. The sample was then centrifuged, the supernatant collected, and SDS-PAGE and immunoblot analysis performed. Beads were collected magnetically.

Intracellular Mucin Granule Isolation from NHBE Cells

Mucin granules were isolated from NHBE cells as reported previously (3, 17). Briefly, NHBE cells were grown in the presence of 10 ng/ml IL-13 to increase the numbers of goblet cells in the cultures and the amount of mucin granules in the cells (18). IL-13 was added basolaterally after the cells had been confluent for 7 days. Cells were collected by physical scraping with 0.3 M sucrose, 5 mM 3-N-morpholinopropane-sulfonic acid (MOPS), 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.2 µg/ml diphenyl-p-phenylenediamine (DPPD), lysed via brief sonication, and centrifuged at $600 \times g$ for 10 minutes. Post-nuclear supernatants (PNS) were diluted with 1.9 vols of 86% Percoll, 0.3 M sucrose, 5 mM MOPS, 1 mM EDTA, and 0.2 µg/ml DPPD, pH 6.8, and then centrifuged at $17,000 \times g$ for 30 minutes. The crude granules were collected from the bottom of the self-formed gradient, diluted with three volumes of 0.3 M sucrose containing 2 mM MOPS, 1 mM EDTA, and 0.2 µg/ml DPPD, and pelleted at $2,000 \times g$ for 15 minutes. The crude granules then were incubated with a custom-produced antibody to the murine gob-5 antigen: ESWKAKPEYTRPKLE (Covance, Denver, PA) overnight at 4°C. After incubation, the antibody–granule complex was applied to Dynal beads coated with protein A. The beads were collected magnetically and washed three times with PBS. Fifty microliters of 2× Laemmli sample buffer was added before boiling for 5 minutes. The sample was then centrifuged, the supernatant collected, and SDS-PAGE and immunoblot analysis performed. Mucin granule isolation was performed using NHBE cells only, as HBE1 cells have few if any apparent mucin granules when viewed microscopically.

Light-Microscopic Immunohistochemistry

To investigate the presence of CSP in goblet cells of the airways *in vivo*, we used sections of lungs derived from mice exposed to human neutrophil elastase (NE), as this procedure induces a goblet cell hyperplasia in murine airways, as opposed to normal mouse airways that contain few if any goblet cells (19). Lungs from male BALB/c mice exposed to NE or PBS (control) via oropharyngeal aspiration (19) were provided by Dr. Bernard Fischer, Duke University Medical Center, Durham, NC. The lungs were washed and inflated with optimum cutting temperature (OCT) medium (Sakura Finetek, Torrance, CA), then fixed in 10% phosphate-buffered formalin overnight at 4°C, and processed to paraffin wax. Before immunolabeling, 5-µm-thick sections were deparaffinized, rehydrated, quenched with H₂O₂, heat-treated by microwaving, and then blocked for 1 hour in PBS containing 10% (vol/vol) normal goat serum. Sections were then washed four times in PBS and incubated for 1 hour at room temperature with the primary anti-CSP antibody (1:1,000 dilution) in PBS plus 0.1% BSA. Samples were

again washed four times and incubated with secondary goat anti-rabbit antibody (1:3,000 dilution; Cell Signaling, Danvers, MA) conjugated with horseradish peroxidase in PBS plus 0.1% BSA. Samples were washed again and the color developed by Dako DAB Chromogen (Dako, Carpinteria, CA). Samples were washed and counterstained with Modified Harris' Hematoxylin (VWR, West Chester, PA) for 1 minute, rinsed in tap water, dehydrated, mounted with permount (Fisher, Pittsburgh, PA), and coverslipped. Control experiments, in which the primary antibody was replaced with chrompure rabbit IgG, were routinely performed to verify specificity of the labeling. Alcian blue and Periodic acid-Schiff staining was used to identify mucus-containing cells within the lung sections.

