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VAMP8 is a vesicle SNARE that regulates mucin secretion in airway goblet cells

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Non-technical summary Mucin secretion in the lung is regulated by the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) exocytotic core, which has not been defined in airway goblet cells. In this study, the SNARE vesicle-associated membrane protein 8 (VAMP8) was found to be expressed in human airway epithelial goblet cells. VAMP8 knockdown by RNA interference techniques reduced airway epithelial mucin secretion induced by PAR agonists, neutrophil elastase and ATP. Basal (non-agonist elicited) mucin secretion was also reduced as a result of VAMP8 knockdown. Importantly, mucin secretion was reduced in the lungs of VAMP8 knockout mice compared to wild-type littermates. Our data suggest that VAMP8 is an essential SNARE in airway mucin granule exocytosis. Reduction of VAMP8 activity/expression may provide a novel therapeutic target to ameliorate airway mucus obstruction in lung diseases.

Abstract Mucin secretion is an innate defence mechanism, which is noxiously upregulated in obstructive lung diseases (e.g. chronic obstructive pulmonary disease (COPD), cystic fibrosis and asthma). Mucin granule exocytosis is regulated by specific protein complexes, but the SNARE exocytotic core has not been defined in airway goblet cells. In this study, we identify VAMP8 as one of the SNAREs regulating mucin granule exocytosis. VAMP8 mRNA was present in human airway and lung epithelial cells, and deep-sequencing and expression analyses of airway epithelial cells revealed that VAMP8 transcripts were expressed at 10 times higher levels than other VAMP mRNAs. In human airway epithelial cell cultures and freshly excised tissues, VAMP8 immunolocalised mainly to goblet cell mucin granules. The function of VAMP8 in airway mucin secretion was tested by RNA interference techniques. Both VAMP8 short interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs) reduced mucin secretion induced by PAR agonists, neutrophil elastase and ATP in two airway epithelial cell culture models. Notably, basal (non-agonist elicited) mucin secretion was also reduced in these experiments. VAMP8 knockdown was also effective in decreasing mucin secretion in airway epithelial cell cultures with induced mucous metaplasia/mucin hypersecretion. Unlike VAMP8 silencing, knockdown of VAMP2 or VAMP3 did not affect mucin secretion. Importantly, in VAMP8 knock-out (KO) mice with IL-13-induced mucous metaplasia, mucin content in the bronchoalveolar lavage (BAL) and ATP-stimulated mucin secretion in the trachea were reduced compared to WT-matched littermates. Our data indicate that VAMP8 is an essential SNARE in airway mucin granule exocytosis. Reduction of VAMP8 activity/expression may provide a novel therapeutic target to ameliorate airway mucus obstruction in lung diseases.

Abbreviations AB-PAS, Alcian blue periodic acid Schiff; ATPγS, adenosine triphosphate-γ-sulfate; BAL, broncho-alveolar lavage; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; COPD, chronic obstructive pulmonary disease; KO, knockout; MARCKS, myristoylated, alanine-rich C-kinase substrate; MCC, mucociliary clearance; PABH, periodic acid biotin hydrazide; PAR, protease activated receptor; qRT-PCR, quantitative RT-PCR; RPKM, read per kilobase of exon model; shRNA, short hairpin RNA; siRNA, short interfering RNA; SMM, supernatant of mucopurulent material from CF lungs; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; t-SNARE, target membrane SNARE;VAMP8, vesicle-associated membrane protein 8; v-SNARE, vesicle membrane SNARE; WD-HBE cells, well-differentiated primary human bronchial epithelial cells.

Introduction

Mucociliary clearance (MCC) is a key innate defence mechanism of human airways (Boucher, 2007). A major element of the MCC mechanism is the thin mucus layer covering the airway surface, which provides hydration, lubrication and clearance of particles and pathogens from the airways. The surface mucus layer is composed of water, electrolytes and macromolecules, but gel-forming mucins (i.e. MUC5AC and MUC5B), which are complex glycosylated macromolecules, are responsible for the biophysical characteristics of the mucus film. Not surprisingly, the secretion of mucins into the airway lumen is a tightly regulated process, occurring via calcium-dependent exocytosis of specialised granules from epithelial goblet (mucous) cells, and dysregulated mucin secretion adversely perturbs MCC activities leading to the development of obstructive lung disease (Kreda *et al.* 2007, 2010*b*; Davis & Dickey, 2008; Evans & Koo, 2009).

Mucin secretion is a multi-step process and requires the recruitment of specific proteins that facilitate the exocytosis of the mucin granule, but only a few of these proteins have been identified in airway goblet cells. Munc 13-2, which is activated by diacylglycerol mobilised during agonist stimulation, is a priming protein for mucin granule exocytosis, and deletion of Munc 13-2 affects tonic mucin secretion in the mouse airways (Zhu *et al.* 2008). Synaptotagmin 2, one of three low-affinity Ca^{2+} sensors that trigger fast synaptic vesicle release in neurons, mediates acute agonist-stimulated mucin secretion in mouse airway goblet cells (Tuvim *et al.* 2009). The myristoylated, alanine-rich C-kinase substrate (MARCKS) protein has also been implicated in the priming of the mucin granule (Li *et al.* 2001), and an inhibitory MARCKS peptide has been shown to decrease airway mucin secretion in an asthma mouse model (Singer *et al.* 2004). However, the SNARE proteins, which associate to form the minimal exocytotic machinery – the 'exocytotic or SNARE core', have not been identified in airway goblet cells. In neuronal and exocrine secretory cells, the exocytotic core is formed by the specific interaction of two or three different t-SNAREs (target membrane SNAREs) or Q-SNAREs with one v-SNARE (vesicle membrane SNARE) or R–SNARE (Burgoyne & Morgan, 2003; Davis & Dickey, 2008).

Endobrevin, or VAMP8 (vesicular associated membrane protein 8), is a v-SNARE. Initially, VAMP8 was identified as an endosomal v-SNARE, but more recent data demonstrated that it might not be essential for endocytosis (Wang *et al.* 2004). Immunohistochemical studies show that VAMP8 is expressed in exocrine tissues such as pancreatic, salivary, lachrymal, sweat, sebaceous, mammary and prostate glands (Wang *et al.* 2007). A major physiological role of VAMP8 has been recently revealed by studies in the VAMP8 knockout (KO) mouse, which indicated that VAMP8 is a crucial vesicular SNARE in regulated exocytosis from exocrine cells (Wang *et al.* 2004). For example, the VAMP8 KO mouse exhibited reduced agonist-elicited secretion of zymogen granules from pancreatic acinar cells and dense granules from salivary and lachrymal acinar cells (Wang *et al.* 2004, 2007). VAMP8 deficiency also affects agonist-stimulated secretion of histamine granules from mast cells *ex vivo* and *in vivo*, and regulated secretion of dense and α-granules from platelets and thrombus formation after injury *in vivo* (Ren *et al.* 2007; Tiwari *et al.* 2008; Graham *et al.* 2009). Regulated secretion from endocrine pancreatic cells or of cytokines/chemokines from mast cells was not affected in the VAMP8 KO mouse (Wang *et al.* 2004; Tiwari *et al.* 2008). Thus, VAMP8 appears to display tissue specificity for regulated exocytotic pathways (Tiwari *et al.* 2008).

We recently discovered that VAMP8 is associated with mucin granules purified from airway goblet cell-like Calu-3 cells (Kreda *et al.* 2010*b*). Moreover, VAMP8 was associated with mucin granules in resting goblet cells, but was diffusely distributed within the cells after agonist-stimulated exocytosis of mucin granules (Kreda *et al.* 2010*b*). Thus, it is plausible that VAMP8 is involved in the regulation of mucin granule exocytosis in the airway goblet cell. In this study, we tested the hypothesis that VAMP8 is a critical element for mucin exocytosis in airway goblet cells using RNA interference techniques. We then explored the role of VAMP8 mediating mucin secretion in the VAMP8 KO mouse. Identification of essential regulators/mediators of mucin granule exocytosis is important for designing effective therapies to ameliorate mucin secretion in mucus hypersecretory and obstructive lung diseases.

Methods

Human cell culture and tissues

Calu-3 cells were cultivated as described in Kreda *et al.* (2007 and 2010*b*). Well-differentiated human bronchial epithelial (WD-HBE) cell cultures were grown on collagen-coated permeable supports (Corning Inc.; Corning, NY, USA) under air–liquid interface conditions, as previously described in Kreda *et al.* (2005 and 2010*b*). For IL-13 studies, WD-HBE cells were incubated with IL–13 (basolateral, $10 \, \text{ng ml}^{-1}$) in growth media for 3–5 days after differentiation as in Okada *et al.* (2011). For supernatant of mucopurulent material (SMM) treatment, WD-HBE cells were treated apically with SMM for 5 days as described previously (Ribeiro *et al.* 2005). Human airway epithelial tissues for immunocytochemistry and cell culture were obtained from excess tissue from cystic fibrosis patients undergoing lung transplant and tissues from normal donors, according to the guidelines of the Institutional Review Board for Protection of Human Rights at the University of North Carolina at Chapel Hill.

VAMP8 knockout animals

The VAMP8 knockout (KO) mouse was obtained from Dr Sidney Whiteheart (University of Kentucky) and has been previously described (Wang *et al.* 2004). Mice were housed at the UNC Chapel Hill animal facility on a 12 h day–night cycle, and they had access to a regular chow diet and water *ad libitum*. Animals heterozygous for the VAMP8 deletion were bred to generate wild-type (WT), heterozygous (HET) and homozygous (KO) littermates. VAMP8 KO were born at Mendelian rates, but exhibited ≤30% mortality within the first 2 weeks of life as previously reported (Wang *et al.* 2004). To induce airway mucous metaplasia, IL-13 treatment was performed on 8–week-old mice by intratracheal instillation of $50 \, \mu\text{l}$ of PBS containing $1 \, \mu\text{g}$ murine recombinant IL-13 or PBS alone as described in Zhu *et al.* (2008). Three days after IL-13 administration, mice were killed by exsanguination under deep avertin (2,2,2-tribromoethanol) anaesthesia administered I.P. ($500 \, \text{mg (kg body weight)}^{-1}$) in accordance with UNC-CH (USA) and UK regulations on animal experimentation; the authors have read, and the experiments comply with the policies and regulations of *The Journal of Physiology* given by Drummond (2009). The chest cavity was opened and tracheas were quickly dissected from the larynx to the point just before the tracheal carina, and kept in ice-cold Hank's buffered saline solution with $1.6 \, \text{mM}$ calcium, $1.8 \, \text{mM}$ magnesium and $25 \, \text{mM}$ Hepes (pH 7.4) (HBSS) until the experiments (see below). After dissecting the trachea, broncho-alveolar lavage (BAL) was performed as described in Mall *et al.* (2008) and Zhu *et al.* (2008).

