Original Paper

Ezrin/Exocyst Complex Regulates Mucin 5AC Secretion Induced by Neutrophil Elastase in Human Airway Epithelial Cells

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Key Words

Ezrin • Sec3 • Mucin 5AC • Neutrophil elastase • Phosphatidylinositol 4,5-bisphosphate

Abstract

Background/Aim: Increased mucin secretion is a characteristic feature of many chronic airway diseases, particularly during periods of exacerbation; however, the exact mechanism of mucin secretion remains unclear. Ezrin, which is a specific marker of apical membranes, is predominantly concentrated in exocyst-rich cell surface structures, crosslinking the actin cytoskeleton with the plasma membrane. In the present study, we examined whether Ezrin is involved in mucin 5AC (MUC5AC) secretion after neutrophil elastase (NE) attack, and we investigated the role of the exocyst complex docking protein Sec3 in this process. **Methods:** NE was used as a stimulator in a 16HBE14o- cell culture model. The expression and location of Ezrin and Sec3 were investigated, and the interaction between Ezrin and Sec3 in 16HBE14o-cells was assayed after treatment with NE, Ezrin siRNA, Sec3 siRNA, neomycin or PIP2-Ab. **Results:** We found that Ezrin was highly expressed in the bronchi of humans with chronic airway diseases. NE induced robust MUC5AC protein secretion. The Ezrin siRNA, Sec3 siRNA, and neomycin treatments led to impaired MUC5AC secretion in cells. Both Ezrin and Sec3 were recruited primarily to the cytoplasmic membrane after NE stimulation, and the neomycin and PIP2-Ab treatments abrogated this effect. Immunoprecipitation analysis revealed that Ezrin and Sec3 combined to form complexes; however, these complexes could not be detected in Ezrin^{Δ1-333} mutant-transfected cells, even when PIP2 was added. **Conclusions:** These results demonstrate that Ezrin/Sec3 complexes are essential for MUC5AC secretion in NE-stimulated airway epithelial cells and that PIP2 is of critical importance in the formation of these complexes.

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Introduction

Physiologically secreted mucus is an important defense barrier in the respiratory system. Mucus can remove foreign substances and maintain the airway microenvironment [1, 2]. However, excess mucus is recruited to the airway during acute exacerbations of chronic obstructive pulmonary disease and severe asthma, particularly during fatal asthma attacks [3-5]; typically, a “burst” of mucus production occurs, forming a mucus plug that can block the airway such that the patient can die of asphyxiation. Studies have found that many factors, such as bacteria, viruses, inflammatory factors and proteases, can induce mucin synthesis and promote exocytosis. These factors simultaneously activate the signaling cascade involved in exocytosis [6-8]. Therefore, we hypothesized that the “mucus burst” under pathological conditions may strongly correlate with mucus exocytosis. Therefore, understanding the signaling mechanisms involved in exocytosis may lead to new treatments for sputum retention, thus preventing airway obstruction.

Ezrin is a specific marker of apical membranes. This protein links the filamentous actin of the cytoskeleton to the apical membrane of cells. Ezrin is primarily distributed in organs that have secretory functions, such as lungs, small intestine, stomach, pancreas, and kidneys, and it is highly expressed in the epidermis and epithelium [9]. This protein is predominantly concentrated in actin-rich cell surface structures and is involved in microvilli formation, cell adhesion, cell motility and secretion [10].

The exocyst is an octameric protein complex involved in targeting post-Golgi secretory vesicles to the plasma membrane. Sec3 is a docking protein of the exocyst complex that is required for secretion. The N-terminus of the exocyst component Sec3 directly interacts with phosphatidylinositol 4,5-bisphosphate (PIP2), and Sec3 and PIP2 combine to play important roles in polarized cell growth and exocytosis. A previous study also found that ERM (Ezrin/Radixin/Moesin) proteins target sites in the plasma membrane that are enriched in lipid rafts and exocyst complexes [11]. However, whether Ezrin and Sec3 interact to regulate mucin secretion in airway epithelial cells remains unclear.