Ultrastructural Immunohistochemistry

Well-differentiated NHBE cells maintained in air-liquid interface culture were fixed in 4% formaldehyde + 0.01% glutaraldehyde in freshly-made 0.1 M Phosphate buffer, pH 7.2 to 7.4 for 1 hour at room temperature. Cells on membranes were embedded in molten 3 to 4% water agar and solidified. The agar-embedded cells were dehydrated and embedded in LR White resin (EM science, Gibbstown, NJ). Ultrathin (80–90 nm) sections were cut and placed on stainless steel grids. The grids were blocked with 0.1 M PBS, pH 7.2 to 7.4 containing 10% FBS for 15 minutes at room temperature and incubated with an anti-CSP polyclonal antibody (1:250 dilution; Chemicon, Temecula, CA) in PBS containing 0.5% BSA at 4°C overnight. After a buffer wash, the grids were incubated with 12 nm gold-labeled secondary antibody (1:50 dilution; Jackson ImmunoResearch, West Grove, PA) in PBS containing 0.5% BSA for 2 hours at room temperature. After incubation, the grids were washed in the same phosphate buffer without BSA for 45 minutes, post-stained with uranyl acetate, dried, and examined in a Philips FEI 208S transmission electron microscope. Chrompure rabbit IgG (Jackson ImmunoResearch) was substituted for the primary antibody as a negative control.

Construction of Plasmid Vectors for siRNAs Targeting MARCKS, HSP70, and CSP

The mammalian expression vector, pSUPER.neo (OligoEngine, Seattle, WA) was used for expression of siRNA for MARCKS and HSP70 in HBE1 cells. The gene-specific insert specifies the 19-bp sequence: 5'-AGCGAACGGACAGGAGAAT-3', corresponding to nucleotides 398 to 416 in human MARCKS (GenBank accession # M68956) mRNA. For HSP70, the 19-bp sequence: 5'-GGTGGAGATCATCGC CAAC-3', corresponding to nucleotides 563 to 581 in human HSP70 (GenBank accession # M11717) mRNA was constructed. An irrelevant (control) siRNA was constructed using a 19-nucleotide sequence (AA TTCTCCGAACGTGTCACGT) with no significant homology to any mammalian gene sequence. These sequences were inserted into the pSUPER.neo after digestion with BglII and HindIII and transformed into XL1-blue supercompetent cells (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Several clones were obtained. Insertion of the siRNA sequences were confirmed by digestion with EcoRI and HindIII and DNA sequencing.

For CSP, using web-based software (Ambion, Austin, TX) we selected the target position start at 242, sequence 5'-GAACGCA ACCTCAGATGAC-3' of CSP (GenBank accession #BC053642). The double-stranded, annealed siRNAs were chemically synthesized using a commercial source (Ambion) and used similarly to the pSuper constructs described above.

siRNA Transfections for Mucin Secretion Studies

HBE1 cells grown in air/liquid interface culture were dissociated with Versene and re-seeded in 12-well plates at 10,000 cells/well. After 24 hours, cells were transfected with double-stranded siRNAs targeting either MARCKS, HSP70, or CSP according to the manufacturer's instructions using FuGene 6 reagent (Roche, Indianapolis, IN). Briefly, a range of concentrations (upper limits of 200 nM or 2 µg) of the siRNA was mixed with FuGene 6 in a total volume of 50 µl serum-free medium. After 15 minutes of incubation, the siRNA/Fugene mix was added to the cells. Cells were cultured for 48 or 72 hours to allow protein expression and then exposed to 500 nM PMA for 30 minutes, and mucin secretion then measured by ELISA. The cells then were harvested and lysed, and protein expression analyzed via Western blotting.

Cytotoxicity Assay

All reagents used in these studies were examined for cytotoxicity by measuring the total release of lactate dehydrogenase from cells. The assays were performed using a Promega Cytotox 96 Kit (Madison, WI) according to the manufacturer's instructions. All experiments were performed with reagents at noncytotoxic concentrations.

Statistical Analysis

Data were analyzed for significance using one-way ANOVA with Bonferroni post-test corrections. Differences between treatments were considered significant at $P < 0.05$.

Figure 1. Airway epithelial cells contain cysteine string protein (CSP). Lungs from mice exposed to neutrophil elastase (NE; A, C, and E) or PBS (control; B, D, and F) were fixed in formalin, embedded in paraffin, sectioned, and mounted on slides. Sections were stained with Alcian blue/PAS (A, B) or incubated with either primary anti-CSP antibody (C, D) or chrompure rabbit IgG (control) followed by secondary goat anti-rabbit antiserum (E, F). Immunoperoxidase labeling shows discrete staining for CSP in the epithelium of the large airways in the elastase-treated mice, apparently in goblet cells, as staining in PBS-exposed control airways, which contain few if any goblet cells, was minimal. Original magnification: $\times 400$. (G) CSP protein is found in normal human bronchial epithelial (NHBE) cells. NHBE cell lysates were immunoblotted with an anti-CSP antibody, demonstrating the presence of CSP in these cells. The CSP in NHBE cells appears to be the CSP1 isoform (molecular weight ~ 37 kD).

