MARCKS and Related Chaperones Bind to Unconventional Myosin V Isoforms in Airway Epithelial Cells

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We have shown previously that myristoylated alanine-rich C kinase substrate (MARCKS) is a key regulatory molecule in the process of mucin secretion by airway epithelial cells, and that part of the secretory mechanism involves intracellular associations of MARCKS with specific chaperones: heat shock protein 70 (Hsp70) and cysteine string protein (CSP). Here, we report that MARCKS also interacts with unconventional myosin isoforms within these cells, and further molecular interactions between MARCKS and these chaperones/cytoskeletal proteins are elucidated. Primary human bronchial epithelial cells and the HBE1 cell line both expressed myosin V and VI proteins, and both MARCKS and CSP were shown to bind to myosin V, specifically Va and Vc. This binding was enhanced by exposing the cells to phorbol-12-myristate-13-acetate, an activator of protein kinase C and stimulator of mucin secretion. Binding of MARCKS, Hsp70, and CSP was further investigated by His-tagged pull down assays of purified recombinant proteins and multiple transfections of HBE1 cells with fusion proteins (MARCKS-HA; Flag-Hsp70; c-Myc-CSP) and immunoprecipitation. The results showed that MARCKS binds directly to Hsp70, and that Hsp70 binds directly to CSP, but that MARCKS binding to CSP appears to require the presence of Hsp70. Interrelated binding(s) of MARCKS, chaperones, and unconventional myosin isoforms may be integral to the mucin secretion process.

Keywords: airway; mucin; MARCKS; chaperones; myosin

The respiratory airways are lined by a thin layer of mucus that protects the lung from inhaled microbes, particulate and gaseous pollutants, and other potentially deleterious substances. Mucin, the major glycoprotein component of mucus, is synthesized and stored within cytoplasmic granules in goblet cells in airway epithelium and glands in the submucosa, and is released from these granules into the airway lumen via an exocytotic process. In a number of studies, we have demonstrated that myristoylated alanine-rich C-kinase substrate (MARCKS) protein, a widely distributed protein kinase C (PKC) substrate, is a central molecule regulating airway mucin secretion (1–4), and that MARCKS association in airway epithelial cells with two specific intracellular chaperones, heat shock protein 70 (Hsp70) and

CLINICAL RELEVANCE

This work delves into mechanisms of mucin secretion in airway epithelial cells and, for the first time, shows that a unique form of myosin is involved in the process, as well as other chaperones.

cysteine string protein (CSP), is integral to the secretory mechanism (5).

However, specific dynamics of this binding, as well as other potential proteins involved, have not been elucidated. Here, we investigated binding of MARCKS and these chaperones to intracellular cytoskeletal proteins in both primary cultures of well-differentiated normal human bronchial epithelial (NHBE) cells and the virally transformed HBE1 cell line. In addition, the molecular nature of the interactions among these proteins was examined further. The results indicate that both MARCKS and CSP bind to the unconventional nonmuscle myosin subtypes, myosin Va and Vc. Since the binding was enhanced by exposure of the cells to the PKC activator phorbol-12-myristate-13-acetate (PMA), a known stimulator of mucin secretion by airway epithelial cells (1–4), these interactions may be important in the secretory process.

MATERIALS AND METHODS

Detailed descriptions are available in the online supplement.

Cell Culture

Air-liquid interface (ALI) culture of normal human bronchial epithelial (NHBE; Lonza, Walkersville, MD) cells was performed as described previously (6). Briefly, cells were seeded on rat-tail collagen (BD Biosciences, San Jose, CA) Transwell inserts (Corning Inc., Corning, NY) in a 1:1 mixture of bronchial epithelial cell growth medium (BEGM, singlequote supplemented; Lonza) and Dulbecco's Modified Eagle's medium (DMEM; Mediatech, Inc., Herndon, VA) containing 0.13 mg/ml bovine pituitary extract, 5×10^{-8} M all-trans retinoic acid (Sigma, St. Louis, MO), and nystatin (Amresco, Solon, OH). Cells were cultured submerged until nearly confluent when ALI was established by removing the apical medium and feeding basolaterally. The human bronchial epithelial cell line (HBE1 [7]) was cultured as described above for NHBE cells. Cells were maintained in a 1:1 mixture of Ham's F-12 and DMEM supplemented as previously detailed (5). Addition of all-trans retinoic acid induced differentiation post-ALI. Experiments were performed with noncytotoxic reagent concentrations as assessed by Promega's CytoTox96 kit (Madison, WI).