All animal studies were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill and performed according to the principles outlined by the Animal Welfare and the National Institutes of Health guidelines for the care and use of animals in biomedical research.

Short interfering RNA (siRNA) transfection of airway epithelial cells

For transient siRNA transfections, Calu-3 cells were dissociated by trypsinization and transfected with four ON-TARGET plus siRNAs for VAMP8, VAMP2 or VAMP3 (Dharmacon; Lafayette CO, USA) using the AMAXA System ($1 \, \mu\text{g}$ RNA per 10^6 cells; Lonza, Walkersville, MD, USA; per manufacturer instructions) as described in Seminario-Vidal *et al.* (2009). GAPDH siRNA or siGlo Green ($1 \, \mu\text{g}$ RNA per 10^6 cells) were used as controls. Cells were cultured for at least 96 h before they were assayed for mRNA and protein expression, and mucin secretion. Effective siRNA sequences for VAMP8 knockdown ('A': GGGAAAACUUGGAACAUCU; 'B': CCACUGGUGCCUUCUCUUA; 'C': GUCCUUAUC UGCGUGAUUG) were cloned as shRNAs (see below).

Short hairpin RNA (shRNA) vector expression in airway epithelial cells

VAMP8 shRNA expression was achieved in Calu-3 cells by infecting partially confluent Calu-3 cells with retroviral pSIREN vectors (Clontech, Mountain View, CA, USA). Cells infected with empty vector or a vector encoding an effective VAMP8 siRNA sequence were selected with puromycin, and used within the first 10 passages. For VAMP8 shRNA expression in WD-HBE cell cultures, passage 1 undifferentiated HBE cells grown on plastic dishes were infected with mock or VAMP8 shRNA-encoding pSIREN vectors. Cells were selected with puromycin, passaged onto collagen-coated permeable supports, and grown under air–liquid interface for 4–6 weeks for epithelial differentiation prior to the experiments.

Quantitative (q)RT-PCR

Quantitative RT-PCR was performed to evaluate mRNA levels, as described previously (Seminario-Vidal *et al.* 2009; Kreda *et al.* 2010*b*). The primers for qRT-PCR were as follows: human VAMP8 forward: 5′-catctccgcaacaagacaga and reverse: 5′-gaccctcctggcacacattt.; human VAMP-2 forward: 5′-ctttccgtcccgggcagcc and reverse: 5′-gcggt agcagacatggcggg; human VAMP-3 forward: 5′-gccgtc ccacccatctccct and reverse: 5′-tcccgattgcccacatcttgc;

primers for human VAMP-7, MUC5AC and MUC5B were purchased from Applied Biosystems (Carlsbad, CA, USA).

mRNA deep sequencing of airway epithelial cells

Total RNA was isolated from Calu–3 cells, as described in Seminario-Vidal *et al.* (2009) and Kreda *et al.* (2010*b*). RNA integrity was evaluated by running 100 ng of RNA on an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies; Santa Clara, CA, USA); the RNA integrity number was 10, the highest quality number. The RNA library was prepared using 2 μg of total RNA and Illumina's mRNA Sequencing Sample Preparation kit (Illumina; San Diego, CA, USA). The resulting library quality was validated on an Agilent Bioanalyzer using an Agilent DNA-1000 chip. The size, purity and concentration of the library met all criteria for mRNA sequencing. The library was sequenced in the UNC High Throughput Sequencing Facility (UNC Chapel Hill), using Illumina 2X36 sequencing. Data were compiled using the updated human genomic database.

Immunofluorescence analyses

Human tissues and WD-HBE cells were fixed in buffered formalin and embedded in paraffin. Tissue and cell culture sections were deparaffinised, subjected to epitope retrieval (Dako, Carpinteria, CA, USA; per manufacturer's instructions) (Kreda & Gentzsch, 2011), and immuno-stained with antibodies against VAMP8 and human MUC5AC, as described previously (Kreda *et al.* 2005, 2010*b*). Immunofluorescence analyses were conducted in a Leica SP5 confocal microscope system with three independent laser sources (Leica; Heidelberg, Germany).

Mucin secretion experiments in airway epithelial cells

Mucin secretion was measured in Calu-3 and WD-HBE cells, as we previously described in Kreda *et al.* (2007 and 2010*b*). Briefly, basal secretion was estimated by incubating cultures in HBSS for at least 2 h at 37°C in the absence of agonist stimulation. Agonist-stimulated mucin secretion was assessed by incubating the cell cultures for an additional 10–20 min with agonists (or vehicle, as a control), as indicated in the figure legends. Cell culture supernatants were collected in basal and agonist-stimulated conditions, and mucin secretion was assessed by slot blot using an antibody against human MUC5AC, as we previously described in Kreda *et al.* (2007 and 2010*b*). In some experiments, slot blots were developed by the periodic acid biotin hydrazide (PABH) method to assess total secreted mucins (Conway *et al.* 2003).

At the end of each experiment, WD-HBE cell cultures were incubated undisturbed for at least 24 h before they were fixed, paraffin-embedded and stained using the Alcian blue periodic acid Schiff (AB-PAS) method (Zhu *et al.* 2008); staining quantification was performed in three to five fields per culture, three cultures per condition, using Adobe Photoshop software. Calu-3 cells, undisturbed for at least 24 h, were fixed and stained with a (human) MUC5AC antibody; staining quantification was performed as described in Kreda *et al.* (2010*b*). Undisturbed cultures (>24 h) were lysed (Kreda *et al.* 2005) and assayed for VAMP and MUC5AC protein levels (Kreda *et al.* 2007, 2010*b*).

Mucin exocytosis in *ex vivo* mouse tracheas

Tracheas quickly removed from WT and VAMP8 HET and VAMP8 KO mice were incubated in HBSS with or without 1 mM ATP for 20 min at 37°C. Tracheas were fixed in buffered formalin, paraffin-embedded, sectioned along the longitudinal axis (5–μm-thick sections), and stained with AB-PAS to label mucous cells using standard histological protocols. Three images per trachea were obtained in a Nikon Microphot SA with a DXM 1200 camera (Melville, NY, USA) using identical conditions throughout the study. AB-PAS staining was quantified using Adobe Photoshop software by thresholding the AB-PAS staining and quantifying the number of pixels per length unit of trachea.

Mucin content in mouse BALs

BAL samples were processed to solubilize mucins and separated in a 1% agarose gel (1 g (100 ml)$^{-1}$) and transferred onto a blotting membrane as described in Livraghi *et al.* (2009). To assess MUC5B, membranes were blotted with polyclonal antibodies that specifically recognize mouse MUC5B (Zhu *et al.* 2008) followed by a fluorescently labelled secondary antibody, and membranes were visualized using a LI-COR infrared scanner system (LI-COR Biosciences) and quantified as previously described (Kreda *et al.* 2010*b*).

Reagents

ATPγS was obtained from Sigma (St Louis, MO, USA); human neutrophil elastase was from Elastin Products (Owensville, MI, USA); human α-thrombin was from Enzyme Research Laboratories (South Bend, IN, USA); IL-13 was from PeproTech (Rocky Hill, NJ, USA); MUC5B antibodies were a kind gift from Dr C. Ehre (Zhu *et al.* 2008), MUC5AC antibodies (Kreda *et al.* 2007) were purchased from LabVision (Fremont, CA, USA). VAMP-8 antibodies (Kreda *et al.* 2010*b*), VAMP2 and

VAMP3 antibodies were from Abcam (USA) and Synaptic Systems (Germany). Secondary antibodies were from Jackson ImmunoResearch Labs (West Grove, PA, USA) and LI-COR (Lincoln, NE, USA). PAR1 and PAR2 peptides were prepared as in Kreda *et al.* (2010*b*). VAMP8, VAMP2 and VAMP3 siRNAs, and siGlo Green were purchased from Dharmacon (Lafayette, CO, USA). Other chemicals were of the highest purity available and from sources previously reported.

Statistics

Student's paired *t* test was performed using Excel 2010; $P < 0.01$ was accepted to indicate statistical significance.

Results

VAMP8 is expressed in airway goblet cells

We have recently reported that VAMP8 was present in mucin granules purified from Calu-3 cells, a cell culture model of airway goblet cells (Kreda *et al.* 2010*b*). Immunostaining studies in well-differentiated primary human bronchial epithelial (WD-HBE) cell cultures indicated that VAMP8 was localised almost exclusively to mucin granules in goblet cells (identified with a MUC5AC antibody; Kreda *et al.* 2005, 2007) (Fig. 1*A*). RT-PCR analysis confirmed that VAMP8 was expressed in native airway epithelium (bronchus and trachea), lung, WD-HBE cells and goblet cell-like Calu-3 cells (Fig. 1*B*).

