MECHANISMS OF REGULATED LUNG SURFACTANT SECRETION

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SURFACTANT SECRETION

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NOMENCLATURE

18S rRNA	18S ribosomal RNA
ABCA1, 3 & 4	ATP binding cassette sub-family A member 1, 3 & 4
α-, β- & γ-SNAP	α-, β- & γ-soluble NSF attachment protein
AIIm	annexin A2 monomer
AIIt	annexin A2 tetramer
ATP	adenosine triphosphate
BAL	bronchoalveolar lavage
BoNT	botulinum neurotoxins
CaMK	Ca^{2+}-calmodulin dependent protein kinase
cAMP	cyclic adenosine monophosphate
cDNA	complimentary DNA
CDP	cytidine 5'-diphosphate
$cPLA_2$	cytosolic phospholipase A_2
CRR	cysteine riched region
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DPPC	dipalmitoylphosphatidylcholine
ECL	enhanced chemiluminescence
EDTA	ethylene diaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ER	endoplasmic reticulum
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

GFP	green fluorescent protein
GLUT-4	glucose transporter 4
GSNO	S-nitrosoglutathione
GST	glutathione *S*-transferase
HDL	high density lipoprotein
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
IEF	isoelectric focusing
IGF1	Insulin-like Growth Factor 1
IgG	immunoglobulin G
IP_3	inositol trisphosphate
IPTG	isopropyl β-D-1-thiogalactopyranoside
Kd	equilibrium dissociation constant
kDa	kilo Dalton
KGF	keratinocyte growth factor
LAMP	lysosome associated membrane proteins
LAR II	Luciferase Assay Reagent II
LB-180	lamellar body protein 180
LDL	low-density lipoprotein
LPS	lipopolysaccharide
MALDI-TOF MS	matrix-assisted laser desorption/ionisation-time of flight mass spectrometry
MEM	minimum Essential Medium
mRNA	messenger RNA
MSDB	mass spectrometry protein sequence database
MVB	multi-vesicular-bodies
NEM	N-ethylmaleimide
NF-κB	nuclear factor kappa B
NP-40	protein solubilizer 40
NSF	N-ethylmaleimide sensitive factor
PA	phosphatidic acid

PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PIP_2	phosphatidylinositol bisphosphate
PLC	phospholipase C
PLD	phospholipase D
PMSF	phenylmethanesulphonyl fluoride
PKA	cAMP-dependent protein kinase A
PKC	protein kinase C
PS	phosphatidylserine
R-18	Octadecyl Rhodamine
RDS	respiratory distress syndrome
RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	reverse transcription polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNAP-25/23	synaptosome associated protein of 25/23 kDa
SNARE	soluble N-ethylmaleimide sensitive factor attachment protein receptor
SP-A, B, C & D	surfactant protein A, B, C & D
SV-40	simian virus 40
TI-VAMP	toxin-insensitive vesicle associated membrane protein
TPA	tetradecanoyl-phorbol-acetate
TTBS	tris-buffered-saline with Tween 20
VAMP	vesicle associated membrane protein

CHAPTER I

INTRODUCTION

1. Retrospective of lung surfactant studies

The lung is the most important component of the respiratory system as it is composed of over 300 million alveoli. Alveoli are sac-like extensions at the distal end of the airway where gas exchange occurs. The walls of alveoli are covered by alveolar epithelium, which is composed of squamous alveolar epithelial type I cells (type I) and cuboidal alveolar epithelial type II cells (type II). Type I cells are large flat cells that comprise about 8% of peripheral lung cells. They cover as much as 95% of the alveolar surface and, along with the underlying basement membrane and capillary endothelial cells, form a thin air-blood barrier. In contrast, type II cells comprise about 15% of lung cells. They are scattered along the alveolar epithelium and form tight junctions with type I cells. Type II cells are the progenitors of type I cells. In normal lungs, type II cells proliferate with some of the daughter cells remaining type II cells and some trans-differentiate into type I cells. The flat type I cells occupy most of the alveolar surface making them the most vulnerable to the damage during acute lung injuries. In response to injury, type II cells spread, proliferate and subsequently differentiate into hyper-plastic type II cells, some of which trans-differentiate into type I cells for the restoration of the damaged alveolar epithelium [1].

Along the alveolar surface, there is a layer of thin liquid termed hypophase. Attraction among these water molecules generates a force, surface tension, which can potentially cause the alveoli to collapse and make the re-expansion of alveoli more difficult during inhalation. To overcome this undesirable consequence, the lung produces a lipoprotein-like material, called surfactant, which forms a monolayer between air and liquid at the air-liquid interface and dramatically reduces the surface tension of alveoli. This reduction subsequently increases lung compliance and prevents alveoli from collapsing [2, 3]. Surfactant deficiency at the alveolar surface is associated with infant and acute respiratory distress syndromes (IRDS & ARDS) [4]. In addition to the primary function of reducing surface tension, surfactant also plays a role in maintaining fluid homeostasis in the alveoli. Surfactant facilitates the clearance of excess fluid in alveoli by directing the net fluid flow across the air-blood barrier, thus preventing the formation of alveolar edema [5, 6]. Lung surfactant also has a role in host defense as first postulated first by Charles Macklin [7] and later by Ulrich Pison [8]. Along with being a physical permeation barrier against certain hazardous substances, surfactant also binds to various pathogens by surfactant protein A and D.

The studies of possible roles for surfactant stem back to as far as 1929, when a Swiss physician, Kurt Von Neergaard, reported pressure-volume studies with excised lungs [9]. His finding strongly suggested the importance of surface tension in alveolar mechanics, but his findings were ignored until Edward Radford carried out similar experiments 25 years later that confirmed Von Neergaard's work [10]. In 1955, R.E. Pattle's work on lung edema first suggested that some material lining along the alveolar surface diminished surface tension to 0 mN/m [3]. It was John A. Clements who first

provided direct evidence for the presence of a surface active material at the alveolar surface [2]. Clements et al. also suggested that dipalmitoylphosphatidylcholine (DPPC) is the major surface active component in surfactant. Their work enlightened the active investigations of surfactant and leading to Mary Avery and James Mead's 1959 discovery that there is an association between infant RDS and the absence or delayed appearance of lung surfactant [11]. A variety of studies from G.C. Liggins [12] showed that corticosteroid treatment accelerated fetal lung maturation and the onset of surfactant production, which led to the first clinical trial in which the administration of corticosteroid to pregnant women prevented the occurrence of IRDS. In 1980, T. Fujiwara carried out a promising clinical trial by treating babies suffering IRDS with exogenous surfactant isolated from bovine lungs [13]. This report gave momentum for studies that aimed to design well-defined surfactant preparations for safe replacement therapy.

Soon after the discovery of surfactant, questions remained as to what cells produced surfactant and how long it stayed in the alveoli. These questions were difficult to answer because the lung contains about 40 different types of cells and surfactant is not a single molecule but a mix of lipids and proteins. As early as 1954, type II cells were postulated to be the makers of surfactant [7]. It was also proposed early on that lamellar bodies of type II cells serve as the storage site of surfactant prior to its secretion into alveolar surface [14, 15]. In 1972, G. Chevialer provided strong evidence that type II cells are the major, if not the only, cells to produce surfactant [16]. Many studies have been carried out to reveal the pathways and regulation of surfactant synthesis, secretion and recycling

thanks to the advances in techniques for type II cell isolation and lamellar body fractionation [17-19].

2. Surfactant biochemistry

Lung surfactant is somewhat like the cell membrane in that it is rich in lipids and proteins. From the numerous biochemical studies of bronchoalveolar lavage (BAL) material, lung surfactant is known to be composed of about 10% of protein and 80-90% of lipid by weight. More than 80% of lipids are phospholipids, whereas cholesterol comprises the largest amount of neutral lipids [20, 21]. Phosphatidylcholine (PC) is the major phospholipid component (60-70%) in surfactant. Other phospholipid components include phosphatidylglycerol (PG) (5-10%), phosphatidylethanolamine (PE) (5-10%), phosphatidylinositol (PI) and phosphatidylserine (PS) (PI + PS = 3-6%). Surfactant contains an unusually high level of saturated-fatty-acid-chained lipids, predominantly DPPC, which contributes substantially to the unique function of surfactant in the reduction of surface tension in the extracellular surfactant film at the air-liquid interface [22]. Phospholipids contain a glycerol backbone, a polar head and a non-polar tail. This structure allows phospholipids to fit in between water molecules to disrupt the formation of hydrogen bonds, thus reducing the surface tension resulted from water molecule interaction.

Besides lipids, surfactant proteins are also required for the formation and maintenance of surfactant film at the alveolar surface [13, 23]. There are four surfactant proteins designated as SP-A, SP-B, SP-C and SP-D. Both SP-A and SP-D are hydrophilic proteins that contain N-terminal collagen-like domains and a C-terminal lectin-like domain. The C-terminal domain harbors calcium and carbohydrate binding sites. SP-A is

able to bind lipids and carbohydrate as well as specific cell receptors and is involved in a variety of lung surfactant functions. SP-A is essential for promoting the transformation of lamellar bodies into tubular myelin [24, 25]. SP-A has the ability to self-aggregate at a physiological extracellular calcium concentration, and this aggregation is believed to be important for the SP-A-induced formation of tubular myelin [26, 27]. In RDS patients, a lack of tubular myelin was found together with a loss of SP-A, which supports the role of SP-A in tubular myelin formation [28]. Another function of SP-A is to regulate surfactant homeostasis. SP-A was reported to mediate the endocytosis of recycling surfactant [29], as well as inhibit surfactant exocytosis [30]. Since SP-A contains the carbohydrate binding site, it also has the ability to recognize and bind lipopolysaccharides (LPS) from Gram-negative bacteria [31], which enhances alveolar macrophage migration and phagocytosis [32]. The main function of SP-D is to modulate innate immunity and pulmonary host defense. Like SP-A, SP-D binds to the surface of pathogens like bacteria [33, 34] and viruses [35] for the facilitation of their elimination by macrophages [36]. Both SP-A and SP-D knockout mice showed abnormal responses against bacterial and viral challenges [37]. One surprising finding about SP-D is that it binds PI in a calcium-dependent manner, which may indicate a role of SP-D in intracellular lipid sorting and/or signal transduction [38, 39].

SP-B and SP-C are highly hydrophobic and are intimately involved in the function of surfactant. They play important roles in the stability and spreading of surfactant along alveoli. SP-B and SP-C are synthesized and packed with phospholipids in lamellar bodies. SP-B is a small protein with 79 amino acid residues that contains 3 characteristic intramolecular disulfide bonds out of its 7 cysteine residues. The one free cysteine

residue forms intermolecular disulfide bond required for its dimerization [40, 41]. The most important function of SP-B is to promote the formation of stable surfactant film by mediating the phospholipid insertion into the air-liquid interface [42, 43]. It is also required for tubular myelin formation [24] and proteolytic processing of SP-C. SP-B knockout mice died of RDS shortly after birth. SP-C has only 35 amino acid residues. In addition to its high content of valine residues, the palmitoylation of the cysteine residues makes SP-C extremely hydrophobic. SP-C is also able to promote the phospholipid insertion into the air-liquid interface in a calcium-dependent manner [43] and regulate phospholipid ordering [44, 45].

3. Biogenesis of surfactant and lamellar bodies

Autoradiographic [16] and immunocytochemical [46] studies have demonstrated that most components of surfactant are synthesized in the endoplasmic reticulum of type II cells. The precursors for synthesis of surfactant lipids in type II cells include glycerol, fatty acids, acyl chains and other head groups. The glycerol backbone used for surfactant lipid synthesis is mainly from circulating glucose [47], while the fatty acids are supplied from several sources: circulation [48], the hydrolysis of recycled phospholipids up-taken from alveoli [49] and synthesis *de novo* [50]. Mammalian tissues do not synthesize free choline and the choline required for PC synthesis is likely from the diet [51]. *Myo*-inositol required for PI formation can be synthesized in the body, while additional supplementation of inositol from the diet has also been reported [52, 53].The first step of surfactant phospholipid synthesis is the formation of phosphatidic acid (PA) from glycerol-3-phosphate derived from glycerol or glucose with two sequential acylation reactions. To synthesize PC, PA is hydrolyzed to diacylglycerol by phosphatidate

phosphatase. Intracellular triacylglycerol might be an additional source by the action of triacylglycerol lipase [54]. With the sequential actions of choline kinase and choline-phosphate cytidylyltransferase, the cytidine 5'-diphosphate (CDP) group is added to free choline. The competent choline group is then transferred to diacylglycerol to form PC by the action of cholinephosphotransferase. For the formation of PG and PI, phosphatidate cytidylyltransferase catalyzes PA to convert into CDP-diacylglycerol, from which PI can be synthesized by the reaction with *myo*-inositol catalyzed by CDP-diacylglycerol-inositol phosphatidyltransferase. CDP-diacylglycerol can also be converted by glycerolphosphate phosphatidyltransferase into phosphatidylglycerolphosphate, which is subsequently dephosphorylated into PG by phosphatidylglycerophosphatase. The synthesis of PE is similar to that of PC, in which an ethanolamine group is added to diacylglycerol by ethanolaminephosphotransferase. PS is likely produced by a head group switch from PC or PE. Most of the cholesterol is from circulating low-density lipoprotein (LDL) or high-density lipoprotein (HDL) and the synthesis in the lung is negligible [55].