RESULTS

Airway Epithelial Cells Contain CSP1

Light microscopic immunohistochemistry. To determine if CSP is present in airway epithelial cells *in vivo*, lung tissues from mice exposed to NE to induce goblet cell hyperplasia were examined via immunohistochemistry. Immunoperoxidase labeling demonstrated immunoreactivity for CSP in epithelial cells lining the larger airways of these mice, but did not appear present in airways of control mice not exposed to elastase, which lack true goblet cells (Figures 1A–1F).

Western blot. To determine whether or not NHBE cells express CSP protein, whole cell lysates were analyzed by immunoblot. CSP was detected in the lysates (Figure 1G). In epithelial cells, CSP is known to consist of at least two isoforms, depending on the makeup of the C-terminal amino acids: CSP1 (33–37 kD; sometimes referred to as CSP α), which is the most abundant, and CSP2 (26–28 kD) (20, 21). NHBE cells appeared to contain the CSP1 isoform based on molecular weight (~ 37 kD) as determined by SDS-PAGE.

CSP Is Associated with Membranes of Cytoplasmic Mucin Granules in NHBE Cells

After determining that CSP is present in airway epithelial cells, we then sought to determine its intracellular localization:

Ultrastructural immunohistochemistry. To examine the subcellular distribution of CSP in airway epithelial cells, more specifically secretory cells, well-differentiated NHBE cells were examined by transmission electron microscopy (TEM). TEM examination showed CSP associated with mucin granules in these cells (Figures 2A and 2B).

Western blot of isolated granule membranes. Isolated mucin granule membranes from NHBE cells were prepared as described in MATERIALS AND METHODS and examined by Western blot for the presence of CSP. As illustrated in Figure 2C, CSP (molecular weight ~ 37 kD) appeared associated with mucin granule membranes. hCLCA1, the human ortholog of

murine gob-5 known to be associated with granule membranes in goblet cells (22), is shown at ~ 120 kD using a commercially available antibody purchased from Imgenex (San Diego, CA). A cleavage product of hCLCA1 is shown at approximately 85 kD (23). The dense band (~ 56 kD, lane C2) is from the secondary antibody interacting with the murine gob-5 antibody used for granule isolation.

siRNA Knockdown of CSP Attenuates Mucin Secretion in Airway Epithelial Cells

siRNA studies in HBE1 cells. Transfection of HBE1 cells with CSP siRNA resulted in a significant decrease in CSP expression ($\sim 80\%$ knockdown at 200nM concentration; Figure 3A) as well as attenuation of mucin secretion in response to PMA (Figure 3B). Treatment with PMA for 30 minutes did not affect expression of CSP in these cells. Transfection with an irrelevant siRNA or FuGene 6 reagent alone did not affect secretion (Figure 3B).

CSP Associates with MARCKS in Airway Epithelial Cells

As illustrated in Figure 4A, co-immunoprecipitation using an anti-MARCKS antibody for IP and an anti-CSP antibody for detection shows that CSP and MARCKS bind each other in NHBE cells.

CSP May Regulate secretion by Serving as a Binding Site for MARCKS on Mucin Granule Membranes

PMA exposure increases mucin secretion and CSP-MARCKS binding. Exposure of NHBE cells to the mucin-secretagogue PMA increased formation of the MARCKS–CSP complex by approximately 50%, as determined by co-immunoprecipitation (Figure 4B). Since PMA activation of PKC results in phosphorylation of MARCKS, increased association of phosphorylated MARCKS with mucin granule membranes (Figure 4C), and increased mucin secretion at this time point (2), the results indicate that CSP-MARCKS binding may be involved in the mucin secretion process.