VAMP8 was the predominant v-SNARE in human airway epithelial cells, as indicated by deep sequencing analysis of Calu-3 cells (Table 1). The analysis of the data expressed as RPKM (read per kilobase of exon model; Mortazavi *et al.* 2008) showed that VAMP8 was expressed ∼5 times higher than VAMP3, 10–14 times higher than VAMP5 and VAMP7, ∼30 times higher than VAMP2, and >100 times higher than VAMP1 and VAMP4 (Table 1). Similar results were observed by deep sequencing of WD-HBE cells (S. Randell, unpublished results). VAMP2 and VAMP3 have been implicated in exocytosis of neuronal, gland acinar and mast cell granules (Deak *et al.* 2004; Wang *et al.* 2004; Pocard *et al.* 2007; Weng *et al.* 2007; Tiwari *et al.* 2009; Hasan *et al.* 2010), while VAMP7 but not VAMP5 has been described to participate in vesicle exocytosis (Hasan *et al.* 2010). Quantitative RT-PCR analysis of WD-HBE and Calu-3 cells confirmed that VAMP8 was more abundantly expressed (10–25 times) that VAMP2, VAMP3 and VAMP7
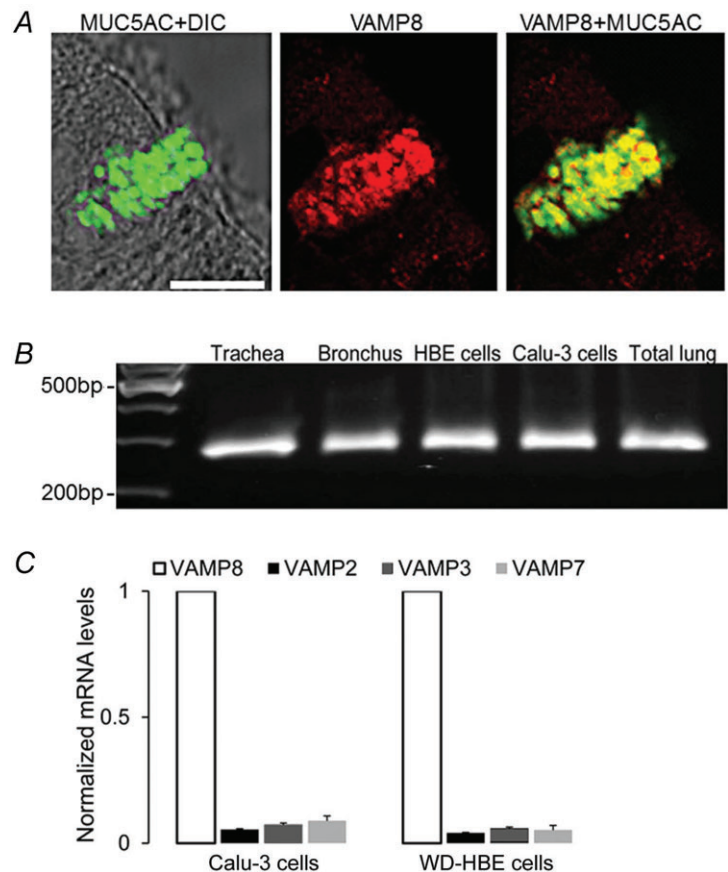

Figure 1. VAMP8 is expressed in lung tissues and airway epithelial cells
A, immunostaining of VAMP8 and MUC5AC in WD-HBE cells. Confocal microscopy images of MUC5AC mucin granule immunostaining (green) overlaid with the DIC/Nomarski channel (grey), VAMP8 immunostaining (red), and overlay of MUC5AC and VAMP8 immunostaining channels (yellow). Both proteins localize in ∼1 μm diameter mucin granules in the goblet cells of WD-HBE cells; scale bar, 10 μm. *B*, RT-PCR analysis. A single PCR product (268 bp), encoding VAMP8 sequence, is present in human airway epithelial cells and native tissues. *C*, relative expression of VAMP2, VAMP3 and VAMP7 to VAMP8 transcripts in Calu-3 and WD-HBE cells. mRNA expression levels of VAMP2, VAMP3, VAMP7 and VAMP8 were measured by qRT-PCR in Calu-3 and WD-HBE cells. The data were normalised to 18S transcript levels and are expressed in relationship to VAMP8 transcript levels (VAMP8 = 1); *n* = 4.

**Table 1. Expression of VAMP transcripts in Calu-3 cells**

	RPKM	RPKM values relative to VAMP8
VAMP1	0.61	0.005
VAMP2	4.00	0.035
VAMP3	28.33	0.248
VAMP4	1.31	0.011
VAMP5	14.34	0.126
VAMP7	8.35	0.073
VAMP8	114.20	1.000

The Calu-3 cell transcriptome was subjected to deep sequencing as described in Methods. Transcripts were identified using the updated version of the human genomic annotation database, and the data were expressed as RPKM (read per kilobase of exon model) as an estimation of the expression levels of each transcript. The relative RPKM values for all VAMPs compared to VAMP8 (VAMP8 = 1) shown in the last column indicate that VAMP8 is the most abundantly expressed VAMP in Calu-3 cells.

(Fig. 1*C*). These data suggest that VAMP8 may have a dominant role in airway goblet cells.

Immunolocalisation studies, using antibodies against human MUC5AC to identify mucous cells in the surface epithelium, demonstrated that VAMP8 was associated with goblet cell mucin granules in human normal bronchial superficial epithelial tissues, while MUC5AC-negative cells (non-goblet cells) displayed low or negligible levels of VAMP8 immunostaining (Fig. 2*Aa*). In cystic fibrosis (CF) airway epithelium, chronic infection and inflammation induces mucous cell metaplasia, and thus, a higher number of goblet cells and MUC5AC mucin granules were observed compared to non-CF airway epithelium (Fig. 2*Ab*), as previously reported (Kreda *et al.* 2005; Evans & Koo, 2009). In CF bronchial epithelium, VAMP8 was also associated with MUC5AC goblet cell granules, but the immunostaining signal was greater than in normal airways (Fig. 2*A*). This increase in VAMP8 immunostaining was consistent with the metaplastic increase of goblet cell numbers present in these tissues.

Evidence for this assertion was generated from studies of WD-HBE cells cultured from non-CF and CF
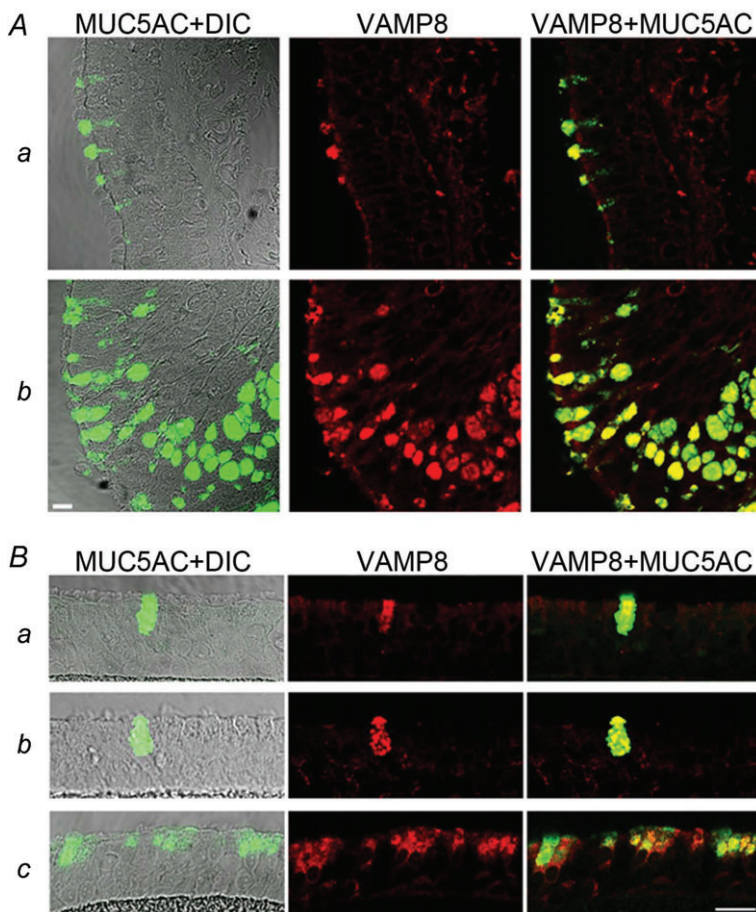
Figure 2. VAMP8 and MUC5AC immunostaining in non-CF and CF human airway epithelia
A, freshly excised human bronchial tissues. Confocal microscopy images of MUC5AC (green) + DIC/Nomarski (grey) overlay, VAMP8 (red), and VAMP8 + MUC5AC overlay. MUC5AC and VAMP8 antibodies labelled the mucin granules of goblet cells within the surface epithelium of bronchial tissues, non-CF (*a*) and CF (*b*) displaying mucous metaplasia (bottom panels); scale bar, 20 μm. *B*, WD-HBE cell cultures. Confocal microscopy images of MUC5AC (green) + DIC/Nomarski (grey) overlay, VAMP8 (red), and VAMP8 + MUC5AC overlay in WD-HBE cell cultures, non-CF (*a*), CF (*b*), and non-CF with IL-13-induced mucous metaplasia (*c*); scale bar, 20 μm. Note, VAMP8 localised with MUC5AC mucin granules, but it was also associated with apical granules within MUC5AC-negative cells of IL-13-induced WD-HBE cells; these cells probably expressed MUC5B granules, which are not recognised by the MUC5AC antibody.

individuals, indicating that similar VAMP8 immuno-staining was observed in both genotypes (Fig. 2*Ba* and *b*, non-CF and CF, respectively). These results reflect the fact that CF airway epithelial cells, in the absence of the native chronic infection/inflammation, differentiate into epithelial cultures with a relative low number of goblet cells, similar to non-CF cultures (Kreda *et al.* 2005). In contrast, non-CF cultures with mucous metaplasia induced by IL-13 treatment (Okada *et al.* 2011) displayed increased VAMP8 immunostaining associated with augmented mucous cell numbers (Fig. 2*Bc*).

Knockdown of VAMP8 expression reduces mucin secretion in Calu-3 cells

To test the function of VAMP8 in mucin secretion, VAMP8 expression was knocked-down using RNA inter-ference techniques. Calu-3 cells were transfected with a mix of four different short interfering RNAs (siRNAs). After 96 h, the mRNA levels of VAMP8 were profoundly reduced (Fig. 3*A*), but the mRNA levels of MUC5AC or other VAMPs expressed in airway epithelium were not affected (mRNA levels in VAMP8 compared to GAPDH siRNA-transfected Calu-3 cells for MUC5AC: $100 \pm 7\%$, VAMP2: $98 \pm 8\%$ and VAMP3: $103 \pm 4\%$). The protein levels of VAMP8 (Fig. 3*B*) were significantly reduced, while the levels of MUC5AC protein (Fig. 3*C*) were unaffected.