Among the 4 surfactant proteins, SP-C is only synthesized in type II cells [56, 57]. SP-A, SP-B and SP-D are synthesized in both bronchiolar Clara cells and type II cells [58-60]. The primary translation product of SP-A preprotein contains a 20 amino acid hydrophobic signal peptide, by which the preprotein attaches and traverses the endoplasmic reticulum. The signal peptide is then cleaved to allow the preprotein into the endoplasmic reticulum lumen. Posttranslational modifications of SP-A include hydroxylation [61], glycosylation [62], sialyation [63], sulfation and sulfhydryl-dependent oligomerization [64]. Mature SP-A is mainly released into alveoli through a

constitutive pathway other than via lamellar bodies [65, 66]. The translated SP-B preprotein is about 42 kDa with a 23 amino acid N-terminal signal peptide. After cleavage of the flanking sequences at both C- and N-termini, the mature SP-B is eventually transported into lamellar bodies where the homodimerization occurs [41]. SP-C cDNA is about 3.5 kb and is translated into a 179 amino acid preprotein. The N-terminus of the preprotein is required for its sorting to the secretion pathway [67], while the C-terminus has been shown to play a role in intracellular transportation [68]. The palmitoylation of cysteine residues takes place in the Golgi apparatus and it is important for lamellar bodies to maintain a dense packing [69]. Like SP-A, SP-D undergoes posttranslational modifications, such as hydroxylation and glycosylation and is capable of forming homo-oligomers [70, 71]. SP-D is localized in the ER but not in lamellar bodies [60, 72], which indicates that it is secreted via a constitutive pathway.

Lamellar bodies are lysosome-related organelles and mature lamellar bodies are one of the largest secretory granules in the cell, ranging from 1 to 2 μm in size. Similar to lysosomes, lamellar bodies contain soluble lysosomal enzymes and proteins, such as acid phosphatase and lysosome associated membrane proteins (LAMP) [73-75] and an acidic interior (pH about 6.1 or below) [76, 77]. In rat lungs, lysozymes were found in lamellar bodies on type II cells [78]. However, lamellar bodies are different from lysosomes in that they are specialized for storage and secretion of surfactant rather than for degradation. Phospholipids are the principle components and are tightly packed within lamellar bodies, being well organized as concentric arrangements of bi-layer membranes. There is a high level of calcium present inside lamellar bodies and part of its significance might be for sequestering acidic phospholipids, which could produce charge repulsion and disrupt

membrane packing. Lamellar body formation requires SP-B and ABCA3. ABCA3 is a member of the ATP Binding Cassette family of ATP-dependent membrane associated transport proteins. ABCA3 is expressed exclusively in alveolar type II cells and localized on lamellar bodies [79-81]. Like ABCA1 and ABCA4, ABCA3 may function as a lipid transporter in type II cells and is essential for the formation of lamellar bodies. ABCA3-knockout mice died of RDS within one hour after birth even with the gross lung developed normally. However, no mature lamellar bodies were found and the surfactant was absent in the alveolar space [82, 83].

Lamellar bodies originate from the endoplasmic reticulum and then travel via the Golgi complex as an intermediate form. Multi-vesicular-bodies (MVBs) are early manifestations. The mature lamellar bodies are transported to apical area of type II cells [84]. By combining electron microscopy and autoradiography techniques, newly synthesized surfactant protein was sequentially detected in the ER, Golgi, MVBs and lamellar bodies in type II cells. After synthesis, surfactant lipids are transferred via the Golgi apparatus to small immature lamellar bodies while proteins are transferred into MVBs [16, 85]. There are two types of MVBs. Large MVBs (l-MVB) with an electron lucent matrix are localized mainly at the apical area and contain SP-A. The smaller MVBs with an electron dense matrix (d-MVB) are mainly localized at the basolateral area and contain lysosomal activity [86]. In addition to MVBs, the composite body, an unusual organelle, contains lipid bilayers and might be derived from the fusion of MVBs and lipid-containing immature lamellar bodies. The fully mature lamellar bodies eventually fuse with the apical plasma membrane and release surfactant into the alveolar space.

The surfactant system is very dynamic. For example, phosphatidylcholine, the predominant component of surfactant, has a half life of only a few hours and about 85% of it is recycled [87]. All components of surfactant, including lipids and all 4 surfactant proteins, have been demonstrated to undergo re-uptake and recycling [88-91]. SP-A, B, C and anionic phospholipids have been reported to stimulate the uptake of isolated type II cells [92, 93] while SP-D is ineffective [88]. At least in the case of SP-A, the uptake is through receptor-mediated endocytosis [29, 94]. The majority of the re-uptake components, especially PC, are incorporated into lamellar bodies via MVBs for reuse, while the rest is degraded in lysosomes for surfactant synthesis or completely out of surfactant system.

4. Surfactant secretion

Surfactant secretion has been studied in various models from intact animals to isolated type II cells [30, 95-97]. The latter is the most utilized. The lipid components of surfactant are secreted through lamellar bodies in a highly regulated pathway. The hydrophobic SP-B and SP-C are secreted with surfactant lipids together, while SP-A and SP-D are through constitutive routes independent of lamellar bodies. Secretion of surfactant is regulated by a variety of chemical or physical stimuli. Distension of the lung is the most physiologically relevant stimulus. This can directly act on type II cells [98-100], or indirectly through type I cells [101-104]. In the latter case, the inflation-induced calcium signal in type I cells can pass on to type II cells through gap junctions [105]. Other pharmacological agents include β-adrenergic and adenosine A_2B receptor agonists, purinergic P_2Y_2 receptor agonists (ATP), forskolin, cholera toxin, tetradecanoyl-phorbol-

acetate (TPA) and calcium ionophores (A23187). There are at least 3 signal transduction pathways involved in the regulation of surfactant secretion.

The first pathway involves the activation of G protein-coupled adenylate cyclase after β-adrenergic or adenosine A_2B receptors are stimulated. Adenylate cyclase generates cAMP and subsequently activates cAMP-dependent protein kinase A (PKA). Cholera toxin directly activates G protein, and forskolin directly activates adenylate cyclase.

The second mechanism involves the activation of the protein kinase C (PKC) pathway. TPA directly activates PKC. ATP binds the P_2Y_2 receptor and activates phospholipase C (PLC), which hydrolyzes phosphatidylinositol bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol trisphosphate (IP_3). The DAG-activated PKC subsequently may activate phospholipase D (PLD), which hydrolyzes PC into choline and PA. Phosphatidate phosphatase converts PA into DAG, which further activates PKC.

The third pathway involves the increase in the intracellular calcium levels. Calcium ionophores promote calcium influx into the cell and the IP_3 from the PIP_2 hydrolysis can stimulate the release of calcium from intracellular compartments, such as the endoplasmic reticulum. The increased calcium level activates a calcium-calmodulin-dependent protein kinase (CaMK). In type II cells, the presence of CaMK II has been demonstrated by Western blotting. Meanwhile, the Ca^{2+} ionophore A23187-stimulated phosphatidylcholine secretion was inhibited by KN-62, a specific CaMK II inhibitor, in a dose-dependent manner in isolated type II cells [106].

After one or multiple activations of the signal transduction pathways, the target proteins are phosphorylated, ultimately leading to surfactant secretion. The precise

mechanisms are still unclear, while certain factors have been reported to play some roles during the process. Calpains are Ca^{2+}-dependent cysteine proteases and have been reported to be activated by secretagogues in type II cells [107]. Calpains partially proteolyze PKA in stimulated type II cells, and this limited proteolysis may convert the initial transient signal into sustained and augmented phosphorylation activity for secretion [108]. Meanwhile, the suppression of calpains significantly inhibited the TPA-stimulated secretion in type II cells [109]. In addition, arachidonic acid has been shown to stimulate secretion in cultured type II cells [110, 111] and promote annexin A2-mediated fusion between isolated lamellar bodies and the plasma membrane [112]. Arachidonic acid is generated through the work of cytosolic phospholipase A_2 ($cPLA_2$) and DAG lipase. The blockage of $cPLA_2$ or DAG lipase activity has been shown to inhibit the secretion in type II cells [113]. These results further confirm the involvement of arachidonic acid in surfactant secretion.

The processes by which lamellar bodies are translocated to the area of the apical plasma membrane are still not known. It has been postulated that actin [114] and microtubules [115] play a role for the transportation of lamellar bodies and intermediate filaments may function as guides [115]. The sub-apical cytoskeletal network of F-actin has to be dissembled for lamellar bodies to be transported to the apical membrane [116]. In stimulated type II cells, the cytoskeleton undergoes disassembly and assembly and annexin A2 may play a role in this process [117]. Once lamellar bodies reach the plasma membrane, annexins may facilitate their fusion with the plasma membrane [118, 119].

The secretion occurs by merging the limiting membrane of lamellar bodies with the plasma membrane and forming a fusion pore, through which the surfactant is released out

of the cells into alveolar lumen [120, 121]. Morphological and functional evidence has demonstrated that fusion pores in type II cells are pretty stable [122, 123]. By using fluorescent dye FM 1-43, fusion pores are found to expand slowly, but the expansion accelerates as calcium concentration increases [124]. Fusion pore structures have been studied on both artificial bilayers [125, 126] and biological membranes [127-130]. A "stalk-pore" hypothesis has been postulated to explain its formation. It starts with the contact between the fusing membranes mediated by tethering molecules, like a viral fusion peptide [131] or a hairpin structure composed of membrane proteins on opposing membranes [132]. The reduction of repulsion between two opposing membrane bilayers may result in a relaxation in the merging of the proximal bilayers. Thus, the proximal monolayers are connected with a highly bent stalk and the distal monolayers are pulled toward each other to form a dimple. This is referred to as hemifusion state and is thought to be a prerequisite for the formation of a fusion pore [133]. Following hemifusion, an expanding fusion pore opens and the content is released. Haller et al. proposed that the diameter of fusion pores might be the rate-limiting step for the surfactant secretion [123]. The observation of increased surfactant secretion in stretched type II cells might be due to widening by direct mechanical forces. Since the molecular composition of fusion pores is still unknown, understanding how fusion pores form remains a challenge.

5. SNARE

One of the fundamental features of eukaryotic cells is the compartmentalization into membrane-bound and biochemically distinct organelles. However, various molecules need to be transported to their correct destinations without compromising the membrane integrity. To achieve this, elaborate mechanisms have been developed to transport

vesicles budding from one compartment and then target and fuse to the destination compartment. Over the last decade, Soluble N-ethylmaleimide-sensitive-factor Attachment protein Receptor (SNARE) proteins have attracted much attention and are considered the most important protein family involved in membrane trafficking, docking and fusion.

The discovery of SNARE proteins followed the identification of SNARE-associated proteins. The Rothman group [134] first isolated an ATPase, which can be inactivated by N-ethylmaleimide and was termed as (NSF). Subsequently, the adaptor proteins for NSF were identified as α-, β-, γ- soluble NSF attachment protein (SNAP) [135]. SNARE proteins were then isolated from brain extract co-precipitated with α-SNAP and NSF [136]. Subsequently, the SNARE protein family has been demonstrated to play a central role not only in neurotransmitter release in synapses, but also in intracellular trafficking events.

Trying to address the molecular mechanisms of membrane fusion, Rothman postulated the so-called SNARE hypothesis [137]. With a substantial body of evidence from a number of research groups, a general principle about how SNARE proteins function in membrane fusion states as follows. The SNAREs from the opposing membrane contain characteristic coiled-coil SNARE motifs, which assemble into a *trans*-SNARE complex and pull the membranes to a close position. After the membrane fusion, the SNARE complex (*cis*-SNARE) is dissembled with the energy from ATP hydrolysis by NSF via the adaptor α-SNAP [138, 139]. The free SNAREs are available for the next round of membrane fusion.

SNARE proteins are membrane proteins which form hetero-oligomeric complexes. According to their subcellular localizations on vesicle or target membranes, SNARE proteins can be classified into vesicle-SNAREs (v-SNAREs) and target SNAREs (t-SNAREs), respectively. v-SNAREs include the vesicle-associated-membrane-protein (VAMP) family, while t-SNAREs include syntaxins and SNAP-25 (synaptosome associated protein of 25 kDa) family. SNARE proteins contain a conserved coiled-coil domain of 60-70 amino acids in length, which is termed as the SNARE motif [140]. Based on whether a conserved glutamine (Q) or arginine (R) is present at the zero-layer of the SNARE motifs, a second classification system has been proposed [141]. In practice, Q-SNAREs are almost always t-SNAREs and R-SNAREs are v-SNAREs. Mostly, a given set of cognate SNARE proteins involved in a certain fusion reaction includes one R-SNARE and three Q-SNARE motifs [142, 143].