Chronic respiratory diseases with mucus hypersecretion are usually accompanied by marked neutrophil infiltration, particularly in cases of fatal asthma. This situation differs from that of conventional asthma, which is characterized by eosinophil infiltration [12, 13]. Neutrophil elastase (NE) generated by neutrophils has been shown to be a potent mucin secretagogue [14]. In the present study, we report that NE exposure increases MUC5AC secretion and that Ezrin and the exocyst complex subunit Sec3 are involved in the secretion process. The data provide the first evidence that Ezrin and Sec3 function together and play important roles in mucus secretion in airways. Our study reveals the molecular mechanism of exocyst complex formation and improves our understanding of mucin secretion in inflammatory airway diseases.

Materials and Methods

Reagents

Rabbit anti-Sec3 polyclonal antibody was obtained from Bethyl Lab Inc. DMEM/Ham's F12 medium, FBS, fetal bovine serum (FBS), neomycin, PIP2, anti- β -actin monoclonal antibody, and anti-Flag tag (M2) antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lipofectamine 2000 and OPTI-MEM reduced-serum medium were purchased from Invitrogen (San Diego, CA, USA). PIP2 antibody was purchased from Abcam (Cambridge, MA, USA). Protein A/G PLUS-Agarose immunoprecipitation reagent, Ezrin siRNA, Sec3 siRNA (h2), control siRNA, rabbit anti-Ezrin (Thr567) polyclonal antibody, mouse anti-Ezrin (3C12) monoclonal antibody and FITC-conjugated fluorescent secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). X-tremeGENE9 DNA transfection reagent was obtained from Roche (Roche, Basel, Switzerland).

Tissues

Normal human lung tissues were obtained from surgical specimens resected at the Department of Thoracic Surgery of the Second Affiliated Hospital (Chongqing Medical University) between 2011 and 2012. The experiments were approved by the institutional ethics committee, and informed consent was obtained before sampling. The samples were collected from 10 patients (8 men and 2 women) with a mean age of 56 years (age range: 45-69 years). At the time of surgery, 6 patients were suffering from mild to moderate COPD or asthma, and 4 patients had no underlying chronic inflammatory diseases of the airway. None of the patients was being chronically treated with theophylline, β -adrenoceptor agonists, corticosteroids, or anticholinergic drugs. Hematoxylin and eosin (HE) staining was used to confirm whether the tissues were non-cancerous.

Immunohistochemistry

Tissue slides were incubated with a mouse anti-Ezrin monoclonal antibody (1:500 dilution) at room temperature for 1 h and then incubated with a rabbit anti-mouse antibody (1:200 dilution) for 1 h. Then, samples were exposed to a streptavidin-peroxidase complex (1:100 dilution) for 45 min. 3,3'-Diaminobenzidine (DAB) was used as a chromogen. Epithelial cells that stained positively for Ezrin were counted; the results are expressed as a percentage of the total number of epithelial cells.

Construction of recombinant Flag-Ezrin and a Flag-Ezrin 1-333 deletion

Total RNA was extracted from 16HBE14o- cells. The following primers were used to amplify the Ezrin sequence by PCR: forward primer, 5'-GCG AAG CTT GAT TAC AAG GAC G *AT GAC AAG ACC GAA ACC AAT CAA TGT C-3'* (the *Bam*H I restriction site is underlined and the Flag sequence is in italics), and reverse primer, 5'-GCG GGA TCC TTA CAG GGC CTC GAA CTC-3' (*Hind*III restriction site is underlined). PCR was performed using Platinum Taq DNA Polymerase II (Invitrogen) to obtain full-length Ezrin cDNA. The pcDNA3.1 vector was cut at unique *Bam*H I and *Hind*III restriction sites, and the purified Ezrin cDNA was subcloned into the linear pcDNA3.1 vector by *Sma*I DNA ligase to obtain pcDNA.3-Flag-Ezrin. The region in Ezrin that is responsible for PLEK binding is in the 1-333 NH2-terminal domain; thus, we constructed an Ezrin 1-333 deletion mutant for study. The pGEX-2T-Ezrin_{Δ1-333} plasmid used to express the Ezrin 1-333 truncated mutant was constructed as described previously [15-17]. The Ezrin_{Δ1-333} fragment was purified and subcloned into the pcDNA3.1-Flag vector to obtain pcDNA.3-Flag-Ezrin_{Δ1-333}.