Next, mucin secretion was evaluated in VAMP8 silenced Calu-3 cells. Airway goblet cells release mucin at a constant rate in the absence of external stimuli; this type of secretion is defined as 'tonic' or 'basal' secretion. Mucin (human MUC5AC) secretion under basal conditions was estimated by incubating cell cultures with vehicle for 2 h in the

absence of external agonist or stimulus (note: mucin secreted levels are higher after 2 h incubation (basal) than after 10 min incubation with vehicle (vehicle), Fig 3*D*). In these conditions, basal mucin secretion was significantly reduced by VAMP8 knockdown. Mucin secretion was significantly increased in cells stimulated with protease activated receptor (PAR) agonists compared to cells incubated in vehicle, as previously reported (Kreda *et al.* 2010*b*). Importantly, agonist-stimulated mucin secretion was profoundly decreased in cells transfected with VAMP8 siRNAs (Fig. 3*D*).

To control for the specificity of the effect of VAMP8 silencing in reducing mucin secretion, similar experiments were conducted to knockdown VAMP2 or VAMP3 expression. VAMP2 and VAMP3 have been implicated in the regulation of exocytosis of mast cell and gland acinar granules (Wang *et al.* 2004; Pocard *et al.* 2007; Weng *et al.* 2007; Tiwari *et al.* 2009; Hasan *et al.* 2010). However, in Calu-3 cells, knockdown of VAMP2 or VAMP3 did not affect basal or agonist-stimulated mucin secretion (Fig. 4). These results suggest that exocytosis of mucin granules from airway goblet cells probably requires VAMP8.

Knockdown of VAMP8 expression reduces mucin secretion in WD-HBE cells

To further investigate the effects of reducing VAMP8 expression on mucin exocytosis, the three most effective VAMP8 siRNA sequences (VAMP8 mRNA knockdown >85%) were individually cloned in retroviral vectors to be expressed as short hairpin RNAs in Calu-3 and WD-HBE cells. Calu-3 cells stably expressing any of the three VAMP8 shRNAs (shRNA A, B and C)
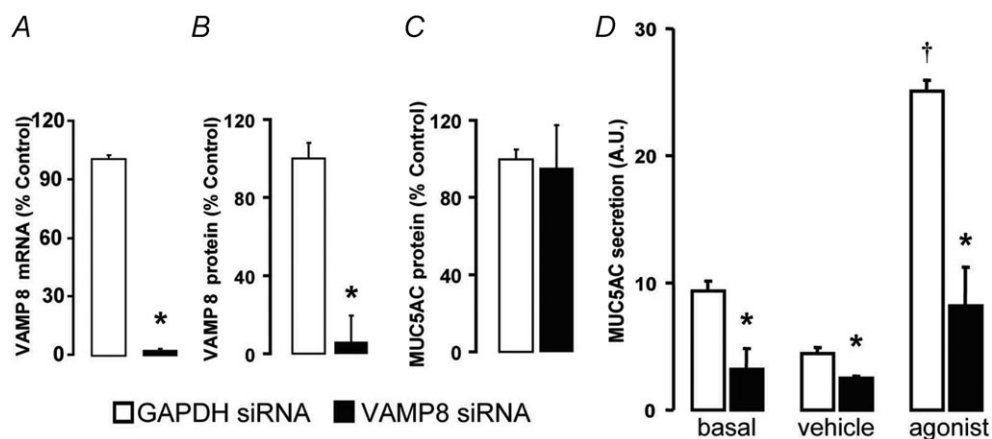

Figure 3. VAMP8 siRNA decreases mucin secretion in Calu-3 cells
Calu-3 cells were transfected with a mix of four VAMP8 siRNA oligos, or GAPDH siRNA (control). After 96 h, VAMP8 mRNA levels were assessed by quantitative RT-PCR (*A*), and protein levels for VAMP8 (*B*) and MUC5AC (*C*), were determined by immuno-slot blot. Mucin secretion was assessed in the extracellular bath after 2 h in resting conditions (basal) and 10 min after adding vehicle or 100 μM PAR1 + 100 μM PAR2 peptide agonists (*D*). The data are the mean $\pm$ SEM of quadruplicate samples ($n = 2$), and are expressed as % of control cells (*A*, *B*, *C*) and arbitrary units (*D*); *$P < 0.01$ VAMP8 *vs.* GAPDH siRNAs (*A*, *B*, *D*), and †$P < 0.01$ agonist *vs.* vehicle (*D*).

displayed a marked reduction in VAMP8 mRNA levels (Fig. 5*A*) (note: shRNA C and shRNA A produced very similar results, therefore shRNA C data are not illustrated in the figures). VAMP8 protein levels were nearly undetectable in shRNA-expressing cells ($2 \pm 2\%$ and $100 \pm 9\%$ in shRNA- and vector-expressing cells, respectively). In contrast, mRNA levels for MUC5AC or other VAMPs were not affected (Fig. 5*A*). Immuno-cytochemical examination of the cell cultures indicated that there were no phenotypic changes, i.e. goblet cell distribution, or intracellular mucin content (MUC5AC staining) in shRNA-expressing compared to mock vector-expressing Calu-3 cells (Fig. 5*B*). MUC5AC protein content was also unchanged ($103 \pm 12\%$ and $100 \pm 8\%$ in shRNA- *vs.* vector-expressing cells, respectively).

Basal secretion appeared to be elevated in Calu-3 cells expressing empty and shRNA-containing vectors (Fig. 5*C*) compared to Calu-3 cells transfected with siRNAs (Figs 3 and 4). These changes in basal secretion may not be related to the expression of viral vectors, since similar variability in the levels of basal mucin secretion is observed in uninfected Calu-3 cells (L. Moussa and S. M. Kreda, unpublished observation). However, like Calu-3 cells expressing VAMP8 siRNAs, VAMP8 shRNA-expressing cells displayed a sharp reduction in mucin secretion in basal conditions, as well as in response to the PAR agonist thrombin and the patho-physiologically relevant mucin secretagogue human neutrophil elastase (Park *et al.* 2005; Kreda *et al.* 2010*a*,*b*) (note, Calu-3 cells do not express P2Y$_2$ receptors; Kreda *et al.* 2007) (Fig. 5*C*).

To validate the results obtained in Calu-3 cells in an alternative and near-physiological cell model system, mucin secretion was investigated in WD-HBE cells infected with the VAMP8 shRNA-encoding retroviral vectors tested in Calu-3 cells. As in Calu-3 cells, WD-HBE cells expressing VAMP8 shRNA displayed a marked decrease in VAMP8 mRNA with no effect on mRNA levels for MUC5AC, MUC5B or other VAMPs (Fig. 6*A*). As expected, VAMP8 protein levels were nearly undetectable in shRNA-expressing cells ($2 \pm 3\%$ and $100 \pm 12\%$ in shRNA- *vs.* vector-expressing cells, respectively). No phenotypic changes, i.e. goblet cell distribution or intracellular mucin content (AB-PAS staining and MUC5AC intracellular protein content), were observed in shRNA-expressing compared to vector-expressing WD-HBE cells (Fig. 6*B* and *C*). Importantly, VAMP8 shRNA-expressing cells displayed a profound reduction in mucin secretion in basal conditions and in response to the potent mucin secretagogues neutrophil elastase, PAR1 and PAR2 agonists, and the P2Y$_2$ receptor agonist ATPγS, a non-hydrolysable ATP molecule (Davis & Lazarowski, 2008) (Fig. 6*D*).

Mucin secretion measured with a MUC5AC antibody (Fig. 6*D*) or by the PABH method (total mucins (MUC5AC + MUC5B) Conway *et al.* 2003) produced identical results (not shown).

VAMP8 knockdown expression is effective in reducing mucin secretion in mucous-metaplastic airway epithelium

Mucous metaplasia with mucin hypersecretion is a feature of obstructive lung diseases (e.g. CF, COPD and asthma). To test whether VAMP8 is essential for mucin exocytosis in airway epithelia exhibiting inflammation-induced
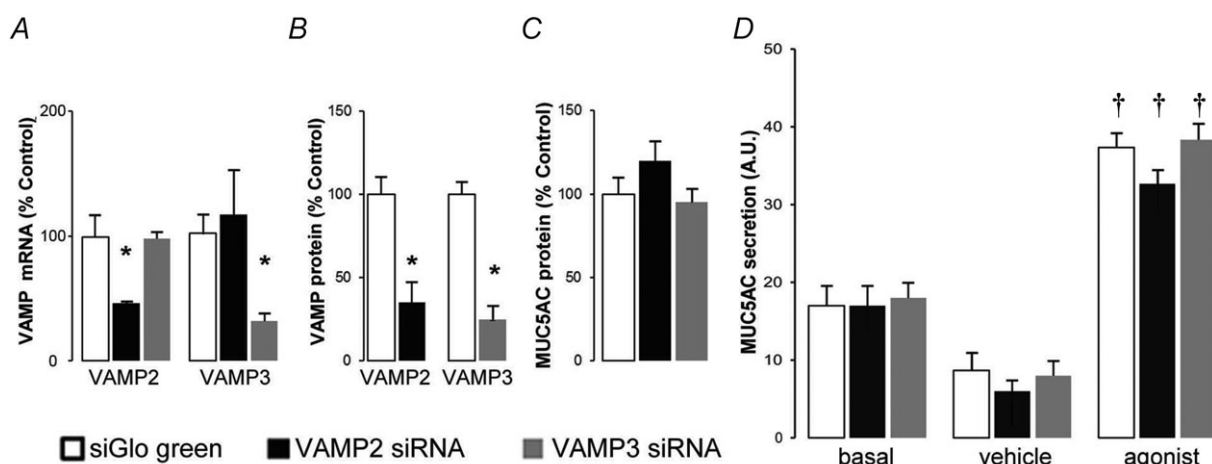

Figure 4. VAMP2 and VAMP3 siRNA does not affect mucin secretion in Calu-3 cells
Calu-3 cells were transfected with a mix of four VAMP2 or VAMP3 siRNA oligos, or siGLO Green (control). After 96 h, VAMP mRNA (*A*) and protein (*B*) levels, intracellular MUC5AC protein levels (*C*) and mucin secretion (*D*) were assessed as in Fig. 3 in VAMP2, VAMP3 and control silenced Calu-3 cells. The data are the mean ± SEM of triplicate samples ($n = 2$), and are expressed as % of control cells (*A*, *B*, *C*) and arbitrary units (*D*); *$P < 0.01$ VAMP siRNA *vs.* siGlo green (*A*, *B*), and †$P < 0.01$ agonist *vs.* control (*D*).

mucous metaplasia, VAMP8 shRNA-expressing WD-HBE cells were challenged with IL-13 (Okada *et al.* 2011) or supernatant of mucopurulent material (SMM) from diseased CF lungs (Ribeiro *et al.* 2005).