There are 15 mammalian syntaxin genes, and syntaxin 1-4 are localized to the plasma membrane and function in exocytotic processes [144]. Besides the coiled-coil SNARE motif, syntaxins contain a transmembrane domain at the C-terminus, through which syntaxins attach to the membrane and a unique N-terminal sequence. Syntaxin 1A has a bundle of three α-helices at the N-terminal sequence in a twisted left-handed up-down bundle, termed as Habc domain [145]. By folding of Habc upon the SNARE motif, syntaxin 1A adopts a closed conformation. n-Sec1/Munc18-1 binds to this closed conformation of syntaxin to prevent the formation of the SNARE complex [146-149]. The activation of the Rab small GTPase could induce the dissociation of n-Sec1/Munc18 and the binding of Munc-13 to syntaxin 1A, which subsequently adopts the open

conformation and renders the SNARE motif available for SNARE complex formation [150].

SNAP-25 is specific in neuronal and neuro-endocrine systems and its non-neuronal homologue SNAP-23 is ubiquitously expressed and localized at the plasma membrane [151, 152]. SNAP-29 is localized in many intracellular membranes and is not thought to be involved in exocytotic regulation [153, 154]. SNAP-25 family members contain two SNARE motifs at both the N- and C-termini, which are connected by a cysteine-rich linker sequence. Unlike syntaxins, the SNAP-25 family does not contain a transmembrane domain and is associated with the membrane via the palmitoylation of the cysteine residues in the linker region [155-158]. SNAP-25 has four cysteine residues, while SNAP-23 contains five. In SNAP-25, one single cysteine mutation could reduce the palmityolation significantly [159]. Brefeldin A, an agent blocking the transport through the Golgi, abolished SNAP-25 palmitoylation [160]. Meanwhile, the plasma membrane is enriched in palmitoylacyl transferase activity, especially in lipid rafts [161, 162], where SNAP-25 and SNAP-23 associate [163, 164]. This indicates that palmitoylation occurs at the lipid raft of the plasma membrane.

The VAMP family has seven members. VAMP proteins contain a single SNARE motif and attach to the vesicle membrane via a C-terminal transmembrane domain. VAMP-2 is initially identified as v-SNARE of synaptic vesicles in neurons and plays an important role in synaptic vesicle exocytosis [165]. Besides in synaptic vesicles, VAMP-2 is also extensively studied in the endocrine system and other non-neuronal tissues and is involved in regulated intracellular transport events, such as in PC12 cells, the adrenal medulla, and the pancreas [163, 166-174]. VAMP-1 is highly homologous to VAMP-2

but has a differential distribution pattern. It is involved in calcium-dependent synaptic vesicle exocytosis [175-177]. VAMP-1 is also expressed in non-neuronal tissues [178], 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 [179], antibody [180], and the cytoplasmic domain [181]. However, the results from VAMP-3 knock-out mice indicate that it is not essential for secretion [182]. VAMP-7 is also termed as TI-VAMP, due to its resistance against tetanus neurotoxin cleavage [183]. VAMP-7 is associated with the late endosomes and involved in endocytosis and intracellular trafficking between ER and Golgi [184, 185]. VAMP-8 is associated with the endocytic pathway and involved in the fusion between early and late endosomes [186, 187]. It has recently been reported to be required in regulated exocytosis in pancreatic acinar cells [188] and platelets [189].

In our laboratory, we demonstrated the presence of syntaxin 2 and SNAP-23 and SNARE associated proteins, NSF and α-SNAP in type II cells and they were also found to be required in the regulated surfactant secretion [190, 191].

6. Annexin A2

Intracellular Ca^{2+} signaling and homeostasis have an essential role in regulating many cellular processes in eukaryotic organisms. Annexins are a family of Ca^{2+}-binding protein which differ from other Ca^{2+}-binding proteins by the unique architecture of their Ca^{2+}-binding sites. This feature enables annexins to peripherally bind to negatively charged membranes in a reversible manner, which links annexins to many membrane

related events, such as membrane organization, membrane-cytoskeleton linkage, membrane transportation and ion conductance across membranes.

As a member of the annexin family, annexin A2 plays an important role in various aspects of vesicular trafficking [192, 193]. It has been shown to be involved in Ca^{2+}-dependent membrane fusion during exocytosis and endocytosis in a variety of cells, including chromaffin cells and alveolar type II cells [194-198]. Among the annexin family, annexin A2 has been shown to require the least Ca^{2+} concentration for membrane binding and lipid vesicle aggregation at sub-μM to a few μM [199]. In our laboratory, annexin A2 has also been demonstrated as the most effective annexin to mediate fusion between lamellar bodies and the plasma membrane [112]. This makes annexin A2 the most attractive candidate for mediating calcium-dependent exocytosis. Annexin A2 exists as a 36 kDa monomer (AIIm) or as a heterotetramer (AIIt) in which two monomers bind to a pair of p11, an S100 family protein [200]. Annexin A2 consists of two functional domains. The C-terminal domain contains the annexin core domain, which harbors the binding sites for Ca^{2+}, phospholipids [201, 202] and F-actin [203, 204]. Unlike other calcium-binding proteins exemplified by calmodulin, which bind calcium via an "E-F hand" helix-loop-helix domain, annexin A2 binds calcium via the core domain. The core domain is conserved within the annexin family and is composed of 4 tandem annexin repeats (except for annexin A6 which has 8), each of which is about 70 amino acids in length. The core domain is a compact, slightly curved disc in shape. Its convex side is the sites for calcium and membrane binding. On the other side, the concave surface points away from the membrane and thus is available for the interactions with other proteins. Therefore, annexin A2 may provide a platform to recruit other components involved in

calcium-dependent membrane events. The N-terminal sequences are highly variable among annexin members. At the N-terminal domain of annexin A2, the first 30 amino acids contain the p11 binding site [201, 205, 206] and serine and tyrosine phosphorylation sites [207, 208]. The presence of the partner-binding and phosphorylation sites in the variable N-terminus suggests that this region renders annexins' characteristic features distinct from each other and is likely to be of regulatory importance. In the case of annexin A2, the N-terminal domain plays a very important role in regulating the Ca^{2+} requirement for annexin A2 activities. The annexin A2 monomer aggregates chromaffin granules with a Kd (Ca^{2+}) of about 1 mM. The proteolytic cleavage of the N-terminal significantly decreases the Kd (Ca^{2+}). Furthermore, the binding of p11 reduces the Ca^{2+} requirement down to about 2 μM [209]. Moreover, the phosphorylation of the tyrosine or serine residue significantly affects the activity of annexin A2 in chromaffin granule aggregation [210] or calcium-evoked secretion in permeabilized chromaffin cells [197]. Annexin A2 mediates the aggregation and fusion of liposomes in the presence of Ca^{2+} [196, 211]. The modifications of cysteine or tyrosine residues of annexin A2 by nitric oxide or peroxynitrite abolished its capability to mediate liposome aggregation [212, 213]. The depletion of annexin A2 from type II cell cytosol reduces its membrane fusion activity [112]. Most recently, we have demonstrated that the silencing of the annexin A2 gene by RNAi significantly decreases the secretion of surfactant in isolated type II cells [214]. These results suggest that annexin A2 participates in the process of regulated surfactant secretion.

7. Purposes and Significance

The presence of lung surfactant at the alveolar space is vital for air exchange. The deficiency of surfactant results in RDS. Although it has been studied for a long time, RDS is still a leading cause of infant death in the United States. Lung surfactant is synthesized and stored in lamellar bodies in type II cells prior to its secretion into the alveolar space. The secretion of surfactant is a highly regulated process and involves the translocation, docking and fusion of lamellar bodies with the apical plasma membrane. However, the mechanisms of surfactant secretion still remain vague.

SNARE proteins play an essential role in intracellular transport and membrane fusion. It has been demonstrated in our laboratory that t-SNAREs, syntaxin 2 and SNAP-23, are present in type II cells and required for regulated surfactant secretion. However, regulated surfactant secretion is a calcium-dependent process, and a role for calcium sensors needs to be fulfilled. Annexin A2 is a calcium-binding protein and it has also been reported to be involved in regulating surfactant secretion. The first part of my project (Chapter II) attempted to investigate whether annexin A2 and SNARE proteins work in the same pathway to regulate lung surfactant. By using different techniques, SNAP-23 was found to be the SNARE protein interacting with annexin A2 physically and functionally. The cysteine rich region of SNAP-23 is essential for the interaction.

The second part of my work (Chapter III) was to characterize v-SNARE proteins in type II cells. VAMP-2, 3 and 8 were found to be expressed in lung and type II cells at both mRNA and protein levels and VAMP-2 was found to be localized on lamellar bodies.

To better understand the molecular mechanisms of lamellar body biogenesis and its translocation, docking and fusion with the plasma membrane, a proteomic analysis of

isolated lamellar bodies was carried out (Chapter IV). Utilizing peptide mass fingerprinting by MALDI-TOF, 44 proteins have been identified and categorized according to their functions, including proteins involved in membrane trafficking, calcium binding and signal transduction. The proteomic profiling of lamellar bodies provides a new perspective for studying surfactant secretion.

CHAPTER II

Interaction of Annexin A2 and SNAP-23 is Required in Annexin A2 Tetramer-Mediated Fusion between Lamellar Bodies and the Plasma Membrane

Abstract

Lung surfactant is secreted through the fusion of large granules, lamellar bodies with the plasma membrane of alveolar epithelial type II cells. Annexin A2, a Ca^{2+}- and phosphlipid-binding protein, promotes the fusion of lamellar bodies with the plasma membrane in vitro. Our previous studies have indicated an essential role of the soluble N-ethylmaleimide-sensitive-factor attachment protein receptors (SNARE) in surfactant secretion. We hypothesized that annexin A2 acts as a Ca^{2+} sensor and mediates membrane fusion via its interaction with SNAREs. Here, we investigated the physical and functional interactions between annexin A2 and SNARE proteins. Glutathione S-Transferase (GST) pull-down experiments showed that SNAP-23 specifically bound with annexin A2 in a Ca^{2+}-dependent manner. Immunofluorescence revealed co-localization of SNAP-23 and annexin A2 in intact type II cells. The deletion study identified the cysteine-rich region (CRR) of SNAP-23 as the binding site for annexin A2. The mutations of cysteine residues in CRR dramatically decreased the binding. SNAP-23 was co-immunoprecipitated with annexin A2, while a SNAP-23 mutant lacking CRR failed to do so. The results indicated a direct interaction between SNAP-23 and annexin A2 in

cells. Furthermore, anti-SNAP-23 antibody significantly inhibited annexin A2-mediated fusion between lamellar bodies and the plasma membrane. These data suggest that annexin A2 and SNAP-23 are involved in the same pathway in the regulation of lung surfactant secretion.

Key Words: Annexin A2, SNARE, membrane fusion, lung surfactant, exocytosis

Introduction

Lung surfactant is a surface active material, which forms a monolayer at the air-liquid interface and reduces the surface tension of alveoli, thus preventing alveoli from collapse. Deficiency of surfactant at the alveolar surface is associated with infant and acute respiratory distress syndromes. Lung surfactant is synthesized and secreted by alveolar type II cells. It is composed of phospholipids, mainly dipalmitoylphosphatidylcholine (DPPC), and surfactant proteins A, B, C, and D. Most components of surfactant are synthesized in endoplasmic reticulum and stored in the specified organelles, lamellar bodies. Secretion of surfactant involves the translocation, docking and fusion of lamellar bodies with the apical plasma membrane. This process is very complicated and the underlying mechanism is still poorly understood [95, 96, 215, 216].

Soluble N-ethylmaleimide-sensitive-factor Attachment protein Receptors (SNAREs) are a protein family, which exist ubiquitously in eukaryotic cells and play crucial roles in membrane targeting, docking, and fusion [132, 137, 217-219]. The vesicle SNARE (v-SNARE), vesicle associated membrane protein (VAMP), is located on the membrane of secretory vesicles while the target SNAREs (t-SNARE), syntaxin and SNAP-25/SNAP-23, are located on the plasma membrane. SNARE proteins contain the characteristic coiled-coil domains, termed as SNARE motifs, which are approximately 60-70 residues in length [140]. The interaction of the SNARE motifs from cognate SNARE proteins in two adjacent membranes forms a trans-SNARE complex to pull the membranes into close apposition, and eventually leads to membrane fusion [220]. Rothman and colleagues reconstituted SNARE proteins into liposomes to simulate the membrane fusion in vitro

and proposed that the SNARE proteins provided the minimal fusion machinery [221-223]. However, the speed was too slow to be physiologically. Furthermore, two recent studies demonstrated that inter-membrane SNARE interactions in the SNARE-reconstituted liposome systems with more native SNARE densities did not drive membrane fusion [224, 225]. An elevation of cytosolic Ca^{2+} concentration is required to trigger regulated exocytosis [226]. Synaptotagmin I was found to increase the fusion of liposomes reconstituted with SNAREs in a Ca^{2+}-dependent manner, demonstrating its role in Ca^{2+}–triggered exocytosis [227]. On the other hand, the hippocampal neurons from synaptotagmin I knock-out mice still can release neurotransmitter although the kinetics of the release was affected [228]. With the studies of the Ca^{2+}-triggered fusion of sea urchin cortical vesicles in vitro, Zimmerberg and colleagues [229-232] proposed that SNARE proteins might just play a modulatory role, acting at the upstream targeting and docking stages. Disruptions of these steps by attacking SNARE proteins with neurotoxins, antibodies or other chemicals would alter the overall exocytotic process, while some other proteins associated with SNARE proteins might actually mediate the final fusion step(s). Mayer and colleagues reported V-ATPase V0 sector as a membrane fusogen acting downstream and independent of trans-SNARE complex formation [233, 234]. These data suggest the possibility that additional proteins exist, which may act as Ca^{2+} sensor and/or fusogenic proteins.