Cell culture

16HBE14o- cells were cultured in RPMI-1640 supplemented with 10% (v/v) fetal bovine serum (FBS). Then, the cells were plated at a density of 1×10^6 - 2×10^6 cells/ml and incubated at 37°C until the cells were 60%-80% confluent. Cells were serum-starved before NE exposure. For inhibition studies, the cells were pre-treated with inhibitors for 1 h before infection.

siRNA transfection

16HBE14o- airway epithelial cells were plated at a density of 1×10^6 - 2×10^6 cells/ml and incubated at 37°C until the cells were 60%-80% confluent. Then, 4 μ l of X-tremeGENE9 DNA transfection reagent was diluted in serum-free DMEM/Ham's F12 medium. After vortexing briefly, Ezrin siRNA or Sec3 siRNA (both from Santa Cruz) was added to the reagent. The mixture was incubated at room temperature for 20 min. Then, the mixture was added dropwise to cells and incubated for 18 h. Next, the cells were washed 3 times with PBS and incubated in DMEM/Ham's F12 medium containing 10% FBS for the following assays.

Real-time PCR and reverse transcription PCR

Total RNA was extracted from each group of 16HBE14o- cells using a Biotek high-purity total RNA extraction kit and verified by 1.5% agarose gel electrophoresis. RNA was primed with oligo(dT) and reverse transcribed using a PrimeScript RT reagent kit. The conditions for the reverse transcription reaction were as follows: denaturing at 94°C for 10 min; followed by 30 cycles of 94°C for 30 s, 60°C for 45 s, and 70°C for 45 s; and a final extension at 72°C for 5 min. The PCR products were resolved by 2% agarose gel electrophoresis and subsequently visualized by ethidium bromide staining. Real-time PCR was performed using a SYBR R Premix EX Taq™ II real time PCR kit. The human Ezrin primer sequences for real-time PCR are listed in Table 1. Relative mRNA expression was determined using a standard curve.

Table 1. Primers for PCR

Gene	Primers
Ezrin	Forward: 5'-AAGGGTTCTGCTCTGACTCCA-3' Reverse: 5'-CCGGAACCTTGAACCTGGAGGG-3'
Ezrin (real-time)	Forward: 5'-CGCTCTAAGGGTTCTGCTCT-3' Reverse: 5'-AACTTGCCCGGAACCTTGAA-3'
GAPDH	Forward: 5'-GAAGGTGAAGGTCGGAGT-3' Reverse: 5'-GAAGATGGTGATGGGATTT-3'

ELISA

ELISA for the MUC5AC protein was performed as described previously [18].

Immunoprecipitation

Protein samples were lysed using RIPA buffer and then collected, and IgG was used to prevent non-specific binding. The anti-Ezrin antibody (1 µg) was added to each sample and then incubated at 4°C overnight. Then, 20 µl of protein A+G agarose was added, and the samples were incubated at 4°C for 3 h. Next, the samples were centrifuged at 2500 rpm for 5 min at 4°C. The pellet was washed with RIPA buffer 5 times, centrifuged, resuspended in 20 µl of buffer solution, and heated at 100°C for 5 min. The immunoprecipitates were fractionated by SDS-PAGE and then transferred onto polyvinylidene difluoride (PVDF) membranes for immunoblotting for Flag-Ezrin and Sec3 proteins.

Immunoblotting

The proteins in cell lysates were extracted and resolved on 15% SDS gels. The gels were equilibrated in transfer buffer containing 25 mM Tris-HCl, 192 mM glycine, and 20% methanol (pH 8.3). Then, the proteins were transferred to PVDF membranes by electrophoresis. The membranes were incubated in 5% milk dissolved in PBS and 0.05% Tween 20 for 1 h at room temperature and subsequently incubated with pEzrin, Ezrin, and Sec3 antibodies overnight at 4°C. After incubation with secondary IgG antibody, the immunoreactivity was visualized using enhanced chemiluminescence detection reagent.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100 in PBS for 30 min. The cells were then blocked with 1% BSA plus 1% normal goat serum and then incubated with a mouse anti-Ezrin monoclonal antibody (1:200) overnight at 4°C. After three 10-min washes in 0.1% Triton X-100/PBS, the cells were incubated with a FITC-conjugated fluorescent secondary antibody (1:200) for 2 h at room temperature, followed by staining of the cell nuclei with diamidino-phenyl-indole (DAPI). The samples were examined using a Leica TCS-SP2 inverted confocal microscope fitted with the appropriate fluorescence filters.