Figure 2. CSP is localized to the membranes of intracellular mucin granules in NHBE cells. (A) Well-differentiated NHBE cells maintained in air–liquid interface culture were processed for ultrastructural immunohistochemistry as described. Thin sections placed on stainless steel grids were incubated with anti-CSP antibody followed by 12 nm gold-labeled secondary antibody. Gold-labeled CSP appears associated with mucin granule membranes (arrows). (B) Negative control for A using the same procedures except chrompure rabbit IgG was substituted for anti-CSP antibody. Nonspecific labeling is quite sparse. (C) Mucin granule membranes were isolated as described in MATERIALS AND METHODS and CSP was detected via immunoblotting. Lane 1: Human hCLCA1 is detected as an uncleaved protein at approximately 120 kD as well as a cleaved product at approximately 85 kD; this demonstrates that the preparation consists of granule membranes (3, 56). Lane 2: CSP is detected via immunoblot at approximately 37 kD. The large band at approximately 56 kD is due to cross reaction of the secondary antibody (donkey anti-rabbit) with the custom produced antibody to murine gob5 used in the isolation of the granule membranes (see MATERIALS AND METHODS).

Figure 3. CSP is involved in mucin secretion by airway epithelial cells. (A) Transfection of HBE1 cells with CSP siRNA down-regulates CSP expression. HBE1 cells were transfected with 200 nM of chemically synthesized double-stranded CSP siRNA or with FuGene 6 (CONT). Cells were cultured for 72 hours to allow expression, and then harvested, lysed, and protein expression analyzed via Western blot. A decrease of about 80% in expression of CSP protein in cells transfected with 200 nM of CSP siRNA is shown. Expression of CSP was not affected by transfection of vehicle alone or transfection of an irrelevant siRNA; transfection with 100 nM CSP siRNA caused a slight decrease in CSP expression (not shown). (B) HBE1 cells cultured for 72 hours after siRNA treatment were exposed to PMA (500 nM) for 30 minutes and mucin secretion measured by enzyme-linked immunosorbent assay (ELISA). Mucin secretion in response to PMA was significantly inhibited by treatment with 200 nM of CSP siRNA. None of the controls affected secretion. Bars 1 and 2: Not transfected (control); bar 3: Vehicle (FuGene-6; mock) alone; bar 4: irrelevant siRNA (200 nM); bar 5: CSP siRNA (100 nM); bar 6: CSP siRNA (200 nM). The control samples contained approximately 5 to 8 ng/ml of mucin.

MARCKS and HSP70 Interact in Airway Epithelial Cells

As shown in Figure 5, human airway epithelial cells express HSP70 protein. In addition, an antibody to MARCKS was able to co-immunoprecipitate HSP70 in HBE1 cell lysates (Figure 5B), suggesting an interaction between the two proteins. To verify that the interaction between MARCKS and HSP70 takes place on mucin granule membranes within airway epithelial cells, co-immunoprecipitation experiments were performed with granule membranes isolated from NHBE cells. Figure 5B illustrates that endogenous MARCKS/HSP70 complexes are immunoprecipitated from mucin granules with an antibody to MARCKS.

MARCKS, HSP70, and CSP Form a Trimeric Complex within Airway Epithelial Cells Associated with Mucin Granule Membranes

Western blot analysis and co-immunoprecipitation of NHBE cell lysates showed that MARCKS, HSP70, and CSP were bound to each other, appearing to form a trimeric complex within the cells (Figure 6A). The trimeric complex appeared associated with mucin granule membranes (Figure 6B).

Figure 4. CSP associates with mrlistolated alanine-rich C kinase substrate (MARCKS) in well-differentiated NHBE cells. (A) CSP complexes with MARCKS protein. Lane 1: CSP was immunoprecipitated from NHBE cell lysates with an anti-MARCKS antibody and immunoblotted with an anti-CSP antibody. Lane 2: Positive control for CSP expression using cell lysate (immunoblot only). (B) PMA (100 nM), a mucin secretagogue, increases association of CSP with MARCKS (~150% versus control) after 5 minutes of exposure. (C). Phosphorylated MARCKS (p-MARCKS) complexes with mucin granule membranes. Mucin granule membranes were isolated as described in MATERIALS AND METHODS, and immunoblots analyzed with anti-phospho-MARCKS and anti-CSP antibodies. Association of phosphorylated MARCKS with mucin granule membranes increased after treatment with PMA (100 nM, 5 min). CSP association with the granule membrane was not affected by PMA during this time period.

siRNA Knockdown of MARCKS or HSP70 Attenuates Mucin Secretion

To further assess the role of MARCKS and HSP70 in mucin secretion, siRNA constructs against each of these proteins were transfected into HBE1 cells as described in MATERIALS AND