Annexin A2 is a Ca^{2+}-dependent phospholipid-binding protein, which plays an important role in various aspects of vesicular trafficking [192, 193]. It has been shown to be involved in Ca^{2+}-dependent membrane fusion during exocytosis and endocytosis in a variety of cells, including chromaffin cells and alveolar type II cells [194-198]. Annexin

A2 exists as a 36 kDa monomer (AIIm) or as a heterotetramer (AIIt) in which two monomers bind to a pair of p11, an S100 family protein [200]. Annexin A2 binds to negatively charged phospholipids and mediates the aggregation and fusion of liposome in the presence of Ca^{2+} [196, 211]. The modifications of cysteine or tyrosine residues of annexin A2 by nitric oxide or peroxynitrite abolished its capability to mediate liposome aggregation [212, 213]. The depletion of annexin A2 from alveolar type II cell cytosol reduced its membrane fusion activity [112]. Most recently, we demonstrated that the silencing of annexin A2 gene by RNAi significantly decreased the secretion of surfactant in isolated alveolar type II cells [214].

Based on our previous studies that SNARE proteins and annexin A2 were required in lung surfactant secretion [112, 118, 190, 191, 196, 214], we proposed that SNARE proteins and annexin A2 function cooperatively in regulating surfactant secretion, with annexin A2 acting as a Ca^{2+} sensor and mediating the final membrane fusion. In this study, we first determined the physical interactions between SNARE proteins and annexin A2 by utilizing the in vitro Glutathione S-Transferase (GST) pull-down assay and co-immunoprecipitation. We also identified the annexin A2 binding site of SNAP-23. We then investigated their functional interactions with the usage of an in vitro biological membrane fusion model. Collectively, our results demonstrated that SNARE proteins and annexin A2 are physically and functionally linked together.

Materials & Methods

Reagents and chemicals: Octadecyl rhodamine B chloride (R18) was obtained from Molecular Probes (Eugene, OR). *Maclura pomifera* agglutinin gel was from EY Laboratories (San Mateo, CA). Fetal bovine serum (FBS), trypsin-EDTA, DMEM, Opti-MEM, and Lipofactamine 2000 were from Invitrogen (Carlsbad, CA). Enhanced chemilluminescence (ECL) reagent, glutathione sepharose 4B beads were from Amersham Pharmacia Biotech (Arlington Heights, IL). N-Ethylmaleimide (NEM) was obtained from Sigma-Aldrich (St. Louis, MO). S-Nitroso-L-glutathione (GSNO) was from Cayman Chemicals (Ann Arbor, MI). Anti-SNAP-23 antibodies were raised using the synthetic peptide corresponding to C-terminal residues 199-210 (CANTRAKKLIDS) of rat SNAP-23 (Genmed Synthesis Inc., South San Francisco, CA). These antibodies were affinity-purified using peptide-conjugated beads, as previously described [190]. Anti-annexin A1, A4, A5, A6 antibodies, and Protein G PLUS-Agarose were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-annexin A2 antibodies were from Santa Cruz Biotechnology and Zymed Laboratories (South San Francisco, CA). Anti-annexin A3 antibody was a kind gift from Dr. J. D. Ernst of the University of California in San Francisco. Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from BD Biosciences (Palo Alto, CA). Anti-green fluorescent protein (GFP) antibody was from Abcam (Cambridge, MA). Anti-FLAG antibody was from Cell Signaling Technology (Danvers, MA). Goat anti-rabbit secondary antibody (horseradish peroxidase-conjugated IgG) was from Bio-Rad Laboratories (Hercules, CA). Rat anti-mouse secondary antibody was from Jackson Immunoresearch Laboratories (West Grove,

PA). *E. coli* BL21 (DE3)pLysS was from EMD Biosciences (Novagen Brand, Madison, WI).

Plasmids and Cells: The pGEX expression vectors encoding GST-tagged SNARE proteins were as follows [171, 235]: cytoplasmic domains of syntaxin 1A (residues 1-265), syntaxin 2 (1-265), syntaxin 3 (1-263), and syntaxin 4 (1-272) were provided as a kind gift from Dr. V. M. Olkkonen (National Public Health Institute, Helsinki, Finland); and full-length SNAP-23 and SNAP-25 were kindly provided from Dr. A. Klip (The Hospital for Sick Children, Toronto, Canada). Cytosolic domains of VAMP-2 (1-94), VAMP-8 (1-75) and full-length Rab14 were from Dr. Richard H. Scheller of the Stanford University. To construct SNAP-23 deletion mutants, various fragments of SNAP-23 were amplified from the plasmid containing full-length SNAP-23 and inserted into the same expression vector. The overexpression vector for annexin A2-GFP (pE/CMV-AII-GFP) was constructed as described [214](Gou et al., 2004). For overexpression of SNAP-23, full-length SNAP-23 or SNAP-23ΔCRR fragments were amplified with FLAG tag added at C-terminus via the 3' primer. The mammalian two-hybrid assay kit from Stratagene was utilized. Full-length SNAP-23, p11 or Rab14 genes was inserted into the bait vector pCMV-BD, respectively. For target construct of pE/CMV-AII-NLS-AD, the GFP gene in pE/CMV-AII-GFP was replaced with the fragment amplified from target vector pCMV-AD, containing SV40 nuclear localization signal, NF-κB activation domain and SV40 polyA (nt 660-1783). All the constructs were confirmed with DNA sequencing. Cell line 293 and A549 were from ATCC.

Purification of bovine annexins: Annexin A1, A2 monomer and tetramer, A4, A5 and A6 were purified from bovine lung tissue through sequential column chromatography by

using DEAE-Sepharose CL6B, Sephacryl S100, and Mono S columns with the method of Khanna et al. [236] as described previously [118].

Preparation of alveolar type II cell lysate: Alveolar type II cells were isolated from 180-200 gram Sprague-Dawley rats, according to the method of Dobbs et al. [237] as described previously [118]. Freshly isolated cells were lysed in lysis buffer (40 mM Hepes, pH 7.0, 100 mM KCl, 1 mM EGTA, 2 mM $MgCl_2$, 1% NP40 and a protease inhibitor cocktail including 1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml benzamidine, and 10 uM pepstatin), followed by sonication on ice for 15 seconds. The lysate was centrifuged at 100,000 ×*g* for 1 hour at 4 ^{o}C, and the supernatant was collected.

Expression and purification of GST-tagged SNARE proteins: The GST-SNARE cDNA clones were transformed into *E. coli* BL21 (DE3)pLysS and GST recombinant proteins were purified. Briefly, a single colony was inoculated in Luria-Bertani medium containing 100 μg/ml of ampicillin. When the A660 reached 0.6-0.8, 1 mM Isopropyl β-D-thiogalactopyranoside (IPTG) was used to induce the protein expression for 4-5 hours at 37 ^{o}C. The cells were harvested by low speed centrifugation and washed with cold phosphate-buffered saline (PBS) for three times. The cells were then sonicated and centrifuged at 20,000 ×*g* for 15 min. The cell lysate was incubated with Glutathione Sepharose 4B beads. The recombinant protein then was eluted with 10 mM reduced glutathione and stored at -80 ^{o}C.

In vitro GST pull-down experiments: Glutathione Sepharose 4B beads were first incubated with 0.5% BSA to block non-specific binding for 3 hrs. 10 μl (bed volume) of the beads were then mixed with 0.2 nmole of GST or GST-SNARE protein in 100 μl of reaction volume and incubated for 4 hours at 4 ^{o}C. The beads were washed with the

binding buffer (40 mM Hepes, pH 7.0, 100 mM KCl, 1 mM EGTA, 2 mM $MgCl_2$, 1% NP40 and varying concentrations of Ca^{2+}) and incubated with either purified AIIt (2, 4, 8, 16, 32, or 64 μg), or other annexins, or alveolar type II cell lysate (500 μg). Following end-to-end rotation at 4 ^{o}C for 4 hours, beads were washed with binding buffer for six times and boiled with SDS sample buffer. The soluble fractions were detected for the presence of annexins by Western blotting. To modify AIIt, 10 μg of AIIt was incubated with 1 mM N-ethylmaleimide (NEM) or 1 mM S-nitroso-L-glutathione (GSNO) in 100 μl of 0.1 M phosphate buffer (pH 7.0) for 15 min. Samples were dialyzed against binding buffer to remove NEM or GSNO. The control was treated in the same way except that NEM and GSNO were omitted. The treated AIIt then was used for pull-down assays.

Immunofluorescence: Freshly isolated alveolar type II cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature. The cells were cyto-spun onto glass slides and stored at 4 ^{o}C until use. Immunocytochemistry was done as described earlier [238]. 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 ^{o}C with monoclonal anti-annexin A2 antibodies (1:50 dilution, BD Biosciences) and polyclonal anti-SNAP-23 antibodies (1:100 dilution). Subsequently, they were 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 and mounted for fluorescence microscopy (Nikon Inc).

Luciferase assay: 0.5 x 10^6 of A549 cells were seeded in 6-well plates in 2 ml of complete DMEM containing 10% FBS and non-essential amino acid. Transfection was performed at 80% cell confluence. 775 ng of total DNA (bait vector pCMV-BD-SNAP-

23, p11 or Rab14, target vector pE/CMV-AII-NLS-AD, and report vector pFR-Luc, 250 ng each; second reporter vector pRL-TK, 25 ng) was used. 48 hours after transfection, luciferase activities were measured with a dual-luciferase reporter assay system (Promega). In brief, cells were washed with PBS and lysed. 7 µl of lysate was mixed with 35 µl of Luciferase Assay Reagent II (LAR II) and firefly (*Photinus pyralis*) luciferase activity was measured. This reaction was stopped and the Renilla (*Renilla reniformis*) luciferase reaction was started simultaneously by adding 35 µl of Stop & Go Reagent to the same sample. The firefly luciferase activity was normalized against that of Renilla luciferase. The luminometer used was FLUOstar OPTIMA from BMG Biotech Inc. (Durham, NC).

Co-immunoprecipitation of annexin A2 and SNAP-23: To overexpress annexin A2-GFP and SNAP-23-FLAG, 0.5 x 10^6 of 293 cells were seeded in 6-well plates in 2 ml of complete DMEM containing 10% FBS and non-essential amino acid. Transfection was performed when the cells reached 80% confluence. 500 ng of plasmid pE/CMV-annexin A2-GFP was mixed with 500 ng of either pE/CMV-SNAP-23-FLAG or pE/CMV-SNAP-23ΔCRR-FLAG in 50 µl of Opti-MEM. The transfection reagent Lipofectamine 2000 was used with a DNA:reagent ratio of 1:3 (mass:volume). 48 hours after transfection, cells were washed with PBS, and lysed in lysis buffer (40 mM Hepes, pH 7.0, 100 mM KCl, 1 mM EGTA, 2 mM $MgCl_2$, 1% NP40 and a protease inhibitor cocktail). 1.5 µl of anti-GFP antibody was added into 150 µl of cell lysate, and $CaCl_2$ was supplemented to a final concentration of 1 mM. The mixture was then incubated at 4 ^oC overnight. 15 µl of Protein G PLUS-Agarose was added and incubated for an additional 4 hours at room

temperature. The resin was then washed with binding buffer for four times, and boiled with SDS sample buffer. The soluble fractions were detected by Western blotting.

Western Blotting: Protein samples were fractionated on 10% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. The blot was visualized with Ponceau S staining and 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 blot was incubated with appropriate primary antibodies (annexin A1, 1:1000; annexin A2, Santa Cruz 1:500; annexin A2, Zymed 1:1000; annexin A3, 1:500; annexin A4, 1:1000; annexin A5, 1:1000; annexin A6, 1:1000; FLAG, 1:1000; and GAPDH, 1:4,000) at 4 ^{o}C overnight, and then with secondary antibody (1:5,000) at room temperature for 1 hour. Finally, the signal was developed with ECL and detected with VersaDoc Imaging system (Bio-Rad). The quantification of Western Blotting was performed by using Quantity One 4.0.3 software (Bio-Rad).

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. [239] and Chattopadhyay et al. [112]. A perfused rat lung was briefly homogenized in 1 M sucrose and 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 ×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 ×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.

Preparation of plasma membrane: The preparation of plasma membrane from rat lung tissue was performed, as described previously [112]. A Sprague-Dawley rat lung was perfused with saline and homogenized in buffer B (10 mM Na-Phosphate, pH 7.4, 30 mM NaCl, 1 mM $MgCl_2$, 5 μM 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 ×g and resuspended in buffer B. To remove the outside-out plasma membrane vesicles, the plasma membrane was incubated with *Maclura pomifera* agglutinin-conjugated beads. To incorporate R-18 into the inside-out plasma membrane vesicles, 1 μl of a 20 mM stock solution (in ethanol) of R-18 was injected by using a 10-μl syringe to 300 μg protein of plasma membrane in 300 μl of buffer B by vortexing. The mixture was incubated for 30 minutes by end-to-end rotation at room temperature, and excessive R-18 was removed by dialysis against 3 liters of buffer B.