Statistical Analysis

The data are presented as the mean ± SEM. The data analysis was performed using SPSS 17.0 software (SPSS Inc., USA). All *in vitro* experiments that used cell lines were performed with materials collected from at least six separate cell cultures in duplicate or triplicate. More than two groups were compared using Student's *t*-test or one-way analysis of variance (ANOVA), followed by Bonferroni analysis with a significance value defined as *P* < 0.05.

Results

Ezrin expression in human bronchial epithelial cells

Ezrin was highly expressed in the mucosal epithelium of bronchi affected by chronic inflammation. This protein was primarily at the apical membrane near the cavitary surface (Fig. 1A-D). The brown areas (positive areas stained by Ezrin antibody) were selected as areas

Fig. 1. Ezrin expression in the bronchial epithelia of subjects with COPD and of control subjects. Tissues were immunostained with a mouse anti-Ezrin monoclonal antibody (1:500 dilution) at room temperature for 1 h and then incubated with a rabbit anti-mouse antibody (1:200 dilution) for 30 min. Then, samples were exposed to a streptavidin-peroxidase complex (1:100 dilution) for another 45 min. 3,3'-Diaminobenzidine (DAB) was used as a chromogen. Epithelial cells that stained positively for Ezrin were counted. The data were analyzed using one-way analysis of variance (ANOVA), followed by Bonferroni analysis. The results are expressed as a percentage of the total number of epithelial cells. Significance was set at $P < 0.05$ (A) Normal controls, (B) COPD group, (C) negative control group (rabbit IgG), (D) blocking peptide group (rabbit Ezrin antibody-specific peptide to block the first antibody), and (E) Ezrin protein expression in the airway epithelium (IOD/area). *: Compared with normal control group, $P < 0.01$.

of interest (AOIs), and the integrated optical density/area (IOD/area) was calculated using Image-Pro Plus 6.0 software. The data were analyzed using one-way analysis of variance (ANOVA), followed by Bonferroni analysis. The results revealed that the intensity (IOD/area) of the positive area for the epithelium samples from patients with chronic inflammation was higher than that for normal epithelium samples (Fig. 1E). Thus, airway epithelial cells under chronic inflammation expressed more Ezrin than normal epithelial cells.

NE induced Ezrin expression and phosphorylation in 16HBE14o- cells

Cells were treated with 0.5 μ M NE. Ezrin mRNA expression was upregulated by NE in a time-dependent manner, with the level of Ezrin mRNA expression increasing at 20 min and 30 min (Fig. 2A and B). We also assayed total Ezrin and phosphorylated Ezrin (pEzrin) protein levels using western blot. β -Actin was used as the loading control. The results showed that NE induced Ezrin phosphorylation in a time-dependent manner; the pEzrin protein level began to increase at 5 min after NE incubation, and the pEzrin protein was greatly increased at 30 min compared with that of the cells at time 0. Additionally, the total Ezrin protein level increased at the 30 min period (Fig. 2C and D). These results revealed that NE, which is a potent agent that leads to mucin secretion, also has the ability to increase the mRNA and protein expression levels of Ezrin and to increase the activated state of the Ezrin protein.

NE stimulated both Ezrin and Sec3 to localize to the plasma membrane

Immunofluorescence analysis showed that Ezrin was predominantly diffuse throughout the cytoplasm in normal cells without any treatment (Fig. 3A). NE stimulation led to the accumulation of Ezrin at the apical membrane (Fig. 3B). Then, we pretreated cells with 10 mM neomycin for 30 min before NE stimulation. Neomycin is a positively charged aminoglycoside

Fig. 2. Ezrin expression and phosphorylation in 16HBE14o- cells after NE incubation. Cells were treated with 0.5 μ M NE. The expression of Ezrin mRNA was assayed at different time points by reverse transcription-PCR and real-time PCR, and the expression of Ezrin protein was assayed at different time points by western blot. The data are expressed as the means $\pm$ standard error (SE), $n=6$. (A) Ezrin mRNA and GAPDH mRNA expression were detected by reverse transcription-PCR. (B) Ezrin mRNA and GAPDH mRNA expression were detected by real-time PCR. The results are expressed as the ratio of Ezrin/GAPDH. *Compared with cells at time 0, $P<0.05$. (C) Total Ezrin protein and phosphorylated Ezrin (pEzrin) protein levels were assayed by western blot. (D) The ratios of pEzrin protein to β -actin protein and of total Ezrin protein to β -actin protein. *: Compared with 0 time points, $P<0.05$; **: compared with cells at time 0, $P<0.01$; Δ : compared with cells at time 0, $P<0.001$.

