Published in final edited form as:

Cell Biol Int. 2012 September ; 36(9): 785–791. doi:10.1042/CBI20110146.

Characterization of VAMP-2 in the Lung: Implication in Lung Surfactant Secretion

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Abstract

Lung surfactant is crucial for reducing the surface tension of alveolar space, thus preventing the alveoli from collapse. Lung surfactant is synthesized in alveolar epithelial type II cells and stored in lamellar bodies before being released via the fusion of lamellar bodies with the apical plasma membrane. The soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) play an essential role in membrane fusion. We have previously demonstrated the requirement of t-SNARE proteins, syntaxin 2 and SNAP-23, in regulated surfactant secretion. Here, we characterized the distribution of vesicle-associated membrane proteins (VAMPs) in rat lung and alveolar type II cells. VAMP-2, 3 and 8 were shown to be present in type II cells at both mRNA and protein levels. VAMP-2 and -8 were enriched in lamellar body fraction. Immunocytochemistry studies indicated that VAMP-2 was co-localized with the lamellar body marker protein, LB-180. Functionally, the cytoplasmic domain of VAMP-2, but not VAMP-8 inhibited surfactant secretion in type II cells. This study suggests that VAMP-2 may be the v-SNARE involved in regulated surfactant secretion.

Keywords

exocytosis; lung surfactant; membrane fusion; SNARE; VAMP

1. Introduction

Lung surfactant forms a monolayer at the air-liquid interface in alveoli to reduce the surface tension, thus preventing the alveoli from collapse. Surfactant is composed of phospholipids and surfactant proteins with most of the components being synthesized in the endoplasmic reticulum in alveolar epithelial type II cells, and stored in the specified organelles of the lamellar bodies. The secretion of surfactant is a highly regulated process, including the translocation, docking, and fusion of lamellar bodies to the apical plasma membrane, and eventually the release of the contents into the alveolar lumen. Our previous studies have demonstrated the involvement of annexin A2, soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE), and some other regulatory factors in the secretion of lung surfactant (Abonyo *et al.*, 2003, 2004; Gou *et al.*, 2004). However, the precise underlying mechanism is still not clear and needs further investigation.

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SNAREs are a protein family which exist ubiquitously in eukaryotic cells and play a crucial role in membrane targeting, docking, and fusion (Rothman, 1994; Chen and Scheller, 2001; Jahn *et al.*, 2003; Brunger, 2005; Jackson and Chapman, 2006). SNARE proteins contain the characteristic coiled-coil domains termed SNARE motifs, which can form a trans-SNARE complex in two adjacent membranes in order to pull the membranes into close apposition, thus leading to membrane fusion (Fasshauer, 2003). There are two classes of SNARE proteins according to their localizations in the cell. The vesicle SNARE (v-SNARE), a vesicle associated membrane protein (VAMP), is located on the membrane of secretory vesicles; while the target SNAREs (t-SNARE), such as syntaxin and SNAP-23/SNAP-25, are located on the plasma membrane. VAMP-2 has been extensively studied in neurons, and has been shown to play a critical role in Ca^{2+} -triggered fusion of synaptic vesicles with the presynaptic membrane. VAMP-2 is also involved in regulating secretion in other cells, such as adipocytes and pancreatic β -cells. We have previously found that t-SNARE proteins, syntaxin 2 and SNAP-23, are required in lung surfactant secretion (Abonyo *et al.*, 2004). Here, we attempted to identify the v-SNARE that is required in this process. We demonstrated the presence of VAMP-2, -3, and -8 in alveolar type II cells at the mRNA and protein levels. Furthermore, VAMP-2 was localized on lamellar bodies. The cytoplasmic domain of VAMP-2 reduced surfactant secretion. This study suggests that VAMP-2 may be the v-SNARE involved in the regulation of lung surfactant secretion.