IL-13 treatment induced mucous metaplasia in both mock vector- and VAMP8 shRNA-expressing WD-HBE cells. Both MUC5AC and MUC5B mRNA levels were significantly elevated in IL-13-treated cell cultures compared to naïve cell cultures; MUC5AC showed a much larger increase in mRNA levels than MUC5B (e.g. ∼20- *vs.* ∼5–fold for MUC5AC and MUC5B, respectively). The number of goblet cells increased similarly (∼7- to 25–fold) in both vector- and shRNA-expressing

WD-HBE cells in response to IL-13 exposure. The intracellular mucin protein content (Fig. 7A) and the histological characteristics (i.e. goblet cell number, AB-PAS staining levels; Fig. 7B) were undistinguishable between VAMP8 shRNA- and mock-expressing cell cultures exposed to IL-13, that were undisturbed for at least 24 h before staining. Importantly, despite the metaplastic increase in mucin-producing cells elicited by IL-13, VAMP8 expression knockdown effectively reduced mucin secretion both in basal conditions and in response to ATPγS (Fig. 7C). Similar results were observed with mucin secretagogues neutrophil elastase and PAR agonists (mucin secretion expressed in arbitrary
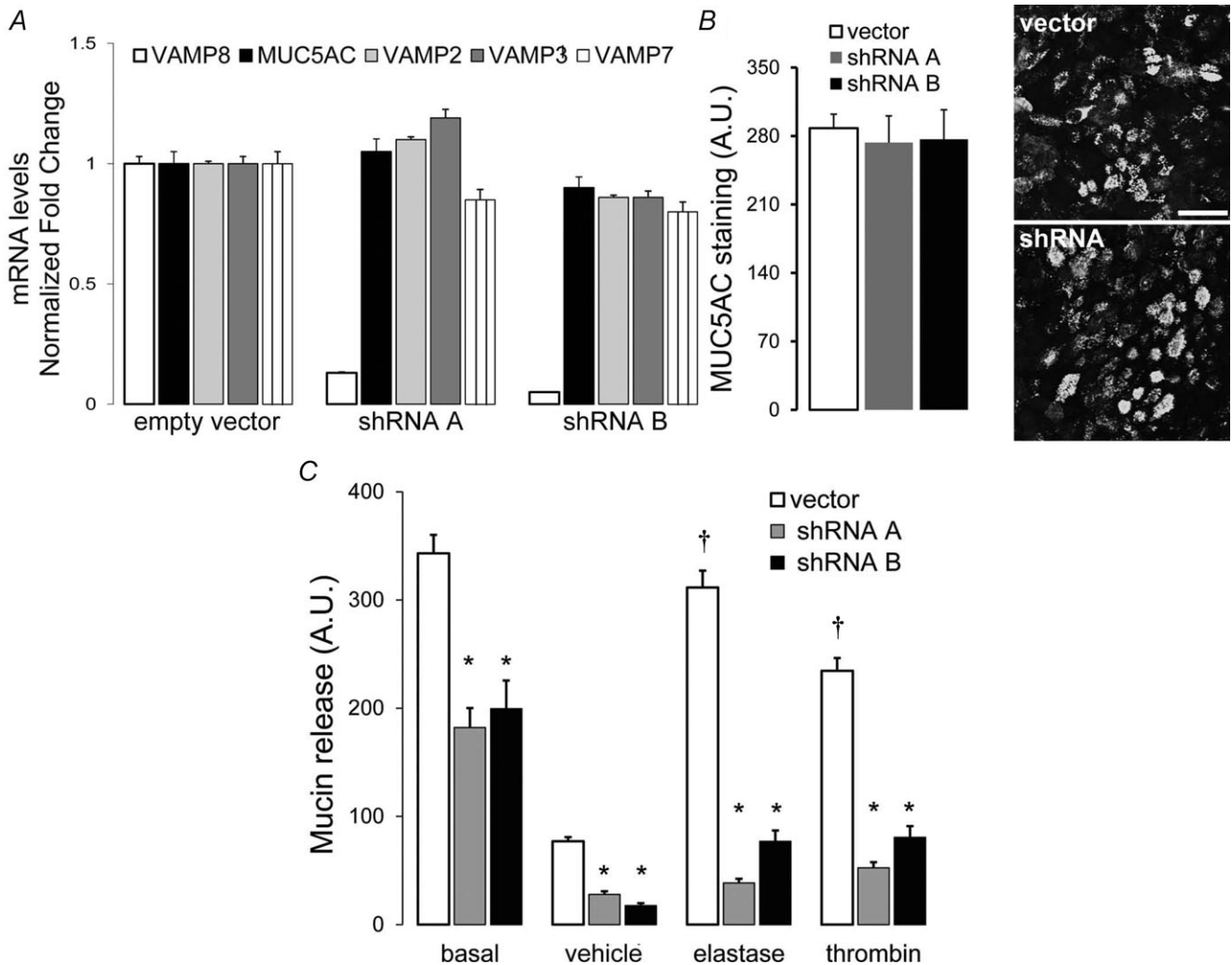

Figure 5. VAMP8 knockdown reduces mucin secretion in Calu-3 cells
Calu-3 cells were infected with retroviral vectors to stably express VAMP8 shRNAs (shRNA A, shRNA B). *A*, mRNA levels assessed by quantitative RT-PCR in Calu-3 cells expressing two independent VAMP8 shRNAs. *B*, confocal microscopy images of MUC5AC mucin granule immunostaining (white) and quantification of MUC5AC immunostaining in Calu-3 cells expressing mock vector or VAMP8 shRNAs that were maintained undisturbed for 24 h; scale bar, 20 μm. *C*, mucin secretion in Calu-3 cells performed as in Fig. 3. Agonists were incubated for 20 min; neutrophil elastase (200 nM), PAR agonist thrombin (50 nM). The data are the mean ± SEM of triplicate samples ($n = 2$), and are expressed in reference to control cells (*A*) and arbitrary units (*B*, *C*); *$P < 0.01$ VAMP8 shRNA *vs.* vector, and †$P < 0.01$ vector agonist *vs.* vector vehicle (*C*).
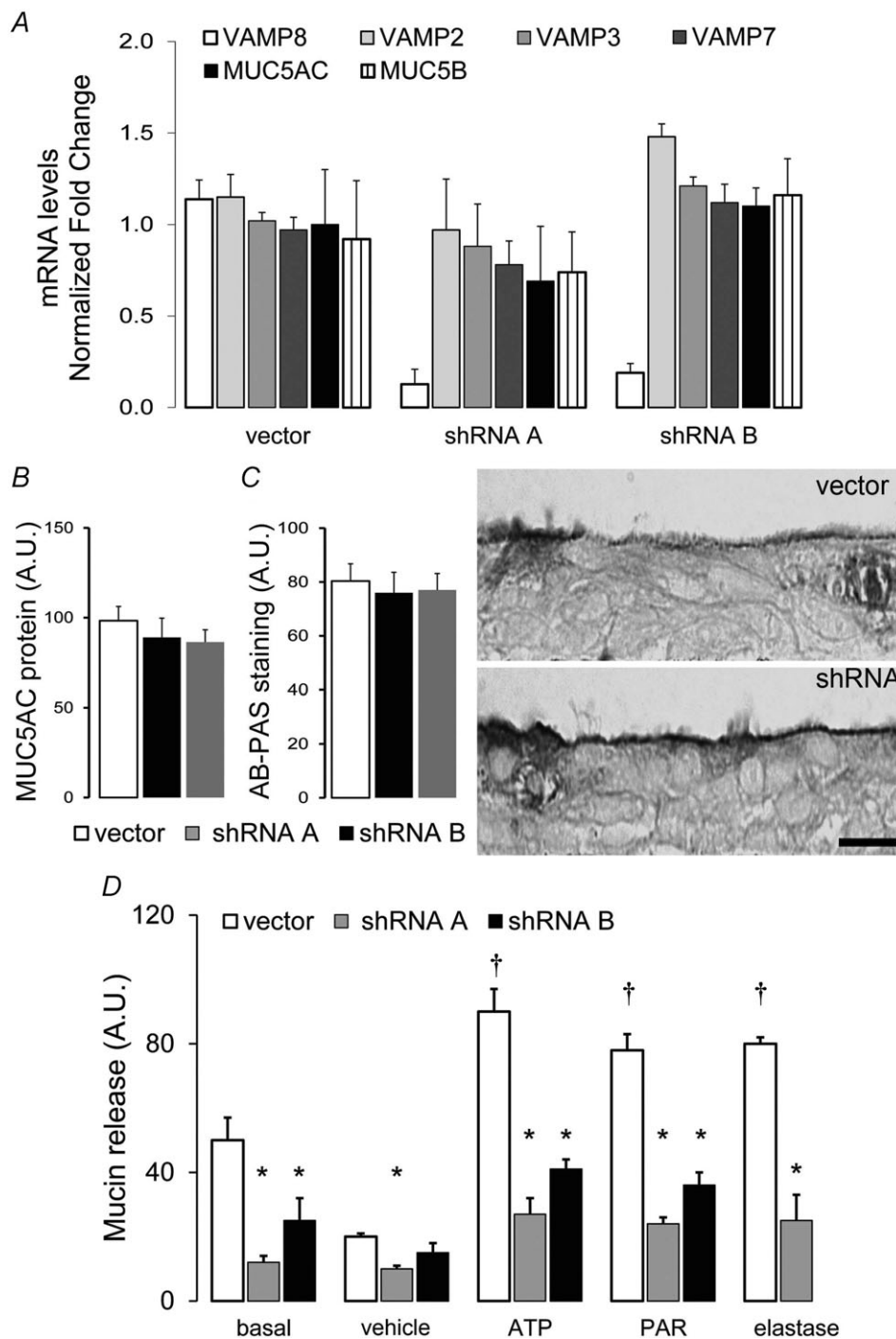
Figure 6. VAMP8 knockdown reduces mucin secretion in WD-HBE cells
HBE cells were independently infected with retroviral vectors encoding two VAMP8 shRNA sequences, antibiotic-selected, and cultivated on permeable supports under air–liquid interface for 21–28 days to produce WD-HBE cell cultures stably expressing VAMP8 shRNAs. *A*, mRNA levels assessed by quantitative RT-PCR in WD-HBE cells expressing VAMP8 shRNAs. *B*, total MUC5AC protein content within cell cultures undisturbed for 24 h. *C*, AB-PAS staining and staining quantification of WD-HBE cells expressing vector and VAMP8 shRNA that were undisturbed for 24 h; scale bar, 10 μm. *D*, mucin secretion in WD-HBE cells performed as in Fig. 3. Agonists were incubated for 20 min; elastase (200 nM), PAR1-peptide (100 μM) + PAR2-peptide (100 μM) and ATPγS (100 μM). The data are the mean ± SEM of triplicate samples and are expressed in arbitrary units; *P < 0.01 VAMP shRNA *vs.* vector, and †P < 0.01 vector agonist *vs.* vector vehicle (*D*). Similar results were obtained in three independent experiments performed with WD-HBE cells cultured from different human subjects and two different VAMP8 shRNAs.