antibiotic known to sequester PIP2 with high affinity. This dose of neomycin was chosen based on a previous study performed by Missiaen et al. [19]. In neomycin-incubated cells, Ezrin was predominantly dispersed in the cytoplasm, which was a significantly different pattern compared with the NE-treated cells not exposed to neomycin (Fig. 3C and D). We also found that Sec3 expression increased in NE-stimulated cells and that this protein showed the same tendency to localize to the cell surface. Neomycin treatment decreased the accumulation of Sec3 at the cell surface, which is a significantly different result compared with that of NE-treated cells ($P<0.05$) (Fig. 3C and D). These results demonstrated that NE could stimulate both Ezrin and Sec3 to localize to the cell surface.

Ezrin and Sec3 levels in the plasma membrane were reduced by neomycin and by PIP2-Ab

Previous analyses of various membrane markers have shown that exocyst components are primarily found in the plasma membrane 2 (P2) fraction [20]. Therefore, the association of the exocyst with the plasma membrane can be assessed by determining the level of this complex in the P2 fraction. The levels of Sec3 and Ezrin in the P2 fraction were determined by immunoblotting. Cell lysates were centrifuged successively at 500 g and 13000 g for 10 min to generate the S1 (cytosol fraction 1), P1 (plasma membrane fraction 1), S2 (cytosol fraction 2) and P2 fractions as previously described [20]. The results revealed that the levels of both Ezrin and Sec3 in the P2 fraction of NE-treated cells were higher compared with those in the P2 fraction of negative control cells ($P<0.01$). When the PIP2-masking reagent neomycin was added, the levels of both Ezrin and Sec3 in the P2 fraction decreased compared with those of the NE-treated group; however, Ezrin and Sec3 protein levels did not show significant changes in the S1 and S2 fractions when the PIP2-masking reagent neomycin was added. Then, we treated cells with 20 μ g/ml of PIP2 antibody (PIP2-Ab), which could reduce PIP2

Fig. 3. Ezrin and Sec3 localization at the membrane was reduced by neomycin treatment. The fluorescence intensities were expressed as IOD/area. IOD/area values were calculated using Image pro-plus-6.0 software. (A) Ezrin and Sec3 expression in normal 16HBE14o- airway epithelial cells. (B) Ezrin and Sec3 expression in NE-treated cells. (C) Ezrin and Sec3 expression in cells preincubated with neomycin and then treated with NE. (D) The fluorescence intensities of Ezrin and Sec3 at the plasma membrane. Δ : Compared with control cells, $P < 0.05$; *: compared with NE-treated cells, $P < 0.05$.

activity in cells [21], and our result indicated that PIP2-Ab treatment also decreased both Ezrin and Sec3 protein levels in the P2 fraction, resulting in significant differences compared with those of the NE-treated cells ($P < 0.01$) (Fig. 4A-D). These results revealed that PIP2 might participate in the translocation of Ezrin and Sec3 to the plasma membrane.

MUC5AC protein secretion inhibited by Ezrin siRNA, Sec3 siRNA, neomycin and PIP2-Ab

Cells were transfected with Ezrin siRNA and then stimulated with NE for 30 min. MUC5AC protein secretion into the supernatant of the cultured cells was decreased compared with that in non-transfected cells; however, no obvious change in the level of MUC5AC in the cytoplasm was observed (Fig. 5A). The MUC5AC protein level in the supernatant was also reduced in Sec3 siRNA-transfected cells compared with that in non-transfected cells (Fig. 5B). To investigate the role of PIP2 in this process, Ezrin siRNA- or Sec3 siRNA-transfected cells were preincubated with 20 μ M PIP2 before NE incubation. The PIP2 dose was chosen according to the study of Kim et al. [21]. The results showed that PIP2 could increase the MUC5AC protein level in the cytoplasm but not in the supernatant of Ezrin siRNA-transfected cells after NE stimulation. An identical effect was detected in Sec3 siRNA-transfected cells preincubated with PIP2 before NE stimulation (Fig. 5A). Thus, the effect of NE on MUC5AC secretion into the supernatant was attenuated in Ezrin- and Sec3-inhibited cells, even when exogenous PIP2 was added. These results indicated that Ezrin and Sec3 were involved in NE-induced MUC5AC protein secretion into the supernatant but not in MUC5AC protein production in the cytoplasm.