2. Materials & Methods

2.1 Reagents and chemicals

Fetal bovine serum (FBS), trypsin-EDTA, and DMEM were from Invitrogen (Carlsbad, CA). Enhanced chemiluminescence (ECL) reagent was from Amersham Pharmacia Biotech (Arlington Heights, IL). Rabbit anti-VAMP-2 antibody was from Stressgen Bioreagents (Ann Arbor, MI). Rabbit anti-VAMP-3 antibody was from Affinity Bioreagents (Golden, CO). Rabbit anti-VAMP-8 antibody was from Abcam (Cambridge, MA). Mouse anti-LB-180 antibody was from Covance (Richmond, CA). Goat anti-SP-C antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit horseradish peroxidase (HRP)-conjugated IgG was from Bio-Rad Laboratories (Hercules, CA). Rat anti-mouse HRP-conjugated IgG was from Jackson Immunoresearch Laboratories (West Grove, PA). Alexa Fluor 488 goat anti-mouse, Alexa Fluor 488 chicken anti-rabbit, Alex Fluor 546 donkey anti-goat and Alexa Fluor 568 goat anti-rabbit antibodies were from Molecular Probe (Eugene, OR). 18S rRNA primers were from Ambion (Austin, TX).

2.2 Isolation of alveolar type II cells

Alveolar type II cells were isolated from 180–200 gram Sprague-Dawley rats, according to the method of Dobbs *et al.* (1986), as described previously (Liu *et al.*, 1996). All of the animal procedures in this study were approved by the Institutional Animal Care and Use Committee at Oklahoma State University.

2.3 Reverse transcription polymerase chain reaction (RT-PCR)

Total RNAs were extracted from rat lung homogenate or freshly isolated type II cells with TRI reagent. 1 μg of total RNA was reverse-transcribed to cDNA by using M-MLV reverse transcriptase and random hexamer primers, followed by PCR amplification with gene specific primers. The primer sequences are listed in Table 1. 18S rRNA was amplified by using classic 18S rRNA primer pairs. The conditions for PCR amplification were as follows: 95°C for 2 min, 35 cycles of 95°C 30 sec, 55°C 30 sec, and 72°C 1 min, followed by 72°C for 8 min. The PCR products were electrophoretically separated on 1% agarose gel.

2.4 Isolation of Lamellar Body

Lamellar bodies were isolated from rat lung by upward flotation on a discontinuous sucrose gradient, as described by Chander *et al.* (1983) and Chattopadhyay *et al.* (2003). A perfused rat lung was briefly homogenized in 1 M sucrose and then loaded at the bottom of a sucrose gradient (0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 M). After centrifugation at 80,000 $\times g$ for 3 hours, the lamellar body fraction was collected at the 0.4 and 0.5 M interface, and diluted to 0.24 M with cold water. Lamellar bodies were then spun down at 20,000 $\times g$ and resuspended in 0.24 M sucrose containing 10 mM Tris and 50 mM Hepes (pH 7.0). The protein concentration of lamellar bodies was determined by Bio-Rad protein assay.

2.5 Preparation of the plasma membrane

The preparation of the plasma membrane from rat lung tissue was performed as described previously (Chattopadhyay *et al.*, 2003). A Sprague-Dawley rat lung was perfused with saline and homogenized in buffer B (10 mM Na-Pi, pH 7.4, 30 mM NaCl, 1 mM MgCl₂, 5 μ M PMSF, and 0.32 M sucrose). Following a discontinuous sucrose gradient (0.5, 0.7, 0.9 and 1.2 M) centrifugation at 95,000 g for 60 minutes, the plasma membrane fraction was collected at the 0.9 and 1.2 M interface and diluted to 0.32 M sucrose with cold buffer A (buffer B without sucrose). The plasma membrane was spun down at 120,000 $\times g$ and resuspended in buffer B.

2.6 Western Blotting

Protein samples were fractionated on 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 5% fat free milk in tris-buffered-saline with Tween 20 (TTBS, 20 mM Tris-HCl, pH 7.6, 150 mM NaCl and 0.1% Tween 20). The membrane was then incubated with appropriate primary antibodies (VAMP-2, 1:100; VAMP-3, 1:500; and VAMP-8, 1:500) at 4 °C overnight, and then with secondary antibody (1:2500) at room temperature for 1 hour. Finally, the signal was developed with ECL reagents.

