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Major vault protein regulates cell growth/survival signalling through oxidative modifications

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Abstract

Major vault protein forms a hollow, barrel-like structure in the cell called the vault, whose functions and regulation are not well understood. The present study reports that major vault protein regulates growth/survival signaling in human airway smooth muscle cells through oxidative modifications. The promotion of protein S-glutathionylation by asthma mediators such as interleukin-22 and platelet-derived growth factor or by knocking down glutaredoxin-1 or thioredoxin activated cell growth signaling. Mass spectrometry identified that major vault protein is glutathionylated. Major vault protein knockdown enhanced cell death and inhibited STAT3 and Akt signaling. We identified a protein partner of major vault protein that is regulated by glutaredoxin-1, namely myosin-9, which was found to serve as a cell death factor. Knocking down myosin-9 or promoting protein S-glutathionylation by knocking down glutaredoxin-1 inhibited the death of airway smooth muscle cells by heating to simulate bronchial thermoplasty, a clinically successful procedure for the treatment of severe asthma. These results establish a novel signaling pathway in which ligand/receptor-mediated oxidation promotes the S-glutathionylation of major vault protein, which in turn binds to myosin-9 to suppress the heating-induced death of airway smooth muscle cells.

Keywords

Airway; Glutathionylation; Major vault protein; Myosin-9; Redox signaling; Smooth muscle cells

Introduction

Major vault protein (MVP) forms a hollow, barrel-like structure in the cytosol called the vault, which seems to occur ubiquitously. While the detailed x-ray structure of the vault has been reported [1], the functions and regulation of MVP remain unclear. MVP is known to be

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identical to lung resistance-related protein [2], a major multidrug resistance protein that was originally found in lung cancer cells [3]. MVP is overexpressed in drug-resistant human cancer cells [3,4], and vaults are thought to export drugs from the nucleus for sequestration in cytosolic vesicles (5–7). In drug-resistant and MVP-overexpressing non-small cell lung cancer cells, MVP is co-localized with anti-tumor drugs [8], and the siRNA knockdown of MVP promotes drug accumulation and cell death [9].

In addition to the deleterious actions of reactive oxygen species (ROS), it is now well accepted that another functional role of ROS is to mediate cell signaling [10]. In airway smooth muscle cells (SMCs), growth and survival signaling induced by ligand/receptor interactions is mediated by ROS [11–14]; however, the mechanism of ROS signaling in airway SMCs has not been well defined. Oxidation can result in protein *S*-glutathionylation, in which glutathione (GSH) is added to cysteine residues. *S*-glutathionylation reactions are regulated by deglutathionylating proteins including glutaredoxin-1 (Grx1) and thioredoxin (Trx) [15]. Understanding the process of the ROS-mediated growth and survival of airway SMCs should thus be important for the regulation of airway remodeling in asthma.

In an effort to understand the mechanism of ROS signaling in airway SMCs, the present study investigated the role of protein *S*-glutathionylation and Grx1. The experiments resulted in the discovery that MVP is expressed in human airway SMCs and that MVP is oxidatively modified via protein *S*-glutathionylation in response to ligand/receptor-mediated cell signaling. We provide evidence that this mechanism serves to inhibit the heating-induced death of airway SMCs, a process that occurs in bronchial thermoplasty, a clinically successful procedure for the treatment of asthma.

Materials and Methods

Cell culture

Human bronchial SMCs from ScienCell Research Laboratories (Carlsbad, CA, USA) were cultured in accordance with the manufacturer's instructions in 5% CO₂ at 37°C. Cells in passages 3–7 were used. Cells were growth-arrested for 2 days in media containing 0.01% fetal bovine serum for proliferation assays or serum-starved overnight for signaling studies. Cells were treated with recombinant human IL-22 (PeproTech, Inc., Rocky Hill, NJ, USA) or human recombinant PDGF-BB (Invitrogen, Carlsbad, CA, USA). For siRNA knockdown, cells were transfected with an siRNA Transfection Reagent and gene silencing siRNAs along with the controls by using a scrambled sequence from Santa Cruz Biotechnology (Dallas, TX, USA). Cells were used for experiments 2 days after transfection.