Fig. 4. The Ezrin and Sec3 levels at the plasma membrane were reduced by neomycin and PIP2-Ab treatments. The data are expressed as the means $\pm$ standard error (SE), $n=6$. (A) Ezrin protein expression in each fraction. (B) Sec3 protein expression in each fraction. S1: cytosol fraction 1; S2: cytosol fraction 2; P2: plasma membrane fraction 2. (C) The relative ratio of Ezrin protein in P2 and S2 fractions. *: Compared with control cells, $P<0.01$; **: compared with NE-treated cells, $P<0.01$. (D) The relative ratio of Sec3 protein in P2 and S2 fractions. *: Compared with control cells, $P<0.01$; **: compared with NE-treated cells, $P<0.01$.

Fig. 5. MUC5AC protein levels in 16HBE14o-epithelial cells and in culture supernatant. The data are expressed as the means $\pm$ standard error (SE), $n=6$. (A) The MUC5AC protein level in the culture supernatant was decreased by transfection with Ezrin and Sec3 siRNAs. *: Compared with NE-treated cells, $P<0.05$. When no endogenous PIP2 was added to Ezrin siRNA- or Sec3 siRNA-transfected cells, the MUC5AC protein level in the supernatant did not increase significantly but remained at a lower level. Compared with NE-treated cells, $P<0.05$. However, the MUC5AC protein level in the cytoplasm increased. **: Compared with Ezrin or Sec3 siRNA-transfected cells without PIP2, $P<0.05$. (B) MUC5AC protein level in the culture supernatant decreased when treated with neomycin and PIP2-Ab. ▲: Compared with NE-treated cells, $P<0.01$; *: compared with NE-treated cells, $P<0.05$.

In the present study, we found that PIP2 could increase MUC5AC protein levels in the cytoplasm under NE stimulation in the Ezrin siRNA- and Sec3 siRNA-transfected cells. To further study the role of PIP2, these cells were preincubated with 10 mM neomycin or 20 μg/ml of PIP2-Ab for 30 min before NE stimulation, and the MUC5AC protein levels in the supernatant and cytoplasm were assayed. The results showed that both neomycin and PIP2-Ab decreased the MUC5AC protein level in both the cell culture supernatant and cytoplasm (Fig. 5B).

The above results revealed that exogenous PIP2 could increase the MUC5AC protein level in the cytoplasm but not in the supernatant of Ezrin siRNA- or Sec3 siRNA-transfected

Fig. 6. Immunoprecipitation of Ezrin and Sec3. (A) 16HBE14o- cells were incubated with 0.5 μ M NE or with normal control medium for 30 min, and Ezrin was immunoprecipitated using an anti-Ezrin antibody. The immunoprecipitates were analyzed by immunoblotting with an antibody against Sec3. (B) Complex formation was confirmed by reverse immunoprecipitation and immunoblotting. (C) Cells were transfected with Flag-Ezrin or Flag-Ezrin Δ 1-333 or transfected with PIP2. Ezrin was immunoprecipitated using an anti-Flag antibody. The immunoprecipitates were analyzed by immunoblotting with an antibody against Sec3.

cells under NE stimulation. The inhibition of PIP2 by neomycin and PIP2-Ab caused decreasing the MUC5AC protein levels in both the culture supernatant and cytoplasm. The effects of neomycin and PIP2-Ab on the MUC5AC protein level in the cytoplasm differed from those of Ezrin siRNA and Sec siRNA. This difference may be because PIP2 is an important signal mediator in a series of mucin hypersecretion events initiated by many stimuli, such as proteases [7, 11] and alcohol [23]. PIP2 may be involved in both MUC5AC protein production and secretion in these processes.