2.7 Immunocytochemistry

Freshly isolated alveolar type II cells were cultured on cover slips overnight and then fixed with 4% paraformaldehyde for 20 minutes at room temperature. Immunocytochemistry was done as described earlier (Chintagari *et al.*, 2006). In brief, cells were permeabilized with 1% Triton X-100 and then blocked with 10% FBS in 50 mM PBS. The slides were then incubated overnight at 4°C with anti-LB-180 antibody (1:1000 dilution) and anti-VAMP-2 or VAMP-8 antibodies (1:100 dilution). They were subsequently washed and incubated with Alexa Fluor 488 goat anti-mouse and Alexa Fluor 568 goat anti-rabbit antibodies at 1:250 dilutions for 1 hour at room temperature. Finally, the slides were washed, mounted and examined with a fluorescence microscope (Nikon Inc).

2.8 Immunohistochemistry

Immunohistochemistry was performed as described earlier (Chintagari *et al.*, 2006). The rat lungs were perfused with PBS and lavaged with normal saline. The lungs were then fixed with 4% paraformaldehyde and afterward paraffin-embedded lungs were sectioned (2 μ m) and placed on glass slides (Fisher Scientific, Pittsburgh, PA). The slides were de-paraffinized with xylene and re-hydrated with graded alcohol and PBS. Inactivation of endogenous peroxidase by incubating slides with 4% hydrogen peroxide and antigen retrieval was done by boiling the slides in citrate buffer (10 mM disodium citrate, pH 6.0, and 0.05% Tween-20) for 20 minutes. The sections were permeabilized with 1% Triton X-100 and blocked with 10% donkey serum in 50 mM PBS. They were then incubated overnight at 4 °C with anti-SP-C antibody (1:100 dilution) and anti-VAMP-2 (1:100

dilution). Subsequently, they were washed and incubated with Alexa Fluor 488 chicken anti-rabbit and Alexa Fluor 546 donkey anti-goat antibodies at 1:250 dilutions for 1 hour at room temperature. Finally, the slides were washed, mounted and examined with a fluorescence microscope.

For fetal lung tissue, ABC staining was used due to the interference of red blood cells with immunofluorescence. The lung tissue sections were dewaxed and rehydrated. The slides were treated with 3% H₂O₂. Antigen retrieval was done by boiling the slides in citric buffer (pH 6.0) for 20 min. The slides were permeabilized in 0.3% Triton X-100 for 10 min and blocked in 10% normal horse serum. The tissue sections were incubated with anti-VAMP-2 (1:100), followed by secondary antibodies (1:100). The slides were incubated in ABC reagents for 30 min. Signals were detected using DAB substrate. The slides were counter-stained with hemeotoxylin.

2.9 Construction of adenoviral vectors

Because of the difficulty in transfecting primary type II cells, we constructed adenoviruses for the overexpression of the cytoplasmic domains of VAMPs. The cytoplasmic domain (amino acids 1-94) of VAMP-2 or the cytoplasmic domain (amino acids 1-75) of VAMP-8 plasmids were kindly provided by Dr. Richard H. Scheller (Stanford U.) and were cloned into the pENTR/CMV-EGFP vector between EGFP and the SV40 polyA terminal sequence (Bhaskaran *et al.*, 2007). The insert was switched to the adenoviral, pAd/PL-DEST vector (Invitrogen) through LR recombination. The vector was then linearized by Pac I digestion and transfected into HEK 293A cells to generate adenoviruses. The virus titer was determined by infecting HEK 293A cells with a serial dilution of virus stock and counting virus-infected cells through GFP fluorescence.

2.10 Surfactant secretion assay

Lung surfactant secretion was determined by monitoring the release of [³H]-labeled PC from type II cells as described (Liu *et al.*, 1996). Freshly isolated type II cells (1×10^6) were infected overnight with adenoviruses at a multiplicity of infection (MOI) of 100 in the presence of 0.6 μ Ci [³H] choline. The cells were washed 6 times and then stimulated with 100 μ M ATP, 100 nM PMA and 10 μ M terbutaline for 2 hrs. At the end of incubation, the lipids in media and cells were extracted with chloroform-methanol. The secretion was calculated as $100 \times (\text{counts in medium} / \text{counts in medium and cells})$.