Annexin A2 tetramer-mediated fusion assay: The fusion between lamellar bodies and the plasma membrane was measured by monitoring the fluorescence changes of R-18, as described by Hoekstra et al. [240] and Chattopadhyay et al. [112]. R-18 was incorporated into the plasma membrane at a self-quenching concentration. Fusion of the plasma membrane with unlabeled lamellar bodies resulted in the dilution of R-18, due to the increased surface area. The relief of the self-quenching of R-18 led to the increase in fluorescence. In our standard assay, 5 μg protein of plasma membrane was mixed with 10 μg protein of lamellar bodies in 1 ml of Ca^{2+}-EGTA buffer (40 mM Hepes, pH 7.0, 100 mM KCl, 1 mM EGTA, 2 mM $MgCl_2$, and 1 mM Ca^{2+}) at 37 ^{o}C with a continuous

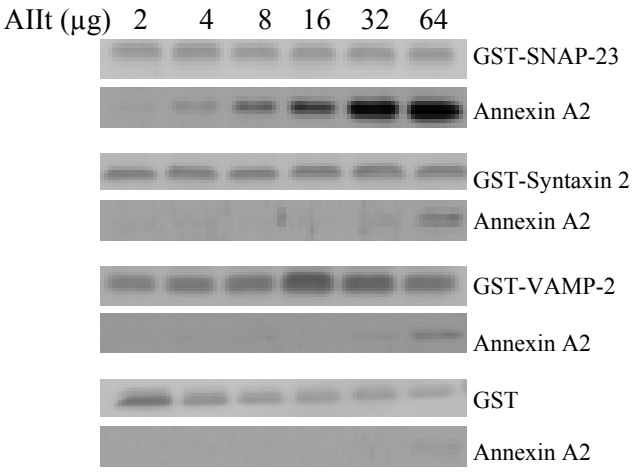
stirring. A basal reading was recorded for 2 minutes, and 10 μg of annexin A2 tetramer was added to initiate the fusion. After an additional 6 minutes of recording, 0.1% (v/v) Triton X-100 was added to achieve the maximal fluorescence. Fusion was calculated as a percentage of the maximal fluorescence. To investigate the functional relationship between SNAREs and annexin A2, the plasma membrane was incubated with anti-SNAP-23 antibodies at room temperature. It was then mixed with lamellar bodies and annexin A2 tetramer was added to initiate the fusion.

Results

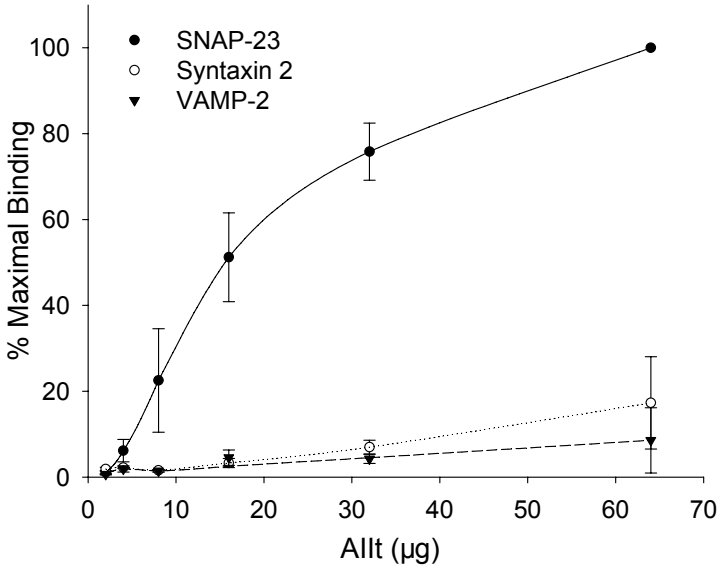
SNAP-23 binds with annexin A2 *in vitro*

To explore the relationship between SNARE proteins and annexin A2, we investigated their physical interactions by using GST pull down assay. SNAP-23, syntaxin 2, and VAMP-2 are the major SNARE isoforms in alveolar type II cells [190, 241] and were tested. We used the same amount (0.2 nmoles) of GST-SNARE fusion proteins and varying doses of purified AIIt in the presence of 1 mM Ca^{2+}. As shown in Fig. II.1 A and B, SNAP-23 had a strong binding with purified AIIt. This binding was AIIt-dose dependent. The increase in binding was unlikely due to the variation of loadings, pull down or transfer of GST-SNAP-23, since Ponceau S staining showed the same amount of GST-SNARE fusion proteins after electrophoretic transferring. In contrast to SNAP-23, syntaxin 2, VAMP-2, or GST control did not show binding with purified AIIt except that a weak binding was noted at very high doses of AIIt. We also tested the ability of other SNARE isoforms to bind with AIIt under the same conditions as above with 8 μg of purified AIIt. As shown in Fig. II.1 C, SNAP-25, the neuronal isoform of SNAP-23, also had a very strong binding, while syntaxin 1A, 2, 3, 4, and VAMP-2, 8 did not have significant binding.

A


AIIt (μg) 2 4 8 16 32 64
GST-SNAP-23
Annexin A2
GST-Syntaxin 2
Annexin A2
GST-VAMP-2
Annexin A2
GST
Annexin A2

B


SNAP-23
Syntaxin 2
VAMP-2
% Maximal Binding
AIIt (μg)
0
20
40
60
80
100
0
10
20
30
40
50
60
70

C

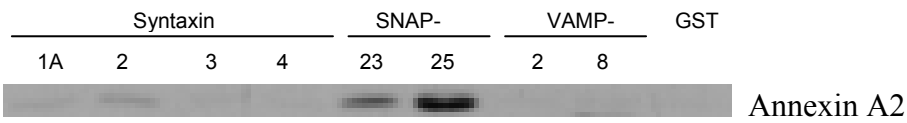

Syntaxin
SNAP-
VAMP-
GST
1A
2
3
4
23
25
2
8
Annexin A2

Fig. II.1 SNAP-23 binds with purified annexin A2 in a dose-dependent manner

(A) Glutathione sepharose beads were incubated with 0.2 nmole of GST-SNAP-23, GST-syntaxin 2, GST-VAMP-2 or GST, followed by the incubation with various amounts of purified annexin A2 tetramer (AIIt, 2-64 μg) in the presence of 1 mM Ca2+. The bound annexin A2 was detected with Western Blotting. GST-SNARE fusion proteins were stained with Ponceau S. (B) Quantitation of the binding. Each band was quantified by densitometry. A linear range was determined by using purified AIIt. The background binding with GST was subtracted from those of GST-SNARE proteins. The results were expressed as a percentage of the maximal binding (the binding of GST-SNAP-23 with 64 μg of AIIt). Data shown are means ± SE (n=3 independent experiments). (C) The binding of AIIt with other SNAREs. AIIt (8 μg) was incubated with glutathione sepharose beads pre-incubated with 0.2 nmole of GST-syntaxin 1A, 2, 3, 4, GST-SNAP-23, 25, or VAMP-2, 8. The bound annexin A2 was detected with Western blotting.

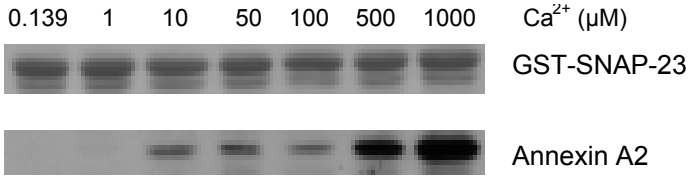
Binding between SNAP-23 and annexin A2 is Ca^{2+}-dependent

Most of the activities of annexin A2, including binding to phospholipids and mediating membrane fusion, are Ca^{2+}-dependent. We further tested the Ca^{2+} requirement for the binding of annexin A2 and SNAP-23. As shown in Fig. II.2, AIIt did not exhibit any binding to SNAP-23 at $\leq$ 1 μM Ca^{2+}. There was weak binding at 10 μM – 100 μM Ca^{2+}. The binding was increased significantly when the Ca^{2+} concentration reached 500 μM.

SNAP-23 does not bind with other annexin isoforms

The experiments above were performed by using purified bovine annexin A2 tetramer. Annexin A2 exists as a monomer without partner p11 as well. To find out whether p11 is required for the binding, we repeated the pull-down experiments with purified annexin A2 monomer. The result showed that annexin A2 monomer bound with GST-SNAP-23 in the presence of 1 mM Ca^{2+}, while GST had no binding (Fig. II.3A). To investigate the binding specificity of SNAP-23, other members of annexin family were tested for the binding with GST-SNAP-23. Same amounts (0.2 μmole) of purified annexin A1, A4, A5 and A6 were incubated with GST-SNAP-23 in the presence of 1 mM Ca^{2+}. As shown in Fig. II.3A, none of these annexins had binding with GST-SNAP-23. Furthermore, we performed the pull-down experiments with rat alveolar type II cell lysate. The results showed that endogenous annexin A2 also bound with GST-SNAP-23 in a Ca^{2+}-dependent manner. The GST control did not bind with annexin A2. Neither annexin A3 nor GAPDH was detected in the GST-SNAP-23 pull-down samples (Fig. II.3B).

A



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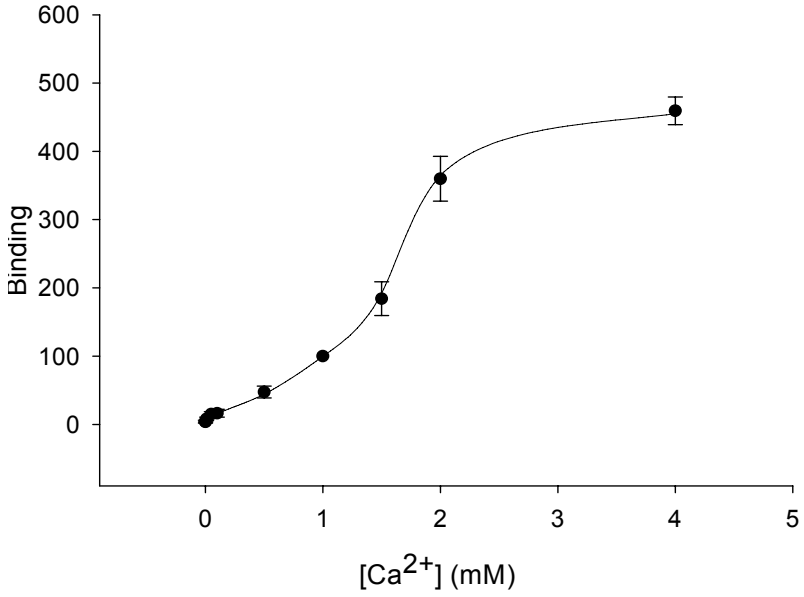
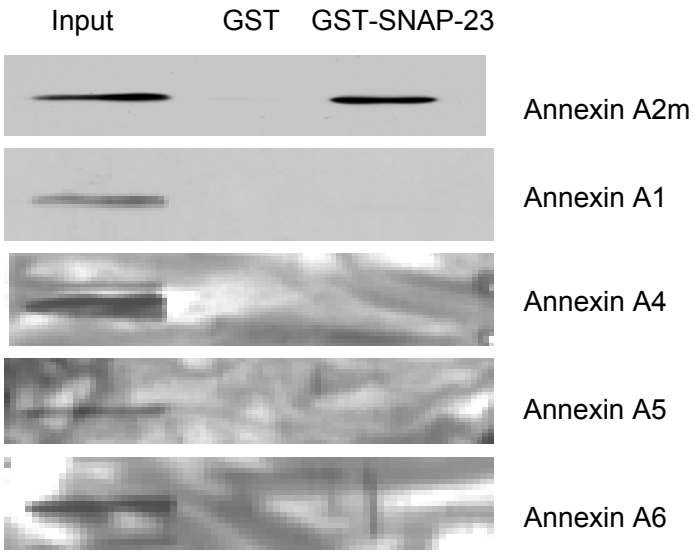


Fig. II.2 Binding between SNAP-23 and annexin A2 tetramer is Ca^{2+}-dependent

GST-SNAP-23 (0.2 nmole) pre-bound to glutathione sepharose beads was incubated with 8 μg of AIIt at various concentrations of Ca^{2+}. GST-SNAP-23 was stained with Ponceau S (A, upper panel) and annexin A2 was detected with Western Blotting (A, lower panel). In (B), the bindings were quantitated by densitometry, and the results were expressed as a percentage of the binding at 1 mM Ca^{2+}. Data shown are means ± SE (n=4 independent experiments).