NE stimulates Ezrin binding to Sec3, and PIP2 is required for Ezrin-Sec3 binding

We showed that NE stimulated both Ezrin and Sec3. We used immunoprecipitation assays to study their interaction further. Confluent 16HBE14o- cells were starved and stimulated with 0.5 μ M NE for 30 min. Ezrin was immunoprecipitated with an anti-Ezrin antibody. The immunoprecipitates were analyzed by immunoblotting with antibodies to Ezrin and Sec3. The immunoprecipitation experiments showed that Ezrin bound to Sec3 (Fig. 6A and B). Then, we transfected cells with Flag-Ezrin or Flag-Ezrin Δ 1-333; some cells were also treated with 20 μ M PIP2 for further evaluation. These cells were immunoprecipitated using a Flag antibody and then immunoblotted with a Sec3 antibody. We detected Sec3 in the samples immunoprecipitated by Flag, indicating that Ezrin and Sec3 interact under these conditions. For cells transfected with Flag-Ezrin and then stimulated with NE, the immunoprecipitation experiments showed that both Flag and Sec3 were expressed but no Sec3 protein was detected in the Flag-Ezrin Δ 1-333-transfected cells, even when PIP2 was added (Fig. 6C). These results indicated that NE stimulates Ezrin binding to Sec3, that PIP2 is required for Ezrin-Sec3 binding, and that the PIP2 binding site 1-333 in Ezrin is indispensable for PIP2 function.

Discussion

Our results indicated that Ezrin was highly expressed in the bronchi of humans with chronic airway diseases. NE induced robust MUC5AC protein secretion into the supernatant and cell lysates in a 16HBE14o- cell culture model. MUC5AC secretion was impaired in culture supernatants when cells were transfected with Ezrin siRNA, and this effect was detected in Sec3 siRNA-transfected cells and in neomycin- or PIP2-Ab-treated cells. Both Ezrin and Sec3 were involved in MUC5AC secretion; this process primarily recruited Ezrin and Sec3 to the cytoplasmic membrane after NE stimulation, where these proteins combined to form complexes. PIP2 is of critical importance in the formation of these complexes and in MUC5AC secretion.

To our knowledge, this study is the first report of Ezrin activation in mucinocytes in airway epithelial cells. Excessive mucin secretion is a prominent feature in chronic airway diseases and can lead to airway obstruction and bacteria colonization [25]. Mucus exocytosis is a complicated process that involves numerous factors, and this process has not been fully elucidated. We do not know precisely how the polarity of the mucus secretory granules develops or how the cell interacts with exocytosis-related proteins. In addition, mucin stored in the cytoplasm cannot increase suddenly. It has been shown that more mucin is stored in the epithelium per unit surface area of nasal mucosa in the bronchi of smokers with airflow obstruction, indicative of the dysregulation of mucin secretion [26]. Mucin tends to exhibit a burst secretion pattern under pathological conditions or upon exposure to certain stimuli; this pattern differs from the basal pattern of secretion under physiological states.

Ezrin is primarily distributed in secretory cells. It is highly expressed in the epidermis and mesothelium. In the present study, we demonstrated that Ezrin is highly expressed in bronchi from patients with chronic inflammation. In addition, we used NE, which is a well-known potent inducer of mucin secretion, to generate the hypersecretion state. NE induced robust MUC5AC protein secretion into the supernatant and cell lysates, which is consistent with the results of previous studies [27]. Ezrin mRNA expression was upregulated by NE stimulation; western blot assays showed that Ezrin activation also increased significantly and that Ezrin was translocated from the cytoplasm to the cell membrane after NE stimulation.

The ERM family belongs to the talin and band 4.1R (erythrocyte membrane-cytoskeleton linker protein band 4.1R or ERM41) superfamily. Ezrin is an important member of the ERM family. This protein physically binds to actin and serves as a crosslinker between the plasma membrane and the cytoskeleton [28, 29]. The Ezrin (NM-003379) gene is on chromosome 6p15.2 and encodes 587 amino acids [30]. Amino acid residues 1-296 in the N-terminus form a 4.1 FERM (Ezrin/Radixin/Moesin) domain, and residues 1-333 contain a phosphatidylinositol 4,5-bisphosphate (PIP2) membrane binding site, which leads to conformational changes that stabilize the activation state of the C-terminus and which result in the localization of Ezrin at the plasma membrane [31, 32]. Residues 297-460 form the consecutive α -helix domain, which is called the central domain. Residues 460-587 are a cluster of proline residues, which could form a hinge region and thus could affect the conformational changes of Ezrin. The C-terminal ERM association domain (C-ERMAD) of Ezrin is formed by amino acid residues 478-586 and is also called the autoinhibitory terminal tail, which has the ability to bind to the plasma membrane. Amino acid residues 551-586 form the F-actin binding domain [33, 34].