Immunoprecipitation

Treated cells were harvested with lysis buffer containing protease/phosphatase inhibitor cocktails (Roche, Indianapolis, IN and Sigma). Sonicated lysates were incubated with antibodies overnight. Protein A

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or G beads were then added and incubated further depending upon selected antibodies. Pre-clearing steps or bridging antibodies were added as needed. Immunocomplex-linked beads were washed, combined with sample buffer, boiled, and separated for analysis via immunoblotting.

Constructs

The construct pcDNA4/TO/MARCKS was generated previously (2). For His-tagged MARCKS constructs, full-length MARCKS was amplified by PCR from pcDNA4/TO/MARCKS and subcloned into a pET-DEST42 vector using a TOPO-TA Cloning kit and Gateway LR-Clonase II (Invitrogen, Carlsbad, CA). The MARCKS-HA construct was amplified from pcDNA4/TO/MARCKS by PCR using primers to add an HA-tag at the C-terminus. For c-Myc-CSP and FLAG-Hsp70 constructs, the targeting sequences were generated from total RNA extracted from NHBE cells by RT-PCR. These PCR products were subcloned into a pGEM-T-EASY vector (Promega), then pCMV3-tag vector (Agilent Technologies, Santa Clara, CA). Sequencing confirmed all constructs.

Protein Expression and Purification

Respective bacterial expression constructs were transformed in the BL21 (DE3) bacterial strain. Recombinant His-tagged MARCKS was extracted from lysates with bacterial protein extraction reagent (Pierce). The expression of fusion proteins was evaluated electrophoretically and confirmed by InVision His-tag Stain (Invitrogen). Native fusion proteins were purified by Ni-NTA agarose affinity columns and cobalt-chelated beads (Qiagen, Valencia, CA; Thermo Fisher, Rockford, IL).

In Vitro Binding Assays

Binding of MARCKS and Hsp70 was evaluated with a ProFound PolyHis Pull-Down Kit (Pierce). Purified, recombinant His-tagged MARCKS was immobilized on a cobalt chelate gel, washed, and then incubated with purified Hsp70 protein (Stressgen Bioreagents, Inc., Victoria, BC, Canada). Samples were washed, eluted, and then analyzed by SDS-PAGE gel electrophoresis and immunoblotting. Binding of GST and Hsp70 was evaluated using a GST-Tag Pull-Down Kit (Thermo Fisher).

Transfections

Cells were dissociated by Versene (Invitrogen) and re-seeded onto collagen-coated plates before transfection. For interaction assays, HBE1 cells were co-transfected with MARCKS-HA, c-Myc-CSP, FLAG-Hsp70, or control DNA using FuGene6 (Roche). Total protein was collected 48 hours after transfection followed by immunoblotting and immunoprecipitation experiments. Efficiency was monitored by transfection of pCMV- β or pcDNA4/TO/lacZ and evaluated by β -gal stain (Roche).