Immunoblotting and immunoprecipitation

Cell lysates were prepared and immunoblotting was performed as previously described [16]. The antibodies used were phospho-STAT3 (Tyr705), phospho-Akt (Ser473), cleaved caspase-3, LC3B (Cell Signaling Technology, Danvers, MA, USA), GAPDH, MVP, myosin-9, GSH (Santa Cruz Biotechnology and EMD Millipore Corporation, Billerica, MA, USA) and p62/SQSTM1 (Syd Labs Inc., Natick, MA). Electrophoresis through an SDS-polyacrylamide gel was followed by electroblotting onto a nitrocellulose membrane. The

Enhanced Chemiluminescence System (Amersham Biosciences, Piscataway, NJ, USA) was used for detection.

For immunoprecipitation, cell lysates were incubated with 1 μ l of antibodies and 10 μ l GammaBind G-Sepharose (Amersham) overnight at 4°C with gentle shaking. After washing twice, the pellet was boiled in Laemmli buffer and centrifuged, and the supernatant was subjected to reducing 12% SDS-polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane. The membrane was then blocked with 5% milk for 1 h at room temperature and incubated overnight at 4°C with antibodies, followed by incubations with HRP-conjugated secondary antibodies and ECL detection.

Mass spectrometry

Protein bands were excised from the Coomassie Blue-stained gels. Mass spectra were recorded by using a matrix-assisted laser desorption/ionization–time of flight, time of flight (MALDI-TOF-TOF) spectrometer (4800 Proteomics Analyzer, Framingham, MA, USA). Peptide masses were compared with the theoretical masses derived from the sequences contained in SWISS-PROT/NCBI databases by using MASCOT.

Statistical analysis

Means $\pm$ SEM were calculated. The comparisons between two groups were analyzed by using a two-tailed Student's *t* test, while those between three or more groups were analyzed by using two-way ANOVA with a Student-Newman-Keuls post-hoc test with the Kolmogorov-Smirnov test for normality. *P* < 0.05 was considered to be significant.

Results

Role of glutathionylation in growth/survival signaling in airway SMCs

Protein S-glutathionylation can be reversed by Grx1 or Trx. STAT3, an important growth/survival signaling molecule, was phosphorylated and activated in response to the siRNA knockdown of Grx1 (Fig. 1A; Supplemental Fig. S1A) or Trx (Fig. 1B; Supplemental Fig. S1B). When these proteins were re-expressed, STAT3 phosphorylation states were normalized (Supplemental Fig. S2). By contrast, the siRNA knockdown of the Trx-interacting protein (TXNIP; Supplemental Fig. S1C), an endogenous inhibitor of Trx, decreased the phosphorylation states of STAT3 (Fig. 1C). Treatment with PDGF or IL-22, a potent cell growth/survival mediator of airway SMCs [14,17], activated STAT3 (Fig. 1D). The GSH antibody from Santa Cruz Biotechnology specifically detected ~110 kDa protein that forms a GSH adduct in response to IL-22 treatment of cells (Fig. 1E).

This antibody was used to identify the glutathionylated protein. Cells were treated with IL-22, cell lysates were immunoprecipitated with the GSH antibody, and glutathionylated protein bands were visualized by Coomassie Blue staining (Fig. 2A) and Silver staining (data not shown). The GSH-protein adduct formation of the band with a molecular weight of ~110 kDa was consistently increased by IL-22 (Fig. 2A). This band was analyzed by mass spectrometry and was identified to contain MVP. The glutathionylation of MVP was confirmed by immunoprecipitating the cell lysate samples with the GSH antibody and

immunoblotting with the MVP antibody. The glutathionylation of MVP was found to occur within 10 min of IL-22 or PDGF treatment (Fig. 2B). MVP glutathionylation was promoted by the siRNA knockdown of Grx1 (Fig. 2C) or Trx (Fig. 2D). The siRNA knockdown of a TRX inhibitor, TXNIP, attenuated MVP glutathionylation induced by IL-22 (Fig. 2E) or PDGF (Fig. 2F).