units was reduced $65 \pm 15\%$ for neutrophil elastase, and $49 \pm 17\%$ for PAR agonists in VAMP8 shRNA- compared to vector-expressing cells treated with elastase and PAR agonists, respectively, in separate experiments).

SMM induced mucous metaplasia with similar efficacy in WD-HBE cell cultures expressing vector or VAMP8 shRNA. The number of goblet cells increased similarly ($\sim$3- to 6–fold) in both vector- and shRNA-expressing WD-HBE cells in response to SMM exposure. MUC5AC mRNA levels increased 8.0 ± 0.5- and 8.6 ± 0.1-fold and MUC5B increased 3.9 ± 0.3- and 4.7 ± 0.2–fold in vector- and shRNA- expressing cells, respectively, after SMM treatment. The levels of MUC5AC protein (Fig. 8*A*), the number of goblet cells/levels of AB-PAS staining (Fig. 8*B*) were similar in mock vector- and shRNA-expressing cell cultures induced with SMM. Importantly, mucin secretion stimulated by ATPγS and under basal conditions was significantly reduced in SMM-inflamed WD-HBE cells with VAMP8 knockdown (Fig. 8*C*).

These results suggest that VAMP8 knockdown can effectively reduce mucin secretion in airway epithelium displaying mucous metaplasia and mucin hypersecretion.

Mucin secretion is reduced in the VAMP8 KO mouse

Next, we tested the hypothesis that VAMP8 is required for physiological mucin secretion from native airways using the VAMP8 KO mouse. In our hands, these animals do not appear to develop lung disease.

Unlike human airways, the main secretory cell type in mouse airways is the Clara cell while very few/no AB-PAS-positive goblet cells are observed and mucin secretion is very low (Davis & Dickey, 2008). In these (base-line) conditions, it is difficult to identify a block/inhibition on mucin secretion. Therefore, the number of goblet cells and the levels of mucin secretion in the airways were increased by inducing mucous metaplasia in these animals
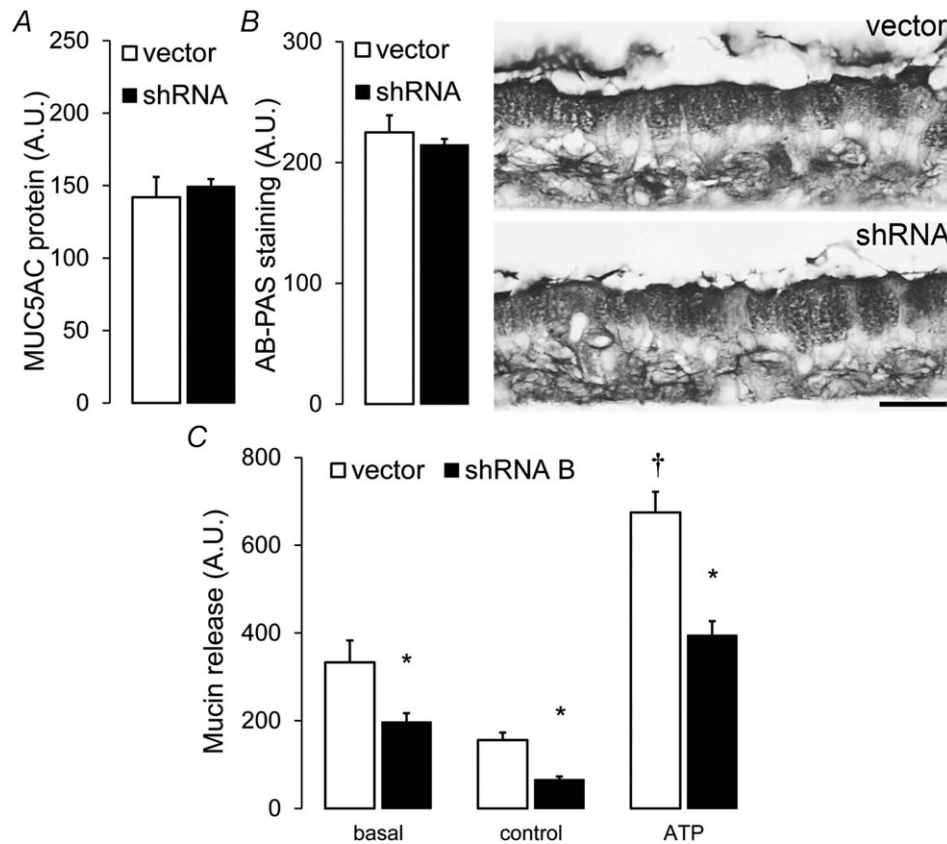

Figure 7. VAMP8 knockdown reduces mucin secretion in WD-HBE cells with IL-13-induced mucous metaplasia
WD-HBE cell cultures stably expressing VAMP8 shRNAs were treated with IL-13 (10 ng day^{-1}, 5 days). *A*, total MUC5AC protein content within cell cultures undisturbed for 24 h. *B*, AB-PAS staining and staining quantification of WD-HBE cells expressing vector and VAMP8 shRNA that were undisturbed for 24 h; scale bar, 20 μm. *C*, mucin secretion in WD-HBE cells performed as in Fig. 3. Agonist ATPγS (100 μM) was incubated for 20 min. The data are the mean $\pm$ SEM of triplicate samples, and are expressed in arbitrary units; *$P < 0.01$ VAMP shRNA *vs.* vector, and †$P < 0.01$ vector agonist *vs.* vector vehicle (*C*). Similar results were obtained in two independent experiments performed with WD-HBE cells obtained from different human subjects and two different VAMP8 shRNAs.

with IL-13. Under these allergic-inflammatory conditions, Clara cells switch to a mucous phenotype and the airways of IL-13-stimulated mice exhibit high numbers of AB–PAS-positive goblet cells and high rates of mucin secretion (Davis & Dickey, 2008; Anagnostopoulou *et al.* 2010). As expected, WT, VAMP8 HET and VAMP8 KO mice treated with IL-13 displayed mucous metaplasia as indicated by high levels of AB-PAS staining in the trachea of these animals (Fig. 9*A*). Quantification of the AB-PAS staining indicated that no differences in the number of goblet cells or intracellular mucin content were evident among the different mouse groups in the absence of a mucin secretagogue (Fig. 9*B*). Tracheas were stimulated *ex vivo* with ATP to induce mucin secretion. WT and HET tracheas displayed low levels of AB-PAS staining after ATP-promoted mucin granule secretion (Fig. 9*A* and *B*). There were no statistically significant differences between

HET *vs.* WT. In contrast, KO tracheas had no significant change in the levels of AB-PAS staining in response to ATP (Fig. 9*A* and *B*). These data suggest that VAMP8 KO mice have an impediment in agonist-regulated mucin exocytosis.