RESULTS

Myosin V and VI Are Expressed in Airway Epithelial Cells; Myosin V Interacts with MARCKS and CSP

Several nonmuscle myosin isoforms, including myosin Va, myosin Vc, and myosin VI, have previously been shown to be expressed at the mRNA level in the lung (8, 9). Here, we show via immunoblotting that these three myosins also are expressed at the protein level in NHBE and HBE1 cells (Figure 1A). To investigate interactions between myosin, MARCKS, and other chaperone proteins, immunoprecipitations with anti-myosin Va, anti-myosin Vc, and anti-myosin VI antibodies were performed on cell lysates either with or without exposure to PMA for 30 minutes. The results show that PMA exposure increases interactions between CSP and both myosin Va and Vc (Figure 1B), but does not affect CSP–myosin VI interactions. As shown in Figure 1C, interactions between MARCKS and myosin Va or Vc also increase after exposure to PMA. Thus, it appears that

Figure 1. Interactions of myosin V with myristoylated alanine-rich C-kinase substrate (MARCKS) and cysteine string protein (CSP) after exposure of airway epithelial cells to phorbol-12-myristate-13-acetate (PMA). (A) Myosin subtypes in NHBE and HBE1 cells revealed by immunoblotting using antibodies against myosin Va, myosin Vc, and myosin VI, all shown at molecular weights slightly lower than 200 kD. (B) Interaction of myosins with CSP after PMA stimulation for 30 minutes. Immunoprecipitation of CSP with anti-myosin subtype antibodies is shown. Arrows represent CSP. (C) Interaction of myosins with MARCKS after exposure to PMA for 30 minutes. Immunoprecipitation of MARCKS by anti-myosin subtype antibodies is shown. Proteins were visualized using immunoblotting with an anti-MARCKS antibody. PMA enhances interactions between myosin Va and Vc and both MARCKS and CSP. Blots shown represent results from three separate experiments.

myosin V, specifically the Va and Vc isoforms, interacts with both MARCKS and CSP.

Purified Recombinant MARCKS Appears to Bind Directly to Hsp70

Since endogenous MARCKS, Hsp70, and CSP have been shown to associate with each other in airway epithelial cells (5), we further investigated whether these protein–protein interactions represent direct binding or require one or more co-factors or adaptor proteins. To address this question, the interactions of purified proteins *in vitro* were investigated. A MARCKS cDNA carrying a His-tag at its C-terminus was constructed in a bacterial expression vector pET-DEST42. The recombinant protein was overexpressed in *Escherichia coli* by isopropyl- β -D-thiogalactopyranoside (IPTG) induction. His-tagged MARCKS protein was

overexpressed and confirmed with a His-tag stain. Bacterially expressed His-tagged MARCKS ran at a molecular weight of 70 kD on an SDS-PAGE gel (the size of overexpressed MARCKS is smaller than endogenous, mammalian MARCKS, probably due to a lack of complete post-translational modifications of MARCKS in the bacterial system). To further purify MARCKS protein, a two-step process was applied. A cobalt-chelated bead column was used after the Ni-IDA column. Purified protein was confirmed by immunoblotting with anti-MARCKS antibody (Figure 2A). Purified MARCKS was then incubated with activity-tested, purified Hsp70 at a 1:1 ratio, and binding evaluated by His-tag pull down assays. The presence of Hsp70 in lane 3 of Figure 2B suggests that the binding of MARCKS and Hsp70 is direct and does not require additional proteins.

Binding Properties of Mammalian Overexpressed MARCKS, Hsp70, and CSP

Since the above results showed that MARCKS seems to bind to Hsp70 directly in a bacterial system, we then investigated how MARCKS, Hsp70, and CSP may bind to each other in a mammalian system. HBE1 cells were co-transfected with mammalian expression vectors of tagged proteins (MARCKS-HA, c-Myc-CSP, and FLAG-Hsp70). The binding properties were analyzed by immunoprecipitation and immunoblotting with specific antibodies. The results indicate that, in this mammalian overexpression system, MARCKS does not bind to CSP directly (Figure 3A). However, both MARCKS (Figure 3B) and CSP (Figure 3C) appear to bind directly to Hsp70.