MVP is a cell growth/survival factor for airway SMCs

To determine the functions of MVP in airway SMCs, siRNA was employed to knockdown MVP. MVP knockdown inhibited basal as well as IL-22- or PDGF-inducible STAT3 phosphorylation (Figs. 3A and 3B). Similarly, MVP knockdown inhibited the phosphorylation and activation of Akt, another cell growth/survival factor (Figs. 3C and 3D). Fig. 3E shows the extent of the siRNA knockdown of MVP in these experiments. The suppression of STAT3 phosphorylation was not observed in cells, in which MVP was re-expressed (Supplemental Fig. S3).

Further, MVP knockdown reduced the number of growing airway SMCs (Fig. 4A; Supplemental Fig. S4). To define the mechanism of cell reduction in response to MVP knockdown, we tested the hypothesis that apoptosis is promoted. MVP knockdown activated the cleavage of caspase-3, a hallmark of apoptosis (Fig. 4B). It also resulted in the activation of autophagy markers, including the formation of LC3B-II (Fig. 4C) and the downregulation of p62 (Fig. 4D). Conversely, the overexpression of MVP upregulated p62 (Fig. 4E).

Identification of the MVP/LRP-binding protein regulated by Grx1

To understand the function of the Grx1-regulated glutathionylation of MVP, cells were treated with siRNA to knockdown Grx1 or to randomly control siRNA. Cell lysates were then immunoprecipitated with MVP and the possible occurrence of Grx1-regulated protein/protein interactions with MVP was examined. We found that a slightly greater than 200 kDa band was consistently upregulated in association with MVP in response to Grx1 knockdown (Fig. 5A). Mass spectrometry identified that the Grx1-regulated MVP-binding protein is myosin 9. Immunoprecipitation/immunoblotting confirmed the mass spectrometry results by showing that Grx1 knockdown indeed caused protein-protein interactions between MVP and myosin-9 (Fig. 5B). While the functions of myosin-9 are not well understood, it has been reported that myosin-9 is cleaved during cell death [18] and that it interacts with vsp34, a protein that regulates beclin-1, a key mediator of autophagy [19]. Thus, we hypothesized that the glutathionylation-dependent interactions of MVP with myosin-9 may function to protect against its cell death actions.

To define the functions of protein S-glutathionylation, MVP, and myosin-9 in the regulation of the death of airway SMCs in clinically relevant scenarios, we established a cell culture model that simulated bronchial thermoplasty, a clinically successful procedure for the treatment of asthma [20]. Bronchial thermoplasty delivers radio frequency energy to heat the airway to the target temperature of 65°C. Killing human bronchial SMCs by heating at this temperature was protected by knocking down myosin-9 (Fig. 5C; Supplemental Fig. S5). The action of myosin-9 seems to be related to autophagic cell death as the heating-induced downregulation of p62 was blocked by knocking down myosin-9 (Fig. 5D). Interestingly,

the role of myosin-9 to promote cell death appears to be specific to heating-induced effects, since myosin-9 knockdown did not inhibit, rather potentiated, cell death as well as p62 downregulation induced by hydrogen peroxide (Supplemental Fig. S6).

Role of S-glutathionylation in the heating-induced death of airway SMCs

To determine whether the heating-induced death of airway SMCs is regulated by S-glutathionylation, Grx1 was knocked down by siRNA. Grx1 knockdown inhibited the heating-induced death of human bronchial SMCs (Fig. 6A; Supplemental Fig. S7). Similarly, knocking down Trx also inhibited the heating-induced death of human bronchial SMCs (Fig. 6B). Grx1 knockdown also attenuated the heating-induced autophagic responses including the formation of LC3B-II (Fig. 6C) and the downregulation of p62 (Fig. 6D).

Discussion

ROS have been implicated in the growth of airway SMCs [11–14]. The present study further extended the role of Grx1-regulated S-glutathionylation in the regulation of cell growth and survival. The major finding is that Grx1 knockdown promotes the S-glutathionylation of a protein called MVP. While the existence of MVP is not well known, this protein is the major component of the largest ribonucleoprotein structure in eukaryotic cells that ubiquitously occurs called the vault, which may have an important biological roles [1]. To our knowledge, this is the first demonstration of the redox regulation of MVP.