3. Results

3.1 VAMP genes are expressed in lung and alveolar type II cells

There are seven members in VAMP family. We utilized the RT-PCR method to examine the expression pattern of VAMP genes in alveolar type II cells. Specific primer pairs were designed, and gene fragments of different VAMP isoforms were amplified from rat lung and highly-purified alveolar type II cells. Brain tissue was used as a reference. As shown in Fig. 1, most of the VAMP isoforms were expressed in type II cells. VAMP-2 mRNA was observed both in lungs and type II cells. High expression of VAMP-3 and VAMP-8 was also observed which appear to be enriched in type II cells in comparison with lungs.

3.2 VAMP proteins are expressed in lamellar body fraction

To explore the distribution of VAMP proteins in alveolar type II cells, we utilized the Western Blotting method to examine the different fractions of type II cells. In addition to the band expressed in brain (18 kDa), there is another band for VAMP-2 with a lower molecular weight found in lung tissue, type II cells, lamellar body fraction, and the plasma membrane. This lower band was dramatically enriched in the lamellar body fraction (Fig. 2). VAMP-8

was detected in both the lamellar body and the plasma membrane fractions, whereas VAMP-3 was mainly present in the plasma membrane fraction and was not detected in the lamellar body fraction.

3.3 Localizations of VAMP-2 in lung tissue and in alveolar type II cells

To examine the cellular localization of VAMP-2 in the lung, we performed immunohistochemistry on adult perfused lung tissue by the dual-immunolabeling technique, using anti-SP-C (a type II cell marker) and anti-VAMP-2 antibodies. The signal of VAMP-2 staining was overlapped with that of SP-C in the lung tissue, indicating that VAMP-2 is localized in type II cells (Fig. 3A). The location of VAMP-2 in the fetal lungs was also determined. As shown in Fig. 3B, VAMP-2 was localized in airway epithelial cells at gestational day 16.

To further study the localization of VAMP-2 protein in alveolar type II cells, we performed dual-immunostaining of VAMP-2 and LB-180, a lamellar body marker protein, in isolated alveolar type II cells. VAMP-2 staining was partially overlapped with LB-180. However, VAMP-8 signal did not overlap with LB-180 (Fig. 4)

3.4 Cytoplasmic domain of VAMP-2 inhibits surfactant secretion

We investigated the role of VAMP-2 in surfactant secretion by overexpressing the cytoplasmic domain (CD) of VAMP-2. The VAMP-2 CD functions as a dominant-negative mutant since it does not contain a membrane domain, thus preventing the trans-SNARE complex formation. We constructed an adenoviral vector (V-2) that contains the CMV promoter and the CD (amino acids 1-94) of VAMP-2. Two controls were included, CMV promoter-driven EGFP (control virus, CV), and CMV promoter-driven CD (amino acids 1-75) of VAMP-8 (V-8). VAMP-2 CD inhibited surfactant secretion, while VAMP-8 CD did not inhibit (Fig. 5), suggesting a role of VAMP-2 in surfactant secretion.

4. Discussion

SNARE proteins play a central role in eukaryotic membrane trafficking events, and the underlying mechanism is conserved among different species. We have previously demonstrated that the t-SNAREs, syntaxin 2, and SNAP-23 are present in alveolar type II cells, and that they are required in surfactant secretion. In this study, we have characterized VAMP proteins in lung tissues and type II cells, and studied its functional role in lung surfactant secretion.