Phosphorylation of MARCKS Affects Its Interaction with CSP and Hsp70

Phosphorylation of MARCKS at different time points after exposure to stimulatory concentrations of PMA, assessed via immunoblotting with a rabbit polyclonal antibody that reacts with residues surrounding Ser152/156 of MARCKS, is indicated in Figure 4A. MARCKS appears to be phosphorylated starting at approximately 1.5 minutes after exposure to PMA; this phosphorylation increases up to 5 minutes, and then appears to decrease (or plateau) before increasing again at 30 minutes. Interactions between MARCKS and CSP at different time points after PMA exposure are shown in Figure 4B. PMA exposure increased formation of the MARCKS–CSP complex in a time-dependent manner up to 30 minutes. To confirm that phosphorylated MARCKS interacts with CSP, co-immunoprecipitation with an anti-CSP antibody and immunoblotting with an anti-phospho-MARCKS antibody were performed. Figure 4C shows that phospho-MARCKS associates with CSP. In Figure 4D, PMA exposure appears to increase formation of the MARCKS–Hsp70 complex. These results indicate that phosphorylation of MARCKS enhances its interaction with both CSP and Hsp70.

DISCUSSION

MARCKS protein has been shown to be a central molecule involved in airway mucin secretion *in vitro* and *in vivo* (2–4). Here, we show that the cytoskeletal protein, myosin V, is expressed in human airway epithelial cells and associates with MARCKS. Exposure of these cells to PMA enhances MARCKS interactions with myosin V, specifically the Va and Vc isoforms. Previously, associations of MARCKS with intracellular chaperones, including Hsp70 and CSP, were shown to be integral to the secretory process (5). In this report, studies using bacterial and mammalian expression systems revealed that, within human

airway epithelial cells, MARCKS appears to bind directly to Hsp70, that Hsp70 binds directly to CSP, but MARCKS does not bind directly to CSP, suggesting that MARCKS–CSP associations are indirect and may require the presence of Hsp70. Interestingly, CSP also binds to myosin V, and exposure of cells to the protein kinase C activator, PMA, enhances interactions of both CSP and MARCKS with myosin V. The results of these studies provide

Figure 2. Purification and binding of recombinant His-tagged MARCKS protein. (A) His-tagged MARCKS protein was purified by nickel-chelated iminodiacetic acid (Ni-IDA), then cobalt-chelated resin, and is shown at approximately 70 kD. Purified His-tagged MARCKS stained with Coomassie Blue is shown in the left panel, and confirmed by immunoblotting with anti-MARCKS antibody (right panel). Arrows indicate purified His-tagged MARCKS. L, protein standard ladder; Co, cobalt chelated resin. The protein was further purified using a cobalt-chelated column, and confirmed as His-tagged MARCKS protein. The figure shown is a composite of multiple blots and the Ni-IDA lane is a composite of two gels. The data are representative of three separate experiments. (B) Binding of Hsp70 to MARCKS *in vitro* was investigated using a His-tagged pull-down assay. Purified recombinant His-tagged MARCKS protein was immobilized in a cobalt-chelated resin and then incubated with the same amount of Hsp70. After washes and elution, samples were analyzed by immunoblotting with specific antibodies (lanes 1 and 2: anti-MARCKS; lanes 3 and 4: anti-Hsp70; lanes 5 and 6: Mouse IgG control antibody). Hsp70 presents in lane 3, indicating that purified Hsp70 binds directly to His-tagged MARCKS. Lanes 5, 6, 7, and 8 represent control experiments for the GST pull-down assay. Lane 9 shows lack of binding between purified GST and purified Hsp70 as a technique control, and lane 10 is an antibody control. The figure shown is a composite of multiple Western blots and represents three separate experiments, as indicated by the white line delineations.