The mucin content in the BAL reflects the amount of mucin already secreted into the airway lumen and a variable amount of mucin being secreted in response to stimuli generated during the BAL collection. Analysis of the BALs from IL-13-treated animals showed that the mucin content (MUC5B) was decreased in VAMP8 KO compared to WT and HET mice (Fig. 9*C* and *D*). (Note: there is no reliable antibody that recognizes mouse MUC5AC; antibodies against human MUC5AC, like the one utilized in the *in vitro* studies (Figs 3–8) are not reliable to detect/quantify mouse MUC5AC.) There were no statistically significant differences between HET *vs.* WT.
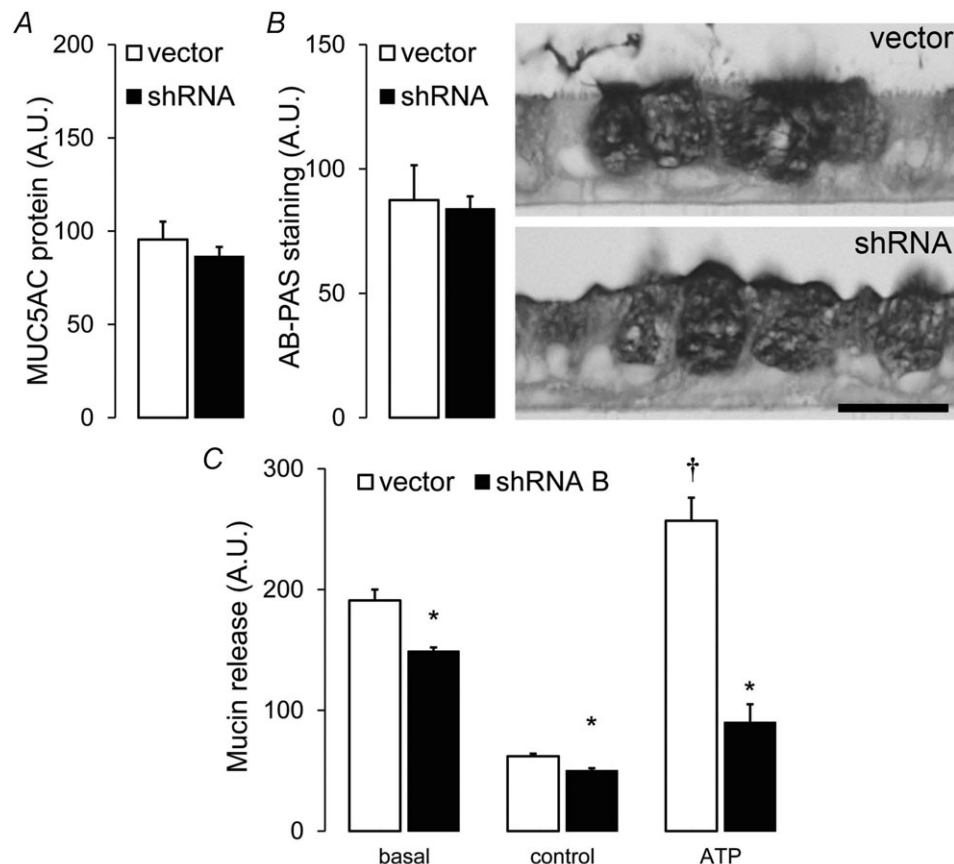

Figure 8. VAMP8 knockdown reduces mucin secretion in WD-HBE cells with SMM-induced inflammation/mucous metaplasia
WD-HBE cell cultures stably expressing VAMP8 shRNAs were treated with SMM (5 days). *A*, total MUC5AC protein content within cell cultures undisturbed for 24 h. *B*, AB-PAS staining and staining quantification of WD-HBE cells expressing vector and VAMP8 shRNA that were undisturbed for 24 h; scale bar, 20 μm. *C*, mucin secretion in WD-HBE cells performed as in Fig. 3. Agonist ATPγS (100 μM) was incubated for 20 min. The data are the mean ± SEM of triplicate samples, and are expressed in arbitrary units; *$P < 0.01$ VAMP shRNA *vs.* vector, and †$P < 0.01$ vector agonist *vs.* vector vehicle (*C*). Similar results were obtained in two independent experiments performed with WD-HBE cells obtained from different human subjects.

A decreased level of BAL mucin in VAMP8 KO mice further suggests that the absence of VAMP8 expression blocks mucin granule exocytosis.

Discussion

The exocytotic release of gel-forming mucins into the airway lumen from specialised granules in goblet cells reflects a tightly regulated process that sustains the thin mucus layer covering the airway surfaces. Our knowledge of the proteins involved in the regulation of airway mucin granule exocytosis remains incomplete. In this study, we identified VAMP8 as a v-SNARE (R–SNARE) in mucin granules of airway goblet cells.

Amongst the 30+ SNARE genes, only seven v-SNAREs or VAMPs have been identified in the human genome (Advani *et al.* 1998; Burgoyne & Morgan, 2003; Brunner *et al.* 2009). Individual SNARE proteins localize to distinct organelles, and VAMPs are differentially expressed in

different tissues, suggesting that each VAMP has a relatively selective role in vesicle trafficking events (Advani *et al.* 1998; Chen & Scheller, 2001; Burgoyne & Morgan, 2003; Brunner *et al.* 2009). VAMP8 was originally associated with endosomal membrane fusion activity (Advani *et al.* 1998; Ho *et al.* 2008), but recent studies in the VAMP8 KO mouse suggest that VAMP8 is critical for regulated exocytosis of secretory granules in pancreatic acini and other exocrine glands (Wang *et al.* 2004, 2007), histamine granules from mast cells, and dense and α-granules from platelets (Ren *et al.* 2007; Tiwari *et al.* 2008; Graham *et al.* 2009).

Similarities between the calcium-dependent exocytosis of mucin granules from airway goblet cells and pancreatic acinar zymogen granule exocytosis have been observed (Burgoyne & Morgan, 2003; Davis & Dickey, 2008). However, the function of VAMP8 in secretory cells of lung epithelia has not been studied. We have previously established that VAMP8 is associated with mucin granules purified from airway goblet cells (Kreda *et al.* 2010*b*), the

Figure 9. Airway mucin secretion is reduced in the VAMP8 KO mouse
A, tracheas dissected from IL-13-induced WT and VAMP8 knockout heterozygous (HET) and homozygous (KO) littermate mice were incubated with vehicle or ATP, fixed and stained with AB-PAS as described in Methods. Tracheas from age-matched mice with no IL-13 treatment (Naïve) are shown for comparison. Images are representative longitudinal views of mouse tracheas, dark purple-blue staining indicates AB-PAS-positive substances (e.g. mucin); scale bar, 30 μm. *B*, AB-PAS staining quantification. *C*, Western blot of mouse BALs to reveal the mucin content using an antibody that recognizes mouse MUC5B; arrowhead indicates the position of MUC5B. *D*, Western blot quantification. The data presented in both graphs are expressed in arbitrary units (A.U.), and represent the mean ± SEM of two experiments (total number of animals: WT ($n = 12$), KO ($n = 12$) and HET ($n = 8$)), *$P < 0.01$.
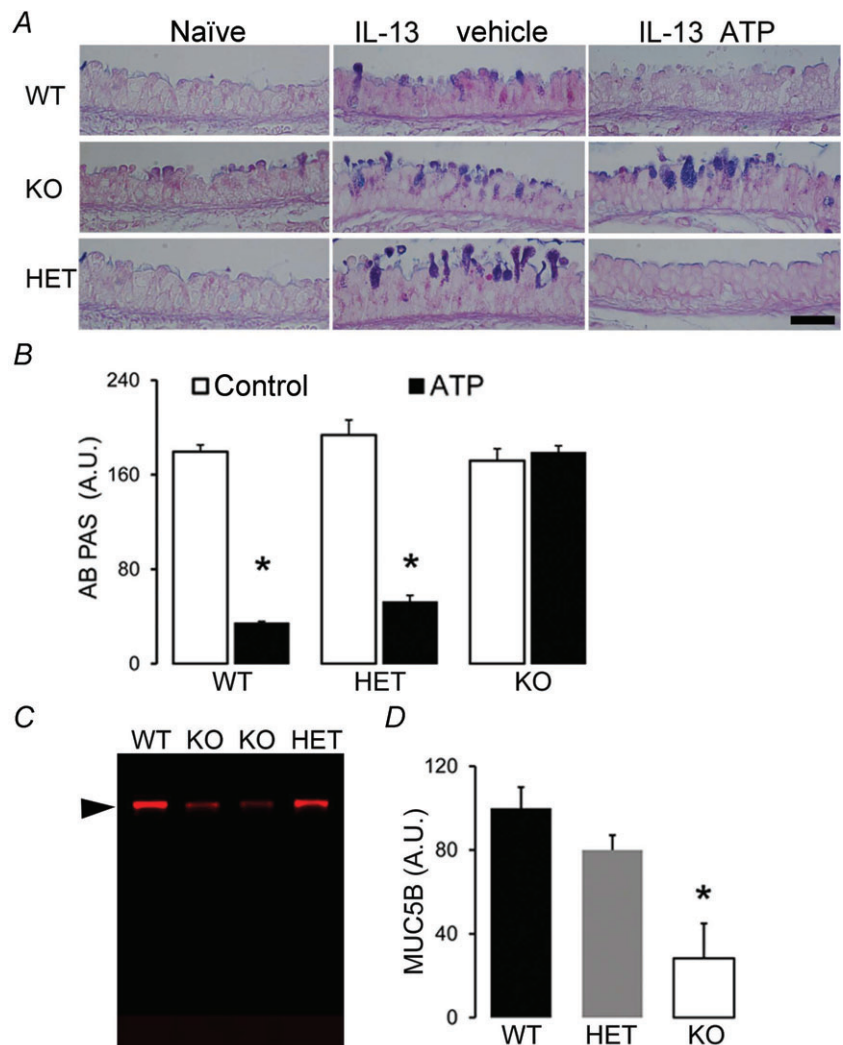
main secretory cell in the superficial epithelium lining the human airways. Our current data indicate that VAMP8 was localised in the mucin granules of goblet cells in native human airway epithelia and in *in vitro* models of human airway epithelium. Moreover, of the seven VAMPs, VAMP8 showed the highest level of expression in airway epithelial cells (Table 1 and Fig. 1*C*). For example, VAMP8 is expressed ∼5–10 times higher than VAMP3, and 10–30 times higher than VAMP2, VAMP5 and VAMP7. Of these VAMP proteins, VAMP2, VAMP3 and VAMP7 (but not VAMP5) have been implicated in vesicle exocytosis of secretory cells (Advani *et al.* 1998; Chen & Scheller, 2001; Burgoyne & Morgan, 2003; Schraw *et al.* 2003; Deak *et al.* 2004; Wang *et al.* 2004; Pocard *et al.* 2007; Weng *et al.* 2007; Tiwari *et al.* 2009; Hasan *et al.* 2010). However, our studies suggest that VAMP8 is the main v-SNARE involved in exocytosis of mucin granules from airway goblet cells.