From the RT-PCR results, we found that various isoforms of VAMP genes are expressed in type II cells. There are seven members in the VAMP family. VAMP-1 is highly homologous to VAMP-2, but has a different cellular distribution pattern. It is involved in calcium-dependent synaptic vesicle exocytosis (Jacobsson *et al.*, 1998; Raptis *et al.*, 2005; Sherry *et al.*, 2003). VAMP-1 is also reported to be expressed in non-neuronal tissues (Rossetto *et al.*, 1996), but its function is not clear. VAMP-3 is ubiquitously expressed and preferentially associated with early/recycling endosomes. VAMP-3 was reported to play an important role in platelet alpha granule secretion by using tetanus toxin (Flaumenhaft *et al.*, 1999), antibody (Feng *et al.*, 2002), and the cytoplasmic domain (Polgar *et al.*, 2002). However, the reduction of VAMP-3 in transgenic mice had no effects on platelet function, indicating it is not essential for platelet releasing reaction in mice (Schraw *et al.*, 2003). VAMP-7 is resistant to tetanus neurotoxin cleavage (Galli *et al.*, 1998). It is associated with the late endosome, and is involved in endocytosis and intracellular trafficking between the ER and Golgi (Braun *et al.*, 2004; Siddiqi *et al.*, 2006). VAMP-8 has been reported to be involved in the fusion between early and late endosomes of the endocytic pathway (Wong *et al.*, 1998; Antonin *et al.*, 2000). It has recently been reported that VAMP-8 is required in the regulated

exocytosis of pancreatic acinar cells and platelets (Wang *et al.*, 2004; Ren *et al.*, 2007). VAMP-2 was initially identified as a v-SNARE of synaptic vesicles in neurons, thus playing an important role in synaptic vesicle exocytosis (Lin and Scheller, 2000). VAMP-2 is also involved in regulated transporting events in non-neuronal systems, such as trafficking of glucose transporter-4 (GLUT-4) to the plasma membrane (Slot *et al.*, 1997; Foster *et al.*, 1998; Foster and Klip, 2000; Bryant *et al.*, 2002; Watson *et al.*, 2004). We found that the VAMP isoforms abundantly expressed in alveolar type II cells were VAMP-2, VAMP-3, and VAMP-8.

We next examined the protein expression pattern of VAMP-2, 3, and 8 in lung and type II cells by Western blotting. Along a band with the same size as that in brain (18 kDa), a band with a lower mass for VAMP-2 was consistently detected by a polyclonal anti-VAMP-2 antibody. This is probably the degradation product of VAMP-2 or another VAMP-2 isoform. VAMP-2 was also detectable in a plasma membrane fraction. An immuno-staining study showed that VAMP-2 was partially co-localized with lamellar body marker, LB-180, indicating that VAMP-2 is localized on lamellar bodies. Based on this association, it is reasonable to consider the putative role of VAMP-2 as a v-SNARE in the fusion of lamellar bodies with the plasma membrane. VAMP-3 was barely detected in lamellar bodies, but was present in type II cells. It appears to be enriched in the lung plasma membrane fraction. Interestingly, a strong band for VAMP-8 was observed on lamellar bodies by Western blot analysis. However, immunostaining only exhibited a faint staining of VAMP-8 on lamellar bodies. This is probably due to the different exposure of the epitopes recognized by the antibody. The presence of more than one VAMP isoform and their distinctive distribution patterns suggest that different VAMP isoforms are involved in different processes in type II cells, rather than simply functional redundancy.

The VAMP-2 cytoplasmic domain (VAMP-2 CD) does not contain its membrane domain. However, it can form a SNARE complex (Hao *et al.*, 1997; Poirier *et al.*, 1998). Once it is expressed in the cells, the VAMP-2 CD incorporates into the SNARE complex to compete with endogenous VAMP-2 effectively, preventing the formation of a functional trans-SNARE complex. Thus, the VAMP-2 CD behaves as a dominant-negative mutant or as a competitive inhibitor. The overexpression of VAMP-2 CD in type II cells inhibited surfactant secretion. This is consistent with a previous study demonstrating that the expression of VAMP-2 CD decreased insulin-stimulated GLUT4 translocation (a process similar to regulated exocytosis) in adipocytes (Olson *et al.*, 1997). VAMP-8 CD had no effects on surfactant secretion. Along with the lamellar body location of VAMP-2, these results indicate that VAMP-2 actively participates in surfactant secretion.