Figure 5. HSP70 is present in cultured human airway epithelial cells, and it interacts with MARCKS. (A) Lane 1: HBE1 cell lysates were immunoblotted with an anti-HSP70 antibody confirming the presence of HSP70. Lane 2: HBE1 cell lysates were immunoprecipitated with an anti-MARCKS antibody, and then immunoblotted with an anti-HSP70 antibody. HSP70 and MARCKS appear to interact in these cells. (B) Well-differentiated NHBE cells maintained in air-liquid interface culture for 2 weeks were lysed and subjected to mucin granule isolation. Granules were immunoprecipitated with an anti-MARCKS antibody, and then immunoblotted with an anti-HSP70 antibody. HSP70 appears to interact with MARCKS in association with mucin granule membranes in NHBE cells.

METHODS. For siRNA studies related to MARCKS, the siRNA construct targeted to MARCKS knocked down expression of MARCKS 48 hours after transfection into HBE1 cells by about 70%, and also attenuated mucin secretion in response to PMA (Figures 7A and 7B). For the HSP70 studies, 2 μ g of the siRNA construct described in MATERIALS AND METHODS knocked down expression of HSP70 in the cells by approximately 55% after 48 hours, and attenuated mucin secretion in response to PMA (Figures 7C and 7D). Exposure of the cells to PMA for 30 minutes did not affect expression of MARCKS or HSP70 protein (data not shown).

In these studies, the effects of the siRNA construct on different HSP70 isoforms were also tested. One well of cells was subjected to RNA extraction and examined by qPCR using different HSP70 isoform primers. The siRNA construct described in MATERIALS AND METHODS above suppressed expression of HspA1A (GenBank accession # NM005345) and HspA1L (GenBank accession # NM005527) by about 50%, and HspA1B (GenBank accession # NM005346) by about 60%. The construct suppressed expression of HspA2 (GenBank accession # NM021979) only slightly (~17%), and had no effect on expression of the HspA5 isoform (GenBank accession# NM005347).

DISCUSSION

Secretion of mucin by airway goblet cells is a regulated exocytotic event involving movement of preformed mucin granules from cytoplasm to cell periphery, fusion of granule membranes with the plasmalemma, and release of the granule contents into the airway lumen. Our previous studies have demonstrated direct involvement of MARCKS in mucin secretion by NHBE cells *in vitro* (2) and in murine airways *in vivo* (3), as a peptide corresponding to the N-terminus of MARCKS (MANS peptide) attenuated mucin secretion in both of these systems. MARCKS has been related to secretion and regulated exo-

Figure 6. Three proteins—MARCKS, HSP70, and CSP—appear to form a trimeric complex in well-differentiated NHBE cells. (A) Lane 1: NHBE cell lysates were immunoprecipitated with an anti-MARCKS antibody and immunoblotted with an anti-HSP70 antibody (~70 kD). Lane 2: NHBE lysates were immunoprecipitated with an anti-HSP70 antibody and immunoblotted with an anti-MARCKS antibody (~90 kD). Both blots were cut around 56 kD to avoid interference from the heavy chain reaction, and CSP was detected simultaneously with an anti-CSP antibody (~40 kD). (B) Isolated granule membranes show a similar reaction. An IP with MARCKS detected HSP70 and CSP proteins (lane 1), and an IP with HSP70 detected MARCKS and CSP proteins (lane 2), indicating the existence of a trimeric complex consisting of MARCKS, HSP70, and CSP that is associated with mucin granule membranes in these cells.

cytosis in a number of other cell types. This includes secretion of oxytocin (24, 25), thyrotropin-releasing hormone (26), catecholamines (27), ACTH (28), insulin (29), pepsinogen (30), noradrenaline (31), serotonin (32), neurotransmitters (33, 34), and neurotensin (35). We have shown recently that MARCKS plays an important role in leukocyte degranulation (36). These published results implicate PKC activation and subsequent phosphorylation of MARCKS in a variety of secretory functions in numerous cell types.

However, identification of other proteins involved in the exocytotic process, and if and how they interact with MARCKS, remain to be determined. It is known that MARCKS translocates from plasma membrane to cytoplasm when it is phosphorylated by PKC (2, 37), and we have presented a hypothetical mechanism whereby phosphorylated MARCKS is targeted to and binds specifically to mucin granule membranes in airway secretory cells (3). Inhibition of mucin secretion by the MANS peptide correlated with its attenuation of binding of MARCKS to mucin granule membranes, indicating that binding of MARCKS to these granule membranes is a key component of the mechanism whereby MARCKS regulates mucin secretion (3). The precise role of MARCKS in the chain of cellular events preceding

exocytosis, and other proteins that may interact with MARCKS in the process, remain unclear.