While the biologic functions of MVP are incompletely understood, MVP was found to be identical to lung resistance-related protein [3]. In contrast to evidence for the role of MVP in multidrug resistance in cancer cells, some studies have reported results that do not support this hypothesis. Siva et al. [21] reported that the upregulation of vaults was not sufficient to induce multidrug resistance. These findings can be explained by the concept that the post-translational activation of MVP/LRP may be needed. Thus, the finding in the present study that MVP is post-translationally modified via oxidation may be important to understand the mechanisms and functions of MVP.

The present study also demonstrated that in airway SMCs, MVP serves as a cell survival factor. Knocking down MVP results in the inhibition of cell survival signaling such as STAT3 and Akt as well as the promotion of cell death that is associated with apoptosis and autophagy. We identified that myosin-9 is a novel protein partner of MVP and that their interactions are regulated in a glutathionylation-dependent manner. Myosin-9 is also known as nonmuscle myosin heavy chain IIa [22], which does not function in muscle contraction. This novel protein has been reported to play roles in migration and cytokinesis. This study provided evidence for a new function of myosin-9, namely to serve as a death factor, as the siRNA knockdown of this protein inhibited cell death. As p62 downregulation (an indicator of the occurrence of autophagy) induced by heating airway SMCs is also inhibited by myosin-9 knockdown, this protein may regulate autophagy. The mechanism of this regulation may involve myosin-9 forming protein-protein interactions with Vps34, which binds to an autophagy regulator beclin-1 [19].

Mechanistic information obtained from the present study may be clinically important in the management of asthma. Most asthma symptoms can be treated by the use of corticosteroids and β_2 -receptor agonists. However, ~5% of asthma patients suffer from persistent symptoms, airflow limitation, frequent exacerbations, and increased medication requirement. In 2010, the FDA approved the use of bronchial thermoplasty to treat these severe asthma patients. This invasive treatment applies thermal energy to the airway wall through a bronchoscope and reduces the airway smooth muscle mass. It has been reported that this treatment resulted in 325 reductions in asthma attacks, an 84% reduction in emergency room visits, a 66% reduction in days lost from work, school, or other daily activities, and a 73% reduction in hospitalizations [20]. However, the current bronchial thermoplasty strategy only partially reduces the airway smooth muscle mass, thus improving this strategy may be helpful in managing this disease. Despite the clinical success of this treatment strategy, mechanisms of how bronchial thermoplasty works, including the mechanism of SMC killing are undefined. The present study used a cell culture model to heat airway SMCs to, in part, simulate bronchial thermoplasty. We found that the oxidative modifications of MVP regulate the resistance of airway SMCs to heating-induced death. We also provided evidence that Grx1 can support heating-induced cell death.

In summary, this study presented a novel redox-regulated mechanism of a protein that comprises the vault, the largest ribonucleoprotein structure in eukaryotic cells, forming a hollow, barrel-like structure. We propose a mechanism in which the ROS-mediated S-glutathionylation of MVP promotes the binding of myosin-9, interfering with the ability of myosin-9 to promote cell death signaling. We also present an idea that Grx1, which can reverse this cell survival/resistance mechanism, can support cell killing strategies to reduce airway smooth muscle thickening.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Growth factors promote *S*-glutathionylation of major vault protein in airway smooth muscle cells.
- Major vault protein mediates cell growth and survival.
- Myosin-9 interacts with major vault protein in an *S*-glutathionylation-dependent fashion.
- Myosin-9 participates in the cell death mechanism that is induced by heating, but not by hydrogen peroxide.
- Glutaredoxin 1 regulates airway smooth muscle cell growth and death.

Fig. 1. Protein S-glutathionylation confers cell growth/survival/signaling

(A – C) Human bronchial SMCs were transfected with control siRNA and siRNAs to knockdown (A) Grx1 (n=6), (B) Trx (n=6), or (C) TXNIP (n=6) to monitor phosphorylated STAT3 (pSTAT3) by immunoblotting. (D) Human bronchial SMC's were treated with IL-22 (10 ng/ml) and PDGF (10 ng/ml) for durations indicated. Cell lysates were subjected to immunoblotting to monitor phosphorylated STAT3 (n=4). (E) Human bronchial SMC's were treated with IL-22 (10 ng/ml) for 10 and 30 min, levels of GSH protein adducts were monitored by nonreducing SDS-PAGE and immunoblotting using the GSH antibody (n=4). Bar graphs represent mean $\pm$ SEM. * denotes values are significantly different from control at $p < 0.05$.