A


Input
GST
GST-SNAP-23
Annexin A2m
Annexin A1
Annexin A4
Annexin A5
Annexin A6

B

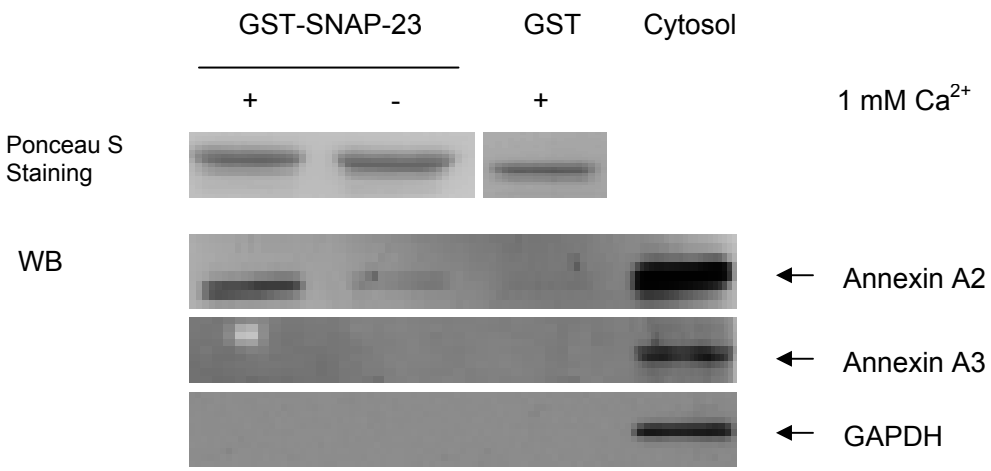

GST-SNAP-23
GST
Cytosol
+
-
+
1 mM Ca^{2+}
Ponceau S Staining
WB
Annexin A2
Annexin A3
GAPDH

Fig. II.3 SNAP-23 does not bind with other annexins

(A) Glutathione sepharose beads were incubated with 0.2 nmole of GST-SNAP-23 or GST, followed by the incubation with 0.2 nmole of various purified annexins (annexin A1, A2 monomer, A4, A5, and A6) in the presence of 1 mM Ca^{2+}. The bound annexins were probed with the corresponding antibodies. 100 ng of each annexin protein was used as a positive control for Western blotting. (B) Freshly isolated rat alveolar type II cells were lysed and centrifuged at 100,000 ×g for one hour. Glutathione sepharose beads bound with GST-SNAP-23 or GST (0.2 nmole) were incubated with the cytosol (500 μg of total protein) in the presence or absence of 1 mM Ca^{2+}. GST-SNAP-23 and GST were stained with Ponceau S. Annexin A2, annexin A3 and GAPDH were detected with Western blotting. Type II cell cytosol (10 μg of protein) was used as a positive control for the Western blotting.

Chemical modification of annexin A2 inhibits its binding with SNAP-23

We have previously shown that the modification of its cysteine residues by NEM or GSNO reduced AIIt-mediated fusion [112, 242]. As shown in Fig. II.4, the binding between AIIt and SNAP-23 were inhibited after the treatment of AIIt with 1 mM NEM (16.1 ± 4.9% of Control) or GSNO (34.8 ± 3.8% of Control).

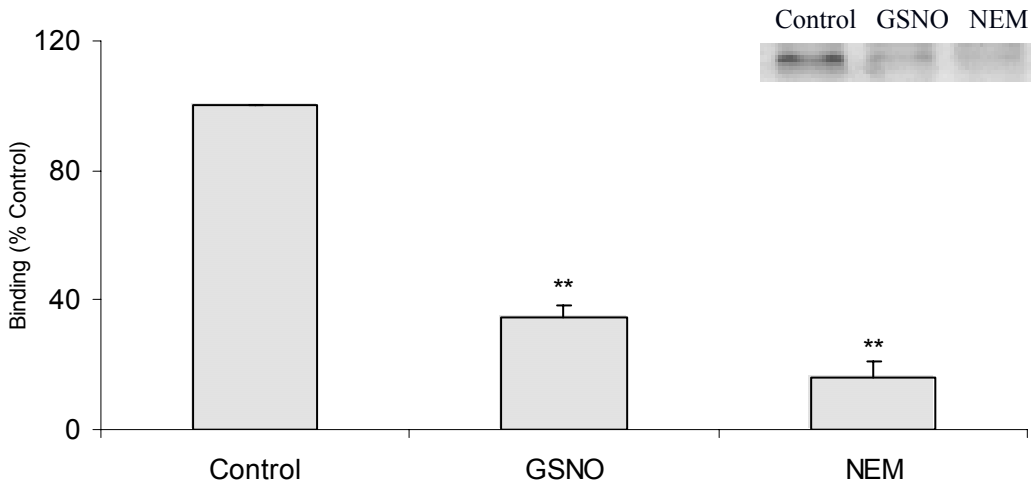


Fig. II.4 Modification of annexin A2 inhibits its binding with SNAP-23

AIIt (8 μg) was treated with 1 mM NEM or 1 mM GSNO for 15 minutes in 100 μl of 0.1 M phosphate buffer, pH 7.0. After the incubation, the mixture was dialyzed against binding buffer overnight to remove NEM or GSNO. The binding assay was performed with 8 μg AIIt, 0.2 nmole of GST-SNAP-23 and 1 mM Ca^{2+}. The results were expressed as a percentage of the untreated control. Data shown are means ± SE. **P< 0.01 versus control (student t test, n=3).

SNAP-23 is co-localized with annexin A2 in alveolar type II cells

To examine whether SNAP-23 and annexin A2 are co-localized in intact cells, we performed dual-labeling experiments on alveolar type II cells using anti-SNAP-23 and anti-annexin A2. As shown in Fig. II.5, the staining patterns of SNAP-23 and annexin A2 overlapped, indicating that they may interact in the cells.

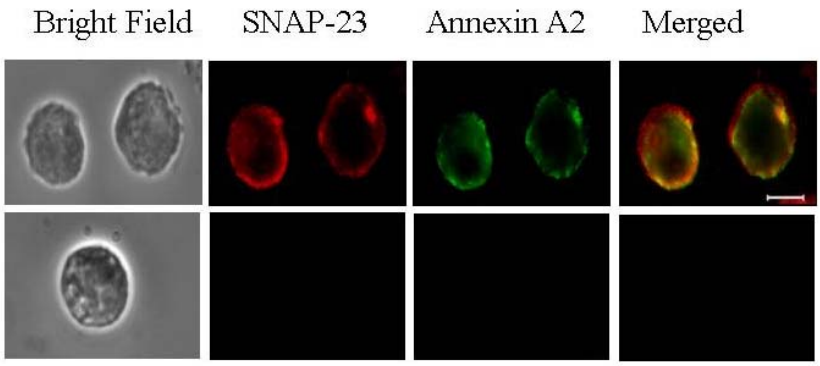


Fig. II.5 Co-localization of SNAP-23 and annexin A2

Alveolar type II cells were double-labeled with anti-SNAP-23/Alexa Fluor 488 anti-mouse and anti-annexin A2/Alexa Fluor 568 anti-rabbit antibodies (upper panels). The controls without primary antibodies are shown in lower panels. Scale bar: 10 micron.

SNAP-23 interacts with annexin A2 as determined by mammalian two-hybrid assay

To investigate the interaction of annexin A2 and SNAP-23 in vivo, we utilized a mammalian two-hybrid system (Stratagene). Annexin A2 and SNAP-23 gene were fused to NF-κB activation domain (AD) and GAL4 DNA binding domain (BD), respectively, resulting plasmids pCMV-AD-AII and pCMV-BD-SNAP-23. However, no fusion protein was expressed from pCMV-AD-AII (data not shown). This was because the NF-κB activation domain was placed at the N-terminus of annexin A2. We therefore constructed a new vector in which the NF-κB activation domain was placed at the C-terminus of annexin A2. This was accomplished by sub-cloning the DNA fragment, containing SV40 nuclear localization signal and NF-κB activation domain, into annexin-GFP expression vector pE/CMV-AII-GFP. Annexin A2 binding partner p11 was fused with BD as a positive control for the interaction. Co-transfection of the resulting pE/CMV-AII-NLS-AD and pCMV-BD-p11 into 293 and A549 cells resulted in a significant increase in luciferase activity, indicating the interaction between annexin A2 and p11 occurred and this modified two-hybrid system can be used to study the interaction of annexin A2 with its binding partners in mammalian cells. Rab14 had no binding with annexin A2 in GST pull-down assay (data not shown) and was fused to BD as a negative control. As shown in Fig. II.6, transfection of pE/CMV-AII-NLS-AD or pCMV-BD-SNAP-23 alone showed only negligible activity (0.051 ± 0.007 and 0.070 ± 0.004, respectively). The luciferase activity was not significantly changed by co-transfection of annexin A2 with Rab14 (0.066 ± 0.006), whereas co-transfection of annexin A2 with SNAP-23 led to a significant increase (0.132 ± 0.014, $p<0.01$) over the control, although a little lower than the positive control of p11 (0.275 ± 0.060, $p<0.01$).

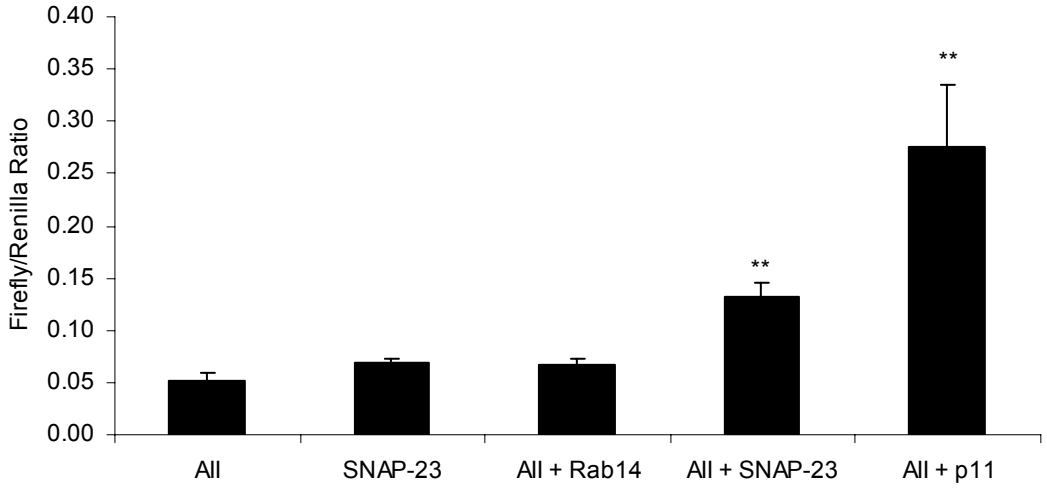


Fig. II.6 SNAP-23 interacts with annexin A2 is determined by mammalian two-hybrid assay

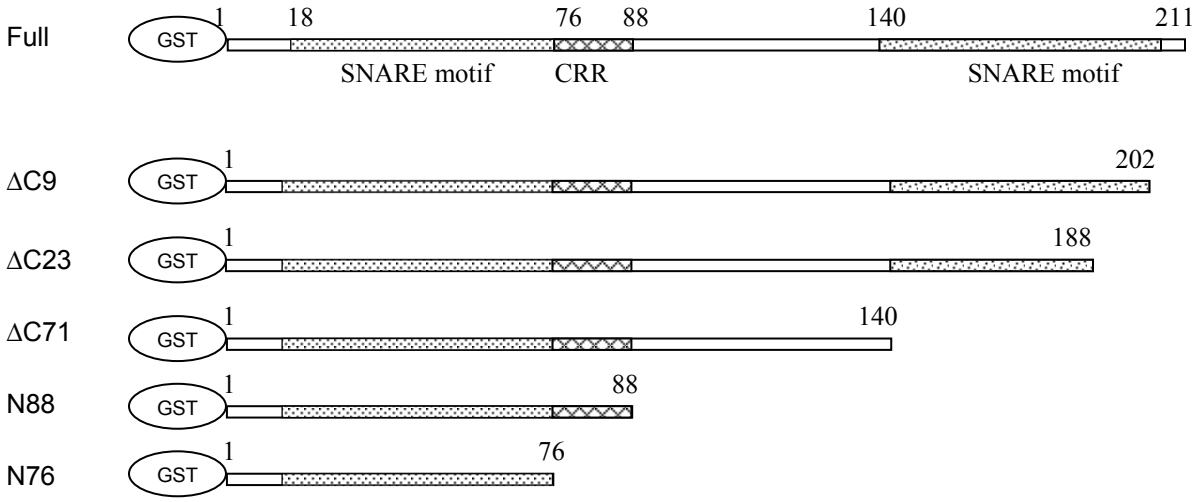
pCMV-BD-SNAP-23 (SNAP-23) and pE/CMV-AII-NLS-AD (AII) were co-transfected into A549 cells. The cells were lysed 48 hours later and applied to a dual luciferase assay. Firefly luciferase activity was measured and quenched, followed by Renilla luciferase activity measurement. pCMV-BD-Rab14 was used as a negative control and pCMV-BD-p11 was a positive control. Data were expressed as a ratio of Firefly/Renilla luciferase activity and shown as means ± SE. **P< 0.01 versus negative control (student t test, n=4).