The first major question addressed in this report related to the character of the binding of MARCKS to granule membranes, specifically other proteins that may be involved in this critical process. In this study, we hypothesized that MARCKS binding to mucin granule membranes, and thus its secretory function, involved interactions with chaperone proteins. This would explain the targeting of MARCKS, after its phosphorylation by PKC and translocation to the cytoplasm, to mucin granules, as well as MARCKS binding to these sites rather than to other intracellular membranes. The first chaperone investigated was CSP, a highly conserved protein made up of four domains: an N-terminal "J" domain (a 70 amino acid region of homology shared with bacterial DnaJ), an adjacent linker, a cysteine-rich domain with a central palmitoylated string of cysteine residues, and a variable C-terminus. Members of the CSP family are involved in exocytosis, and they bind to secretory vesicle membranes (e.g., synaptic vesicles, chromaffin granules, zymogen granules) via their hydrophobic side chains of palmitoyl (6, 7, 38). CSP was first discovered in retina and brain of *Drosophila* as a neuronal-specific protein (39) and, since then, wide expression of several CSP isoforms has been shown in both neuronal and nonneuronal tissues. Airway and mammary epithelial cells have been reported previously to express CSP (9, 21).

Figure 7. siRNA silencing of MARCKS or HSP70 attenuates PMA-stimulated mucin secretion in airway epithelial cells. HBE1 cells were transfected with siRNA against MARCKS or HSP70. After 48 hours of transfection, the cells were lysed and analyzed on a SDS-PAGE gel followed by immunoblot analysis with anti-MARCKS (A, B) or anti-HSP70 (C, D) antibody, with β -actin as a loading control. (A) Western blot showed a decrease of about 70% in MARCKS protein expression in cells transfected with 2.0 μ g siRNA compared with cells transfected with the control pSUPER vector. Results are representative of three independent experiments. (B) Knockdown of MARCKS results in attenuation of PMA-stimulated mucin secretion. After 48 hours of transfection, cells were exposed to medium alone (bar 1), or to PMA (500 nM) (bars 2–8) for 30 minutes, at which time mucin secretion was analyzed by ELISA. Bars 1 and 2: Control (no transfection); bar 3: Vector control (pSUPER vector, 2 μ g); bar 4: Irrelevant siRNA control (pSUPER/irrelevant siRNA; 2.0 μ g); bars 5–8: pSUPER/MARCKS siRNA: 0.25, 0.5, 1.0, and 2.0 μ g, respectively. Data are presented as mean $\pm$ SEM ($n = 4$ at each point). *Significantly different from medium control ($P < 0.05$); †significantly different from PMA exposure ($P < 0.05$). (C) siRNA silencing of HSP70. Western blot showed a decrease of approximately 55% in HSP70 protein expression compared with cells transfected with the control pSUPER vector. Data are representative of three independent experiments. (D) Knock down of HSP70 results in attenuation of PMA-stimulated mucin secretion. Cells were exposed to medium alone (bar 1) or 500 nM PMA (bars 2–8) for 30 minutes, at which time mucin secretion was analyzed by ELISA. Bars 1 and 2: Control (no transfection); bar 3: Vector control (pSUPER vector, 2 μ g); bar 4: Irrelevant siRNA control (pSUPER/irrelevant siRNA; 2.0 μ g); bars 5–8: pSUPER/HSP70 siRNA: 0.25, 0.5, 1.0, and 2.0 μ g, respectively. Data are presented as mean $\pm$ SEM ($n = 4$ at each point). *Significantly different from medium control ($P < 0.05$); †significantly different from PMA stimulation ($P < 0.05$). Control samples contained approximately 5 to 8 ng/ml of mucin.

CSP exists in two isoforms in epithelial cells, CSP1 (also referred to recently as CSP α) and CSP2, with CSP2 lacking a stretch of 31 amino acids in its C-terminus that is present in CSP1 (9). Differential expression studies of CSP isoforms in *Drosophila* indicated that the smaller CSP2 is present in neuronal tissues, while CSP1 is detected in nonneuronal tissue (40). CSP1 is expressed on large dense-core granules of chromaffin cells (6), insulin-containing secretory granules (41), pancreatic zymogen granules (7), and secretory granules of the neurohypophysis (38), indicating potential involvement of CSP1 in exocytosis of large dense-core granules. Our data, showing CSP1 expression in NHBE cells, fit in well with these studies.