Fig. 2. MVP is glutathionylated

(A) Human bronchial SMCs were treated with or without IL-22 (10 ng/ml) for 10 min, and cell lysates were immunoprecipitated with GSH antibody and subjected to SDS-PAGE. GSH-protein adducts were visualized with Coomassie Blue staining. The band indicated underwent mass spectrometry analysis, and the protein was identified to be MVP. (B) Cells were treated with IL-22 or PDGF (10 ng/ml) for 10, 30 and 60 min, lysates were immunoprecipitated with the GSH antibody, followed by nonreducing SDS-PAGE and immunoblotting with the MVP antibody (n=3). (C & D) Cells were transfected with control siRNA and siRNA to knockdown Grx1 or TRX (n=6). (E) Cells were transfected with TXNIP siRNA and treated with IL-22 or PDGF (10 ng/ml), cell lysates were immunoprecipitated with the GSH antibody, followed by immunoblotting with MVP (n=3). Bar graphs represent means $\pm$ SEM. * denotes values significantly different from control, and ** denotes values significantly different from each other at $P < 0.05$.

Fig. 3. MVP mediates airway smooth muscle cell growth/survival signaling

Human bronchial SMC's were transfected with control siRNA and MVP siRNA for 48 h. Cells were then treated with 10 ng/ml IL-22 (A & C) or PDGF (B & D) for 10 and 30 min. Cell lysates were subjected to immunoblotting to monitor phosphorylated STAT3 (A & B) and phosphorylated Akt (C & D). (E) Cell lysates were subjected to immunoblotting to show the extent of MVP knockdown. Bar graphs represent means $\pm$ SEM (n=6). * denotes values significantly different from each other.

Fig. 4. MVP protects against cell death

(A – D) Human bronchial SMCs were transfected with control siRNA or siRNA to knockdown MVP. The cell number was determined by counting on a hemocytometer. Cell lysates were subjected to immunoblotting to monitor cleaved caspase-3, LC3B-II expression, and p62 expression. (E) Cells were treated with adenovirus (Ad) expressing MVP, and the p62 expression was monitored. Bar graphs represent means $\pm$ SEM (n=6). * denotes values significantly different from control at $P < 0.05$.

Fig. 5. MVP interacts with myosin 9 to mediate cell death in a Grx1 dependent manner
Human bronchial SMCs were transfected with control or Grx1 siRNA. Cell lysates were immunoprecipitated with the MVP antibody and subjected to SDS-PAGE and Coomassie Blue staining. The band around 200kDa (indicated by an arrow) was found to be increased in a Grx1 dependent fashion ($n = 3$). Mass spectrometry identified this protein to be myosin 9. (B) The MVP interacting band that was detected as myosin 9 was confirmed using immunoblotting with myosin 9 antibody. (C) Human bronchial SMCs transfected with control siRNA or siRNA to knockdown myosin 9 siRNA. Cells were then heated at 65°C for 10 sec and incubated at 37°C for 2 h. The cell number was determined by counting on a hemocytometer ($n = 4$). (D) Cell lysates were subjected to immunoblotting to monitor the expression of p62 ($n=4$). Bar graphs represent means $\pm$ SEM. * denotes values significantly different from each other.

Fig. 6. Role of protein S-glutathionylation in regulating heating-induced death of airway SMCs
 (A & B) Human bronchial SMCs were transfected with control siRNA and siRNA to knockdown Grx1 or TRX. Cells were then heated at 65°C for 10 sec and incubated at 37°C for 2 h. Cell number was determined by counting on a hemocytometer. (C) Cells were transfected with control siRNA and siRNA to knockdown Grx1. Cells were then heated at 65°C for 10 sec and incubated at 37°C for 2 h. Cell lysates were subjected to immunoblotting to monitor the expression of LC3B-II. (D) These cell lysates were subjected to immunoblotting to monitor p62 expression. Bar graphs represent means $\pm$ SEM (n = 4). * denotes values significantly different from each other.