Cysteine-rich region of SNAP-23 is required for the binding with annexin A2:

SNAP-23 has two SNARE motifs at its N- and C- termini and a cysteine-rich region (CRR) between them. To identify the domains of SNAP-23 essential for its binding with annexin A2, we constructed various SNAP-23 deletion mutants (Fig. II.7A). The recombinant GST fusion proteins were expressed in *E. coli* and purified. The purity of these recombinant proteins was shown in Fig. II.7B. The N76 mutant has a deletion of the C-terminal 77-211 amino acids (CRR and the C-terminal SNARE motif). The ability of the N76 mutant to bind with annexin A2 tetramer was almost abolished in comparison with that of full-length SNAP-23 (7.5 ± 5.9%). However, the mutant N88, which retains the additional 12-amino acid CRR, had a binding of 95.4 ± 16.8% (Fig. II.7C, D). The deletion of C-terminal SNARE motif (ΔC71) had no significant effect on the binding. Interestingly, the ΔC23 mutant, which is equivalent to the botulinum neurotoxin (BoNT) E cleavage, slightly increased the binding (128.0 ± 32.0%). However, the ΔC9 mutant, which is equivalent to the BoNT A cleavage, showed a decreased binding (37.9 ± 13.0%). The deletion of N-terminal 18, 32 amino acids or the N-terminal SNARE motif (1-76 amino acids) renders the recombinant proteins prone to degrade. However, these mixtures containing intact and partially-degraded recombinant proteins still bound to annexin A2 (data not shown). We also tested the ability of annexin A2 monomer to bind with these mutants as well and the same binding pattern as annexin A2 tetramer was observed (data not shown). To further confirm the importance of the cysteine-rich region, we deleted CRR domain from full-length SNAP-23 (ΔCRR) (Fig. II.8A). Similar to N76 mutant, the ΔCRR mutant failed to bind with annexin A2 (6.1 ± 2.8%) (Fig. II.8C, D). There are five cysteine residues within the CRR domain. Using site-directed mutagenesis, we

substituted one (C83S), two (C85,87S) and three cysteine residues (C83,85,87S) with serine in the N88 mutant. The substitution of cysteine residues in the mutant N88 dramatically decreased its binding with annexin A2 from 95.4 ± 16.8% (N88) to 39.9 ± 5.1% (C83S), 13.6 ± 3.1% (C85,87S) and 7.6 ± 3.7% (C83,85,87S) (Fig. II.8C, D), respectively.

A



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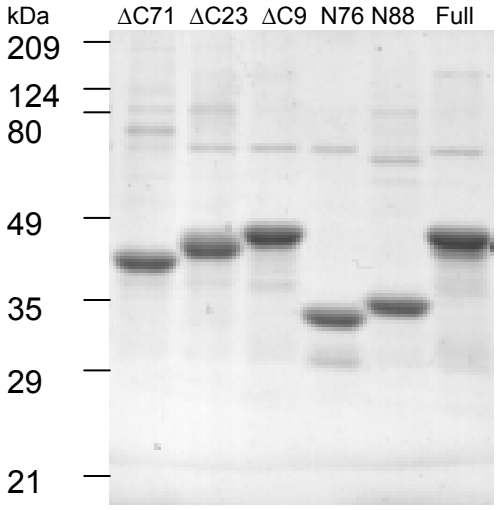
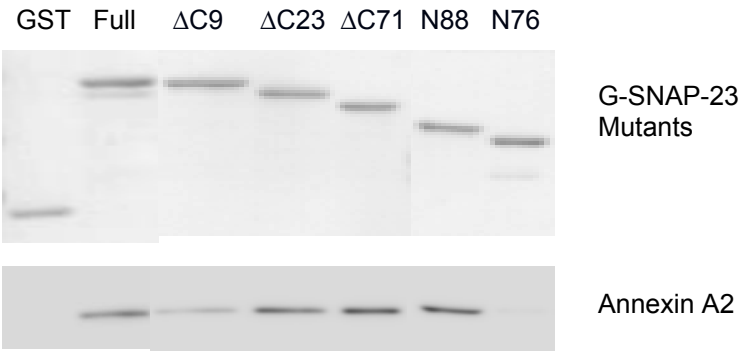


C



D

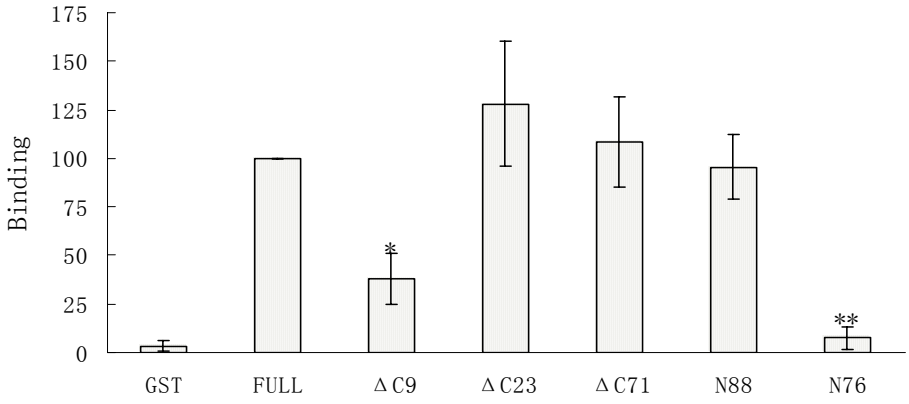
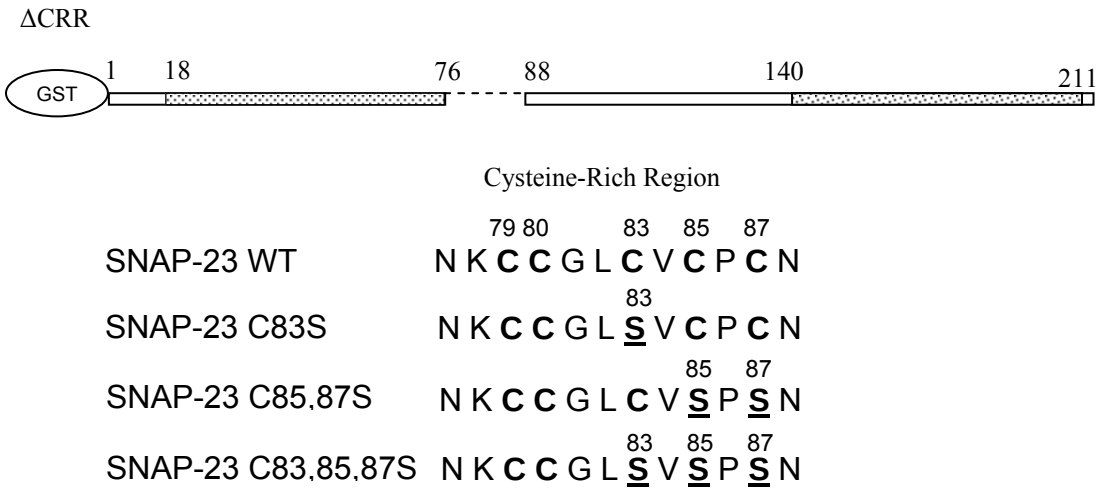


Fig. II.7 Cysteine-rich region of SNAP-23 is required for the binding with annexin A2

(A) Diagrammatic representations of SNAP-23 mutants. The top diagram represents full-length SNAP-23. The cysteine-rich region (CRR) between residues 76 and 88 (crossed) and two SNARE motifs (dotted) are shown. The numbers on the fusion proteins indicate the amino acid positions of SNAP-23. (B) The purity of recombinant proteins as visualized by Coomassie Brilliant Blue R-250 staining. (C) 0.2 nmole of GST-tagged SNAP-23 mutant protein was bound to glutathione sepharose beads and was incubated with 8 μg of AIIt and 1 mM Ca^{2+}. Recombinant proteins were stained with

Ponceau S (upper panel) and annexin A2 was detected with Western Blotting (lower panel). (D) The bindings were quantitated by VersaDoc Imaging system (Bio-Rad), and the results were expressed as a percentage of full-length SNAP-23 recombinant protein. Data shown are means ± SE. *P< 0.05, **P< 0.01 versus Full-length (student t test, n=3 independent experiments).

A



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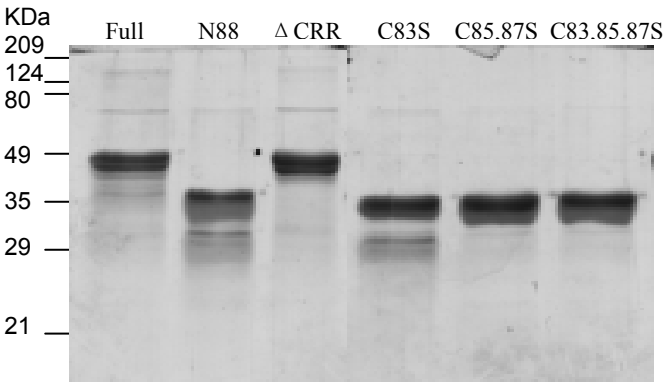

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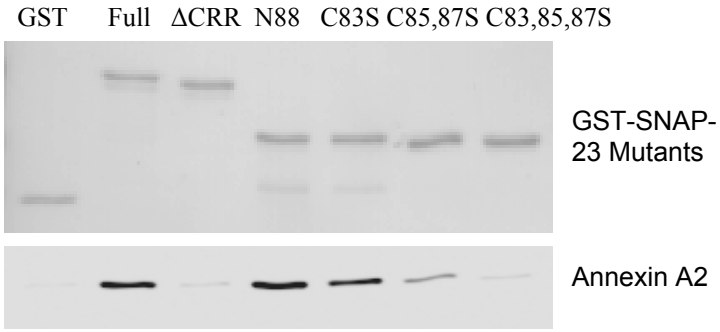

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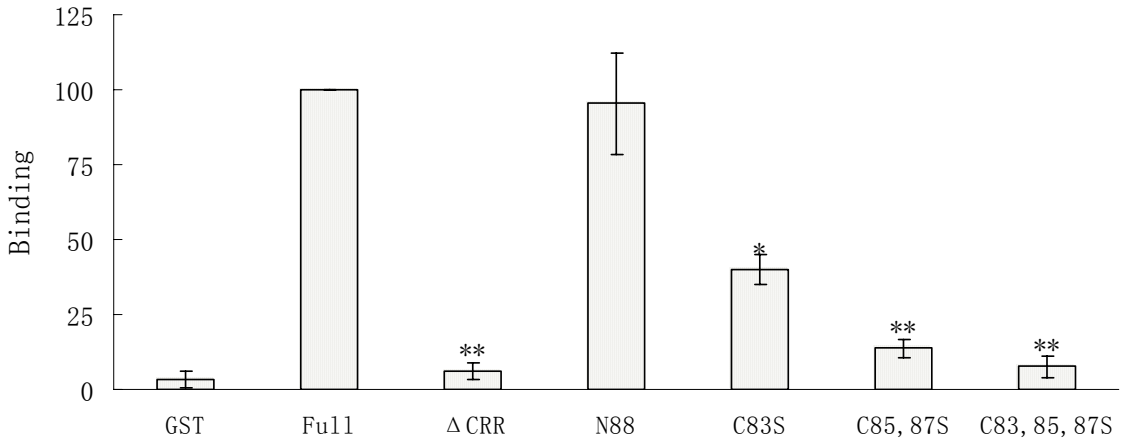


Fig. II.8 Cysteine residues in cysteine-rich region of SNAP-23 are required for binding between SNAP-23 and annexin A2

(A) The diagrams show full-length SNAP-23 with the CRR deletion (ΔCRR) and various site-directed mutations of cysteine residues in CRR. The sequence in SNAP-23WT represents CRR from wild type SNAP-23. The underlined S represents cysteine residues that were mutated into serine residues in SNAP-23 mutants. (B) The purity of mutant proteins as visualized with Coomassie blue staining. (C) Pull-down experiments were performed with 0.2 nmole of SNAP-23 mutant proteins incubated with 8 μg of AIIt

in the presence of 1 mM Ca^{2+}. Recombinant proteins were stained with Ponceau S (upper panel) and annexin A2 was detected with Western Blotting (lower panel). (D) The bindings were quantitated by VersaDoc Imaging system (Bio-Rad), and the results were expressed as a percentage of full-length SNAP-23 recombinant protein. Data shown are means ± SE. *P< 0.05, **P< 0.01 versus Full-length or N88 (student t test, n=4 independent experiments).

SNAP-23 is co-immunoprecipitated with annexin A2

To investigate the interaction of annexin A2 and SNAP-23 in cells, we co-expressed annexin A2-GFP fusion protein with FLAG-tagged SNAP-23 wild type or SNAP-23 ΔCRR mutant in 293 cells. Immunoprecipitation was performed by using anti-GFP antibody. As shown in Fig. II.9, a similar amount of annexin A2-GFP fusion protein was immunoprecipitated from SNAP-23 wild type- and SNAP-23 ΔCRR- expressed cells. However, only SNAP-23 wild type, but not the CRR deletion mutant was co-precipitated with annexin A2. The control pre-immune serum did not pull down annexin A2, SNAP-23 or SNAP-23 ΔCRR. The results are consistent with our in vitro study.