VAMP8 is the primary SNARE for acute agonist-regulated mucin secretion from airway goblet cells

Mucin granules, like other dense-core secretory granules, are released exocytotically by basal/tonic and acute regulation by Ca^{2+}-mobilizing extracellular agonists (Burgoyne & Morgan, 2003; Kreda *et al.* 2007; Davis & Dickey, 2008). The functional role of VAMP8 in agonist-regulated mucin granule exocytosis was investigated *in vitro* by specifically knocking-down VAMP8 expression, using siRNA and shRNA approaches, in two different models of goblet cell-containing airway epithelium, i.e. Calu-3 cells and WD-HBE cells. Short interfering RNAs (siRNAs and shRNAs) targeting VAMP8 efficiently reduced VAMP8 expression levels without affecting the expression levels of mucins (e.g. MUC5AC), or that of other VAMPs that are significantly expressed in airway epithelial cells and have been associated with exocytosis (i.e. VAMP2, VAMP3 and VAMP7). Importantly, VAMP8 silencing was effective in reducing mucin secretion stimulated by physiologically relevant mucin secretagogues. For example, nucleotides that activate apically expressed $P2Y_2$ receptors (Davis & Lazarowski, 2008), proteases and synthetic peptides that activate basolaterally expressed PARs (Kreda *et al.* 2010*b*), and basolateral neutrophil elastase (through an undefined mechanism) (Kreda *et al.* 2010*a*) stimulated airway epithelial mucin secretion in a VAMP8-dependent manner (Figs 3, 5 and 6).

The findings in the *in vitro* studies using human airway epithelial cells were further explored in the VAMP8 KO mouse with IL-13-induced mucous metaplasia. In VAMP8 KO mice, unlike their WT and (VAMP8) HET-matched controls, tracheal mucin granule secretion was reduced

post *ex vivo* stimulation with ATP as shown in Fig. 9. Thus, regulated exocytosis of mucin granules in airway goblet cells *in vivo* also requires VAMP8.

In neurons, VAMP2 but not VAMP8 is essential for rapid synaptic vesicle release (Deak *et al.* 2004). VAMP8 is also not crucial for regulated exocytosis of hormone-containing granules from pancreatic cells or cytokines-containing granules from mast cells (Wang *et al.* 2004; Tiwari *et al.* 2008). Moreover, proteomic analyses of secretory organelles from different tissues indicated that VAMP8 was present in some but not all secretory vesicles (Brunner *et al.* 2009). Thus, VAMP8 appears to display selectivity with respect to regulated exocytotic pathways (Tiwari *et al.* 2008). In our *in vitro* studies, although the expression of VAMP8 was abolished, the expression levels of other VAMPs were not different in VAMP8 knocked-down *vs.* normal cells. Moreover, knockdown expression of VAMP2 or VAMP3 did not affect mucin secretion. Thus, most probably, VAMP8, but not other VAMPs, was responsible for the reduction in mucin secretion observed in VAMP8-silenced cells. Similarly, VAMP8 and no other VAMPs appears to be essential in agonist-regulated exocytosis of secretory granules from exocrine secretory cells (Wang *et al.* 2004, 2007), platelets (Ren *et al.* 2007), mast cells (Tiwari *et al.* 2008) and macrophages (Pushparaj *et al.* 2009).

Our data indicate that VAMP8 is the primary v-SNARE in the exocytic core assembled during agonist-regulated exocytosis of airway mucin granules. Although further studies are needed, we could speculate about the identity of VAMP8 partners in the SNARE core of airway goblet cells. For example, it has been postulated that SNAP23 or SNAP25 is one of the t–SNARES in the exocytotic core of mucin granules (Davis & Dickey, 2008; Evans & Koo, 2009). SNAP23 has been shown to regulate apical exocytosis in polarised epithelial MDCK cells (Low *et al.* 1998). Deep sequencing mRNA data showed that SNAP23 but not SNAP25 is highly expressed in both Calu-3 cells (data expressed in RPKM, 10.60 ± 0.13 *vs.* 0.37 ± 0.03 for SNAP23 and SNAP25, respectively) and in WD-HBE cells (S. Randell, unpublished results). Thus, it is plausible that SNAP23 is the t-SNARE partner of VAMP8 in goblet cells. The second t-SNARE in the core complex could be syntaxin 3. We have previously established that syntaxin 3 is specifically localised apically in airway goblet cells (Kreda *et al.* 2007), and syntaxin 3 has been implicated in regulating apical vesicle exocytosis in polarised MDCK cells (Low *et al.* 1998; Kreitzer *et al.* 2003).

VAMP8 regulates the basal secretion of mucins

In the absence of acute agonist stimulation, a constant, low rate secretion of mucin is observed in the airway epithelial surface, which is defined as basal secretion (Davis & Dickey, 2008; Evans & Koo, 2009; Zhu *et al.* 2008). Basal

mucin secretion most probably reflects low, but constant tonic stimulation of goblet cells. It is critical to maintain the properties of the mucus layer bathing the airway lumen and support proper functioning of the MCC activities in the lung (Boucher, 2007; Kreda *et al.* 2007, 2010*b*; Davis & Dickey, 2008; Evans & Koo, 2009; Tuvim *et al.* 2009).

Whether the same SNARE and regulatory proteins participate in the exocytosis of the mucin granule in response to tonic *vs.* acute agonist stimulation has not been defined (Davis & Dickey, 2008; Zhu *et al.* 2008). For example, deletion of Munc13–2 indicated that this exocytotic priming protein is not essential for agonist-stimulated mucin secretion, but it does appear to regulate a tonically active, baseline mucin secretory pathway in the airways (Zhu *et al.* 2008). In the exocrine glands of the VAMP8 KO mouse, agonist-stimulated secretion of zymogen granules was reduced, while basal secretion was preserved (Wang *et al.* 2004, 2007).

Tonically active basal mucin secretion cannot be unambiguously measured *in vivo* in the airways but can be estimated by histological, BAL mucin content and *ex vivo* mucin secretion analyses. Our studies in the IL-13-induced VAMP8 KO mouse revealed that the histological evaluation of tracheas (without ATP stimulation) was not discriminatory amongst genotypes. In the intact mouse, BAL mucin content was reduced in the IL-13-induced VAMP8 KO mouse compared to WT and (VAMP8) HET-matched controls. These data could suggest a regulatory role of VAMP8 in tonically active basal secretion *in vivo* in the airways. However, the mucin content in the BAL reflects not only basal secretion, but also mucin secreted into the lumen in response to the manoeuvres of BAL collection.

Unlike the *in vivo* studies in the lung, *in vitro* studies in cultured airway epithelial cells allowed direct evaluation of the role of VAMP8 in mediating basal mucin secretion. Our *in vitro* studies in airway epithelial cells indicated that VAMP8 knockdown produced not only a reduction in the agonist-stimulated secretion of mucins, but also in the tonically active basal secretion as well. Despite a reduction in basal secretion, the mucin content within goblet cells was not significantly increased in VAMP8-deficient cell cultures that were undisturbed for at least 24 h. This finding is in contrast to observations in the airway epithelia of Munc13–2 knockout mice showing increased intracellular mucins with reduced basal secretion (Zhu *et al.* 2008). The lack of mucin accumulation within VAMP8-deficient airway epithelial cell cultures could be explained by a slow rate of mucin granule synthesis in these cell cultures; thus, accumulation of granules would not be noticeably higher over 24 h even if the basal secretion is decreased in VAMP8-deficient *vs.* normal cell cultures. Alternatively, only a small pool of mucin granules may participate in basal secretion while a much larger pool of granules would be available for secretion in response to an acute agonist; thus, in resting cell cultures it may be difficult to detect significant intracellular accumulation of mucins.

Further study will be necessary to unambiguously understand the role of VAMP8 regulating tonically active basal secretion of mucins *in vivo* in the lung.

VAMP8 in mucus-hypersecretory lung diseases

Lung infection and inflammation induces airway epithelial remodelling, producing a goblet cell-dominated epithelium with increased mucin secretion and defective MCC activities. Thus, lung mucin hypersecretion, leading to mucus obstruction of the airways, is a major pathological feature of chronic lung diseases (Davis & Dickey, 2008; Evans & Koo, 2009; Fahy & Dickey, 2010). Current therapies focus on the use of anti-inflammatory agents such as corticosteroids to reduce mucous metaplasia (a consequence of epithelial inflammation), aerosolised hypertonic saline solution, DNA severing enzymes and reducing agents to decrease mucus viscosity, and beta-agonists to stimulate ciliary action and improve mucociliary clearance. However, these treatments have limited efficacy (Evans & Koo, 2009).

Inhibition of mucin secretion is a desirable therapeutic strategy and recent developments in the field reveal potential targets for blocking this activity in the diseased lung (Singer *et al.* 2004; Davis & Dickey, 2008; Evans & Koo, 2009; Tuvim *et al.* 2009). Our studies in models of airway epithelium with induced inflammation and goblet cell metaplasia (i.e. chronic treatment with IL-13 and SMM of VAMP8-silenced cell cultures and *in vivo* IL-13-treatment of VAMP8 KO mice) indicated that reduction of VAMP8 expression can efficiently reduce both tonically- and agonist-regulated secretion of mucins in inflamed/hypersecretory airway epithelia. Thus, targeting VAMP8 to reduce its expression/activity in airway goblet cells may be a viable therapeutic approach for improvement of obstructive lung diseases. SNARE proteins are targets of potent natural toxins that exhibit relative specificities (Dolly *et al.* 2009; Fletcher *et al.* 2010). For example, botulinum toxins, mainly type A (and to a lesser extent type B), are being used clinically with notable success for the treatment of numerous human disorders involving over-activity of cholinergic nerves innervating muscles and glands (Dolly *et al.* 2009). Emergence of additional toxin-based therapeutics is anticipated based on in-depth knowledge of botulinum toxin serotypes and other toxins (Fletcher *et al.* 2010) that target SNARES to devise new engineered versions for inhibitory actions in non-neuronal cell types. It is plausible that such engineered toxins could be targeted to VAMP8 to disrupt mucin granule exocytosis specifically in the airways to improve mucus obstruction and MCC in chronic lung diseases.

In summary, the present study identifies VAMP8 as a v-SNARE for mucin granules in airway epithelial goblet cells. VAMP8 is associated with mucin granules in the goblet cells of human airway epithelia and is a critical SNARE for mucin granule exocytosis in response to acute agonist regulation. Basal secretion of mucins is also regulated by VAMP8. Since reduction in mucin secretion is a desirable therapeutic approach for many severe lung diseases, VAMP8 may be a potential target for drug development.

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