Acknowledgments

This work was supported by NIH R01 HL-052146 and AHA GRNT 7450069. We thank Ms. Tazia Cook for her editorial assistance.

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Fig. 1. RT-PCR amplification of VAMP isoform genes in alveolar type II cells

Total RNA was extracted from whole lung tissue (L) and freshly isolated alveolar type II cells (> 95% purity, T2) and brain (B). After reverse-transcription with random hexamer primers, VAMP isoform genes were amplified with specific primers for 35 cycles with standard PCR protocols. 18S rRNA was used for normalization. PCR products were electrophoretically separated on 1% agarose gels. A reaction without cDNA template was used as a negative control (-'ve).

Fig. 2. The expression of VAMP proteins in alveolar type II cells

Freshly isolated type II cells (T₂), lung tissue homogenate (L), lamellar body fraction (LB), and the plasma membrane fraction (PM) isolated from lungs were lysed. Same amounts of total protein were separated by 12% SDS-PAGE, and probed with anti-VAMP-2 antibodies, anti-VAMP-3 and -8 antibodies, respectively. Brain (B) was used as a reference.

Fig. 3. VAMP-2 localization in lung tissues

(A) Adult lungs: Paraformaldehyde-fixed and Triton X-100-permeabilized lung tissue sections were blocked and incubated with rabbit anti-VAMP-2 and goat anti-SP-C antibodies, followed by incubation with Alexa 546-conjugated donkey anti-goat and Alexa 488-conjugated chicken anti-rabbit antibodies. The images were taken at $\times 400$ (B) Fetal lung tissues at gestational day 16 (D16) were immunostained with anti-VAMP-2 antibodies and ABC reagents. The images were taken at $\times 100$.

Fig. 4. VAMP-2 is localized on lamellar bodies in alveolar type II cells

Freshly isolated type II cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. The cells were blocked and incubated with rabbit anti-VAMP-2 or VAMP-8 antibodies and mouse anti-LB-180 antibodies. They were then incubated with Alexa 546-conjugated goat anti-rabbit and Alexa 488-conjugated rabbit anti-mouse antibodies.

Fig. 5. The role of VAMP-2 in surfactant secretion

Freshly isolated type II cells were infected with adenoviruses containing EGFP (control virus, CV), VAMP-2 cytoplasmic domain (V-2) or VAMP-8 cytoplasmic domain (V-8) at a multiplicity of infection (MOI) of 100. After a 24-hr infection, type II cells were stimulated with 100 μ M ATP, 100 nM PMA and 10 μ M terbutaline for 2 hrs, and surfactant secretion was measured. The results were expressed as a percentage of blank control (BC). * $P < 0.05$ v.s. BC (n=4 cell preparations, student *t* test).

Table 1

PCR primers for VAMP gene amplification

Gene Name	Primer Sequences	Product Size (bp)
VAMP-1	5'-AGGGACCAGAAGTTGTCAGAGT-3'	301
GeneID: 25624	5'-GCCATCTCCATACCTCTGCA-3'	
VAMP-2	5'-CTGGTGTGTAAGTGTCTTGGAG-3'	299
GeneID: 24803	5'-CAGAGATTTCAGGCAGGAATTA-3'	
VAMP-3	5'-CTGATGTGGTTTCTTCCTAGA-3'	301
GeneID: 29528	5'-AGGCATGTCTTCAACACTTG-3'	
VAMP-4	5'-GAATCAGGTGGACGAAGTTAT-3'	301
GeneID: 364033	5'-TGTTATTGTCCCAGATCTTGTT-3'	
VAMP-5	5'-CAGACCAAGTGACGGAATC-3'	251
GeneID: 89818	5'-TCCACTCGGAAGAAAGATGA-3'	
VAMP-7	5'-GGATTGTGTATCTTTCATCA-3'	301
GeneID: 85491	5'-TCTATCAGCAATTCTAGCCTTT-3'	
VAMP-8	5'-GAAATGACCGAGTCAGGAAC-3'	263
GeneID: 83730	5'-CGTAGCAAAGAGTATGATGAGG-3'	