Figure 3. Binding of MARCKS, Hsp70, and CSP with fusion proteins in transiently co-transfected HBE1 cells. Different combinations of MARCKS-HA, c-Myc-CSP, or FLAG-Hsp70 expression vectors were co-transfected into HBE1 cells, and their binding analyzed by immunoprecipitation (IP) and immunoblotting (IB). (A) Cells were co-transfected with MARCKS-HA and c-Myc-CSP expression vectors. Lysates were immunoprecipitated with anti-HA Ab and immunoblotted with c-Myc Ab. No c-Myc CSP bands are shown. (B) Cells were co-transfected with MARCKS-HA and FLAG-Hsp70 expression vectors. Lysates were immunoprecipitated with anti-HA Ab and IB with anti-FLAG Ab. Arrow indicates FLAG-Hsp70. (C) Cells were co-transfected with c-myc-CSP and FLAG-Hsp70 expression vectors. Lysates were immunoprecipitated with anti-c-Myc Ab and immunoblotted with anti-FLAG Ab. Arrow indicates FLAG-Hsp70. CTL, control Abs. Immunoprecipitation with MARCKS-HA does not detect c-Myc-CSP protein, immunoprecipitation with MARCKS-HA detects FLAG-Hsp70, and immunoprecipitation with c-Myc-CSP detects FLAG-Hsp70. This suggests that MARCKS and CSP bind directly to Hsp70, but that MARCKS does not bind directly to CSP. The blots shown represent results of three to four separate experiments.

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additional evidence of interactions between MARCKS and the chaperones, Hsp70 and CSP, and provide the first evidence that MARCKS interacts specifically with the cytoskeletal protein myosin V.

Previous work from our laboratory, using a radiolabeled immunoprecipitation assay and matrix-assisted laser desorption ionization/time of flight mass spectrometry/internal sequence analysis (MALDI-TOF), showed that MARCKS associates with myosin in the cytoplasm of NHBE cells (2). Mass spectrometric proteomic analysis of tryptic peptides from isolated mucin granule membranes from airway epithelial cells also implicated myosin as one of the proteins that associates with these granules (10). Since MARCKS is an actin-binding and crosslinking protein, actin/myosin-dependent contraction in concert with MARCKS may mediate granule movement to the cell periphery as an initial step in the exocytotic release of mucin.

The myosin investigated in airway epithelium here, unconventional myosin V, is a nonmuscle type, F-actin-based motor protein. There are three distinct subclasses of myosin V in vertebrates: myosins Va, Vb, and Vc (8, 11). Myosin V is a dimeric protein (12) and myosin Va is known to have a high affinity for F-actin (13). Myosin Va has been implicated in regulating traffic of synaptic vesicles in neurons, secretory granules in neuroendocrine cells, insulin granules in β -cells, and melanosome trafficking in melanocytes (14–19). Myosin Vc is a newly discovered subtype abundantly expressed in epithelial and secretory tissues (8). It is present on zymogen granules of pancreatic acinar cells, and could play a role in exocrine secretion from this organ (20). Myosin VI is another unconventional myosin implicated in organelle trafficking (21–23). In our studies, myosin Va, myosin Vc, and myosin VI all were expressed in both NHBE and HBE1 cells (Figure 1A). Both MARCKS and CSP were shown to bind to myosin Va and Vc, and this binding was enhanced by exposure to PMA (Figures 1B and 1C). As myosin Vc has been shown to be physically associated with mucin granule membranes in airway epithelial cells via mass spectrometric analysis (10), it may represent an important MARCKS-binding protein that could be involved in airway mucin secretion. Binding of MARCKS to myosin isoforms might involve the tail domain of the myosin, as this region of the molecule has been shown previously to be involved in exocytosis of large dense core vesicles in neurons (24). Studies involving silencing of the different myosin isoforms or transfection of dominant negative mutants of these proteins are presently underway in our laboratory to determine the role of MARCKS-myosin interactions in the process of mucin secretion.

The results of this study also support our previous findings that MARCKS, Hsp70, and CSP associate in airway epithelial cells. The binding solutions used here include nonionic NP-40 and modified RIPA buffer. Since RIPA buffer is a strong solution for immunoprecipitation, the fact that MARCKS, Hsp70, and CSP still associate after treatment with RIPA suggests strong interactions between these proteins. To determine whether these protein-protein interactions are truly specific interactions, and to understand how these proteins actually bind, *in vitro* binding assays of purified recombinant proteins and co-immunoprecipitation of overexpressed tagged proteins in mammalian cells were performed. Apparent direct binding between MARCKS and Hsp70 *in vitro* was observed by generating purified recombinant proteins and analyzing their binding properties using His-tagged pull-down assays (Figure 2). Purified GST protein did not bind to purified Hsp70, which not only provided a negative “technique” control for the binding assay, but also supports the idea that the binding between MARCKS and Hsp70 could be direct and specific.