PIP2 can bind to Ezrin primarily through residues 253, 254, 262 and 263 in the NH2-terminus [35]. PIP2 binds to Ezrin, and the phosphorylation of Thr567 is essential for Ezrin to translocate to the apical membrane [36, 37]. We transfected cells with Ezrin siRNA to determine whether Ezrin and PIP2 are involved in NE-induced MUC5AC secretion. Ezrin siRNA transfection reduced the MUC5AC protein level in the culture supernatant but not the intracellular protein level, indicating that Ezrin was involved in NE-induced MUC5AC

protein secretion but not in MUC5AC protein production in the cytoplasm. Then, when exogenous PIP2 was added to Ezrin siRNA-transfected cells before NE stimulation, we found that the MUC5AC protein level in the supernatant remained at a relatively lower level but that the MUC5AC protein level in the cytoplasm increased. Thus, PIP2 participated in Ezrin-mediated MUC5AC protein secretion into the supernatant and might be involved in MUC5AC production in the cytoplasm, which was independent of Ezrin. This finding may be because PIP2 is an important signal mediator in a series of mucin hypersecretion events initiated by many stimuli, such as proteases [22] and cold [23]. In addition, we found that neomycin, which is a PIP2-masking reagent, and PIP2-Ab led to impaired MUC5AC secretion in cells. These results indicate that Ezrin is involved in MUC5AC secretion in 16HBE14o- cells and that PIP2 binding could be necessary for this process.

The exocyst complex is essential for many exocystic events because this complex mediates the transport of secretory vesicles to the plasma membrane for fusion [38], as we described for mucin secretion. The exocyst is a conserved multi-subunit complex involved in the docking of post-Golgi transport vesicles to sites of membrane remodeling during cellular processes such as polarization, migration, and division [39, 40]. The exocyst complex is composed of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84. Sec3 and Exo70 interact with Rho family small GTPases, which are key regulators of polarized cell growth in yeast [41]. Sec3 is regarded as a spatial landmark for the development of polarity in budding yeast and in epithelial cells. This protein has been implicated in coordinating the membrane targeting of the remaining members of the exocyst complex that travel on secretory granules [43].

Sec3 is a docking protein of the exocyst complex that is required for secretion. The PH-like domain, which is formed by amino acids 1-133 at the NH2-terminus, has the ability to bind PIP2, phospholipids, GTPases (Rho1/Cdc42), and the β/γ subunit of G proteins [44]. Therefore, we also studied the Sec3 protein in airway epithelial cells to evaluate whether Ezrin is associated with exocyst-related proteins. Immunofluorescence and immunoblotting assays showed that Sec3 was recruited primarily to the plasma membrane after NE stimulation, which is consistent with the localization of Ezrin. This result is also consistent with those of another study [45]. The PIP2-masking reagent neomycin abrogated this effect. Furthermore, we used an Ezrin deletion (Ezrin $_{\Delta 1-333}$) mutant in this study to confirm the roles of PIP2 in regulating the function of Ezrin. Immunoprecipitation experiments revealed that Ezrin and Sec3 could not form complexes; however, these complexes could not be detected in cells transfected with the Ezrin $_{\Delta 1-333}$ mutant that lacks the PIP2 binding domain, even when exogenous PIP2 was added. These results demonstrate that Ezrin/Sec3 formed a complex and functioned together, contributing to MUC5AC secretion. These proteins did not interact directly but acted as a linker and was required for complex formation and for MUC5AC secretion.

In summary, we presented evidence that the docking protein Sec3 is targeted to plasma membrane and remains enriched in Ezrin after NE stimulation and that PIP2 is required for Sec3 binding to Ezrin. These events were followed by a higher level of MUC5AC secretion into the supernatant than occurs in normal static cells, indicating the involvement of these proteins in the mucus secretion process. The specific functions and co-localization of Ezrin and Sec3 provide new potential therapeutic targets for the intervention of mucus hypersecretion in airway diseases.

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