Once established as present in airway epithelial cells and associated with mucin granule membranes, a functional role for CSP in mucin secretion was demonstrated, as an siRNA corresponding to CSP decreased CSP protein levels in HBE1 cells and substantially attenuated mucin secretion induced by PMA stimulation (Figures 3A and 3B). If MARCKS–CSP interactions on granule membranes are important in secretion, an obvious question to address was how MARCKS, after phosphorylation and translocation to the cytoplasm, is targeted to mucin granules and CSP in these cells rather than to other intracellular membranes. It has been suggested previously that MARCKS may be directed to bind to specific sites on membranes via protein–protein interactions. For example, a protein with a MARCKS-like membrane-binding domain, p60^{v-src}, has been shown to associate with membranes via a 32-kD receptor (42), and MARCKS was reported to associate with a protein factor in the cytoplasm of bovine brain cells (43). In investigating potential MARCKS-binding proteins related to airway mucin secretion, an interesting possibility was HSP70 (HSP70 is the inducible form and HSC70 the constitutive form of the

protein). HSP70 seemed a likely targeting candidate as it is a well-known chaperone associated with various exocytotic processes, including mucus secretion by gastric epithelial cells (44). Of additional interest, the conserved “J”-domain of CSP contains a motif, HPD, which is known to specifically bind to HSP70-like proteins and to stimulate HSP70 ATPase activity (the interaction of CSP with heat shock proteins is confined to HSP70/Hsc70, and it does not interact with other heat shock proteins such as HSP60 or HSP90) (10, 11, 45). In fact, interactions of CSP with HSP70 proteins have been suggested to play a role in exocytotic secretion (46–48); specifically CSP, in concert with HSP/HSC70-like proteins, may coordinate sequential protein–protein interactions to mediate various steps in the exocytotic process in synaptic vesicles (49), oocytes (47), and pancreatic β cells (50, 51).

HSP70 was shown to be involved in mucin secretion in airway epithelial cells via siRNA studies (Figure 7). We then investigated whether or not HSP70 associates with MARCKS in airway epithelial cells. Both HSP70 and MARCKS were detected by immunoprecipitation in a complex associated with mucin granule membranes isolated from NHBE cells, as well as in a complex from HBE1 whole cell lysates. We then investigated potential interactions between HSP70 and CSP, and the possibility that these proteins, together with MARCKS, formed a trimeric complex, apparently in association with mucin granule membranes, that could be related to the secretory process. Formation of similar complexes involving some of these chaperones has been reported in other secretory systems. For example, formation of a trimeric complex involving glutamate decarboxylase (GAD), HSC70, and CSP on synaptic vesicles membranes is very similar to the proposed trimeric complex of MARCKS–HSP70–CSP alluded to herein. GAD exists in either a soluble or vesicle membrane-bound form in the mammalian brain, and it associates with synaptic vesicle membranes through first binding to HSC70, followed by interactions with CSP located on vesicle membranes (52). Similarly, a trimeric protein complex consisting of HSC70, CSP and the small glutamine-rich tetratricopeptide repeat-containing protein (SGT) is involved integrally in neurosecretion (53, 54). Relatedly, Phillips and coworkers (55) described a complex of proteins around presynaptic vesicles in synaptic junctions from the rat cerebral cortex, and found that HSC70 was part of the complex, together with various docking and fusion proteins. As illustrated in Figure 6, MARCKS, HSP70, and CSP indeed form a trimeric complex within NHBE cells, and this complex may play an important role in the secretory process.

In conclusion, the data presented in this report show that CSP in airway epithelial cells associates with membranes of mucin granules and is involved in attachment of MARCKS to granule membranes during the secretory process. A second chaperone, HSP70, interacts with MARCKS and CSP and also appears involved in the secretory pathway. Interestingly, silencing of each of these proteins attenuates mucin secretion. Clearly, numerous protein–protein and protein–granule interactions are involved in the exocytotic process in airway epithelial cells. The exact temporal and kinetic character of these interactions and how they may relate structurally and functionally with a plethora of relevant fusion and docking proteins in what is clearly a complex mechanism of granule translocation and exocytotic release remain to be determined.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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