We further confirmed binding of these proteins in a mammalian overexpression system. Stepwise binding events were examined by multiple transfections of HBE1 cells with specifically designed fusion proteins (MARCKS-HA; Flag-Hsp70; c-Myc-CSP) and immunoprecipitations. Since the amount of overexpressed fusion proteins is much higher than other endogenous proteins, the positive results from these immunoprecipitation experiments strongly indicate direct binding of the proteins. As mentioned above, the results indicate that MARCKS-Hsp70 binding is direct, as is Hsp70-CSP binding, but MARCKS binding to CSP appears to be indirect (Figure 3). It should be pointed out that Figure 3A reflects results in HBE1 cell lysates in which tagged MARCKS and CSP are highly overexpressed, with probably hundredfold increase over endogenous levels of the proteins and of the Hsp70 that is present in these cells. The data thus support a mechanism whereby interaction of MARCKS and CSP requires the presence of Hsp70; cytosolic MARCKS may bind to Hsp70, and this MARCKS/Hsp70 complex then may be targeted to cytoplasmic mucin granules through interactions with granule-associated CSP.

HBE1 cells exposed to the PKC activator and mucin secretagogue, PMA, show phosphorylation of MARCKS in a concentration-dependent manner (Figure 4A). The formation of MARCKS-CSP and MARCKS-Hsp70 complexes was increased by exposure to PMA, and CSP was shown to interact with phosphorylated MARCKS (Figures 4B, 4C, and 4D). The data

Figure 4. Phosphorylation increases MARCKS interactions with Hsp70 and CSP in airway epithelial cells. (A) HBE1 cells were exposed to PMA for 0, 1.5, 3, 5, 15, or 30 minutes. Total proteins were collected and analyzed by immunoblotting with rabbit polyclonal anti-phospho-MARCKS and mouse monoclonal anti-MARCKS antibodies. (B) Co-immunoprecipitations of MARCKS from HBE1 cells by anti-CSP antibody following exposure to PMA for 0, 1.5, 3, 5, 15, or 30 minutes. Bands shown in immunoblot represent MARCKS interacting with CSP (upper panel). (C) Immunoprecipitation of phospho-MARCKS from HBE1 cells by anti-CSP antibody after PMA stimulation for 30 minutes. Phospho-MARCKS appears at approximately 80 kDa. (D) Immunoprecipitation of Hsp70 from HBE1 cells by anti-MARCKS antibody after exposure to PMA for 0 or 30 minutes. Upper panel represents Hsp70 that interacts with MARCKS. PMA enhances interactions between MARCKS and Hsp70 (54% increase based on densitometric analysis of blots; data not shown) and subsequently, CSP (25% increase over 30 min). In these experiments, Hsp70 is present in the total protein isolated and therefore available to bind with p-MARCKS and CSP, thus enabling their interaction. Blots shown represent results from three separate experiments.

shown in Figure 4 reflect results of experiments performed in whole cell lysates with only endogenous levels of proteins expressed. Therefore, it is probable that endogenously present Hsp70 enables CSP to interact with MARCKS, although not directly as indicated in the purified protein experiments shown above. In addition, Figure 4 shows that as PMA increases the phosphorylation of MARCKS (panel A), the levels of Hsp70 are also increased (panel D). Consequently, the levels of CSP that interact with MARCKS are also increased, perhaps due to the increased availability of Hsp70 acting as the common binding target for both MARCKS and CSP (Figures 4B and 4C).

These findings suggest a functional role for interactions between MARCKS, Hsp70, and CSP in several cellular events,

and support a mechanism of mucin secretion involving MARCKS phosphorylation and important roles for these chaperones in the secretory process (1, 5, 25). Additional proteins and intracellular signaling steps involved in the process remain to be elucidated.

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