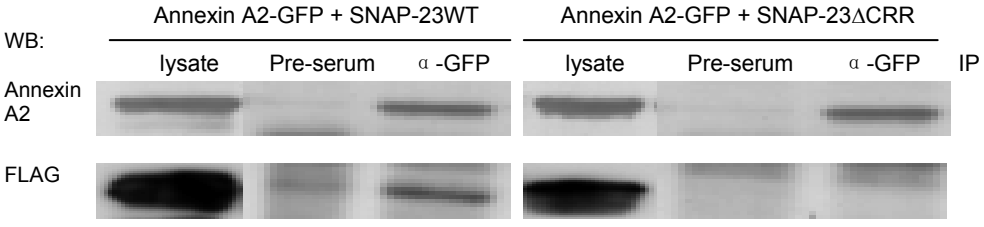


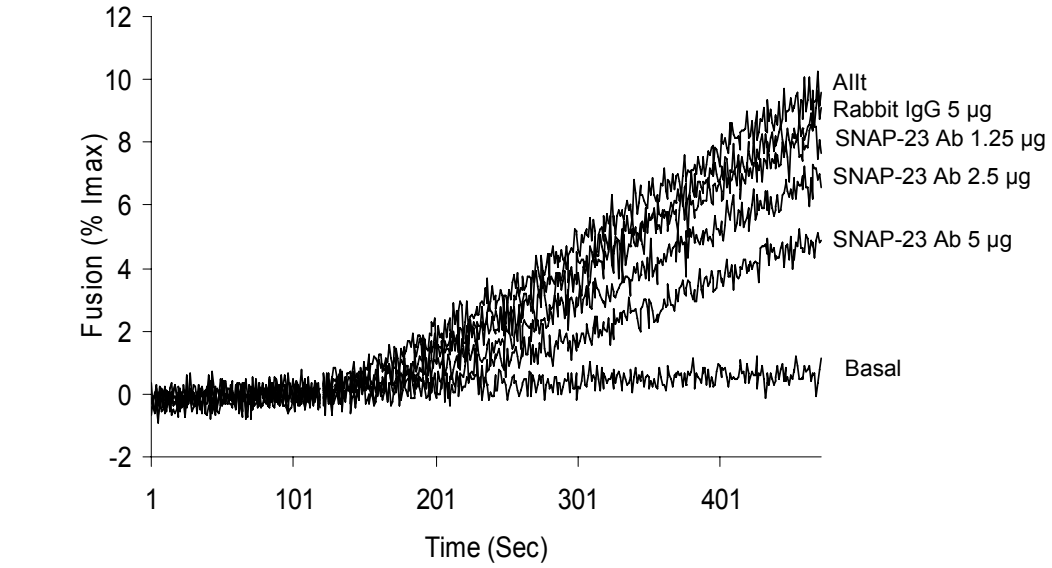
Fig. II.9 Co-immunoprecipitation of SNAP-23 with annexin A2

Annexin A2-GFP and FLAG-tagged SNAP-23WT or SNAP-23ΔCRR fusion proteins were co-expressed in 293 cells, and cells were lysed 48 hours after transfection. Annexin A2-GFP was immunoprecipitated with anti-GFP antibody (α-GFP) or pre-immune serum. The immunoprecipitates were probed with anti-annexin A2 and anti-FLAG antibodies. The cell lysate was used as a positive control for western blotting. The blot shown is a representative from 3 independent experiments.

Anti-SNAP-23 antibody inhibits AIIt-mediated fusion

To investigate the functional interaction of annexin A2 and SNAP-23, we utilized an in vitro biological membrane fusion model, in which the isolated plasma membrane and lamellar bodies were induced to fuse by purified annexin II tetramer [112]. Because SNAP-23 showed a direct interaction with annexin A2, we blocked SNAP-23 on the plasma membrane with an affinity-purified antibody and tested AIIt-mediated fusion between lamellar bodies and the plasma membrane. This antibody was raised against C-terminal residues 199-210 of rat SNAP-23. It recognized SNAP-23, but not SNAP-25 or syntaxins 1-4 [190]. As shown in Fig. II.10, anti-SNAP-23 antibody inhibited the fusion in a dose-dependent manner, while rabbit IgG did not show any effects. These results indicated that SNAP-23 was required for AIIt-mediated fusion between lamellar bodies and the plasma membrane.

A



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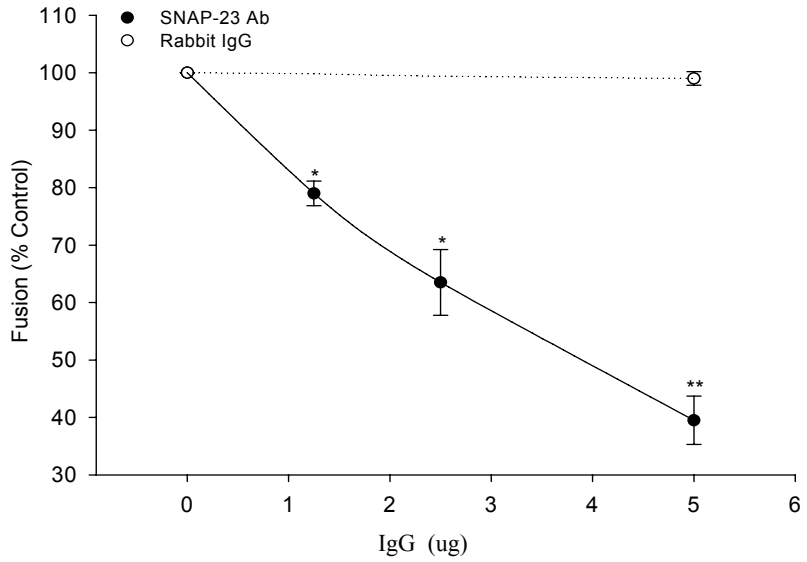

Fig. II.10 Anti-SNAP-23 antibody inhibits AIIt-mediated fusion between lamellar bodies and the plasma membrane

Five μg protein of R-18-labeled plasma membrane were incubated with various amounts of anti-SNAP-23 antibody or rabbit IgG control at room temperature for 30 min and then mixed with 10 μg protein of lamellar bodies in 1 ml of Ca^{2+}-EGTA buffer (1 mM free Ca^{2+}). Ten μg of AIIt were added to initiate the fusion. The fusion was expressed as a percentage of the maximal fluorescence (Imax), achieved by adding 0.1% Triton X-100. (A) A representative of the fusion curves. (B) Data were expressed as a percentage of the control (the fusion content during 6 min incubation) and shown as means ± SE. *P< 0.05, **P< 0.01 versus control (student t test, n=3).

Discussion

SNARE proteins play an important role in intracellular vesicular trafficking events in eukaryotic cells, and are required for lung surfactant secretion from alveolar type II cells. In this study, we provided evidence for the physical and functional interactions between annexin A2 and SNAP-23. Our results indicated that annexin A2 may interact with SNAP-23, functioning as a Ca^{2+} sensor and/or fusogenic protein.

Many proteins have been reported to interact with SNARE proteins, thus regulating and fine-tuning the processes of intracellular transportation. For example, the interaction of n-Sec1 and syntaxin 1 [243], Snapin and SNAP-23 [244], or synaptophysin and VAMP-2 [245] modulates the assembly/disassembly of SNARE complex. Rabphilin directly interacts with SNAP-25 via its C2B domain [246]. Expression of the rabphilin mutant lacking the C2B domain in PC12 cells decreases the number of docked vesicle or fusing at the plasma membrane. Synaptotagmin I binds to SNAP-25 in a Ca^{2+}-dependent manner and the cleavage of the C-terminus of SNAP-25 by BoNT E abolished this binding [247]. Functionally, synaptotagmin I stimulates SNARE-mediated membrane fusion in the presence of Ca^{2+} [227]. Among all of the SNARE partners reported, it appears that only synaptotagmin I requires Ca^{2+} for its interaction. However, synaptotagmin I-depleted neurons still can release neurotransmitter with different kinetics [228], suggesting that other additional Ca^{2+}-binding proteins, that behave as Ca^{2+} sensors, may exist.

Annexins are a superfamily of Ca^{2+}-dependent phospholipid binding proteins. Each annexin has a conserved C-terminal core domain, which harbors the Ca^{2+}/membrane binding sites. This feature confers annexins as potential Ca^{2+} sensors, monitoring the

changes in cytoplasmic Ca^{2+} concentration. The sequential conformational changes upon the binding with Ca^{2+} may regulate their functions. Annexin A7 has been reported to work as a Ca^{2+} sensor in exocytotic secretion in chromaffin cells [248]. The insertion of the N-terminal domain of annexin A1 into its C-terminal core domain in the absence of Ca^{2+} represents the inactive form of the protein, while the conformational switch in the presence of Ca^{2+} may trigger the exposure of some domains for additional interactions [249]. Besides the conserved C-terminal domain, annexin A2 contains the phosphorylation sites and p11 binding sites at the N-terminal domain. Our data showed that annexin A2 specifically interacted with SNAP-23 in a Ca^{2+}-dependent manner, suggesting that annexin A2 may be a candidate of the Ca^{2+} sensor. This Ca^{2+} requirement may be due to a similar exposure of binding site of annexin A2 induced by its binding with Ca^{2+}. Our previous study has demonstrated that only annexin A2, but not annexin A1, A3, A4, A5, or A6 is able to promote the fusion of lamellar bodies with the plasma membrane [112] and to reconstitute surfactant secretion in the permeabilized type II cells [118]. In this study, we also found that only annexin A2, but not other annexins interacted with SNAP-23. This suggests that the specific interaction between annexin A2 and SNAP-23 may be functionally relevant.

In this study, the deletion of various segments of SNAP-23 identified cysteine-rich region as the binding site of SNAP-23 to annexin A2. There are no reports about direct interactions of SNAP-25/23 with their possible regulatory proteins within this region so far. Although the cysteine-rich region is the site for palmitoylation and may facilitate the association of SNAP-25/23 to the plasma membrane [155, 157, 159], an alternative mechanism for SNAP-25/23 association with membrane via the binding of syntaxin has

been proposed [250-252]. Therefore, the cysteine-rich region is still likely available for the interaction with additional proteins in vivo. The cysteine residues play a role in fine-tuning the affinities of SNAP-25/23 for plasma membrane and the mutations of these residues only restore the exocytosis to different extents in a permeabilized cell system [253, 254]. Another report has shown that the cysteine residue mutant of SNAP-25 still can form SDS-resistant complex with syntaxin 1A and VAMP-2. However, this complex can not be disrupted by NSF. Furthermore, these mutants are not able to rescue the exocytosis [251]. SNAP-23 has five cysteine residues within this region. The mutation of cysteine residue at 83 of SNAP-23 resulted in a significant decrease in its binding to annexin A2, and the mutations of three cysteine residues (83,85,87) almost abolished the binding. These results suggest that the cysteine residues of SNAP-23 may control its interaction with annexin A2 in the process of exocytosis.

BoNT A and BoNT E cleave the C-terminal 9 and 23 amino acids of SNAP-23, respectively. Over-expression of the SNAP-25 mutant ΔC9 decreases the fast component of the exocytotic burst in bovine chromaffin cells even though the over-expressed mutant still can assemble into SNARE complexes in vivo [255]. Interestingly, deletion of the C-terminal 9 amino acids, but not the 23 amino acids markedly reduced its binding with annexin A2. The reason for this is unclear, but could be due to the different conformational changes caused by the two deletions.

By using a modified mammalian two-hybrid system, we analyzed the interaction in vivo, and the annexin A2 binding partner p11 was used as the positive control. We first tried 293 cell line originated from kidney. Unlike the positive control p11, co-transfection of SNAP-23 with annexin A2 only yielded negligible luciferase activity as negative

control (Data not shown). We then used the lung-originated cell line A549, in which significant increase in luciferase activity was observed with co-transfection of SNAP-23/annexin A2. This suggests that some cell specific factors, which may exclusively required in processes in lungs, facilitated that interaction. The luciferase activity of SNAP-23/annexin A2 was lower than that of p11/annexin A2, which may reflect the Ca^{2+} dependence of the interaction between SNAP-23 and annexin A2.

To carry out the functional study, we used a specific antibody against the C-terminal last 11 amino acids of rat SNAP-23 to block its interaction with annexin A2. This antibody recognizes SNAP-23 specifically and inhibits surfactant secretion in the permeabilized alveolar type II cells [190]. In this study, when the plasma membrane was pre-incubated with the anti-SNAP-23 antibody, AIIt-mediated fusion of lamellar bodies with the plasma membrane was decreased. One possible reason is that the antibody blocks the direct interaction of SNAP-23 and annexin A2. However, we can not rule out the possibility that the spatial hindrance from the binding of antibody interferes the formation of functional SNARE complexes between lamellar bodies and the plasma membrane.

In summary, annexin A2 may function as a Ca^{2+} sensor and/or a fusogenic protein via its interaction with SNAP-23 in the surfactant secretion of alveolar type II cells. Annexin A2 has been reported to be involved into the Ca^{2+}-evoked exocytosis in chromaffin cells [194, 197]. Since annexin A2 also binds with SNAP-25, this interaction may not be unique to alveolar type II cells and annexin A2 may function in a similar way in the exocytotic secretion in some endocrine cells, such as chromaffin cells.

Acknowledgements:

Contributions of co-authors: Chintagari N.R. performed immunocytochemistry; Gou DM. performed luciferase assay. Liu L. is the PI.

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CHAPTER III

Characterization of VAMP Proteins in Alveolar Type II Cells

Abstract

Lung surfactant is crucial to reduce the surface tension at alveolar space, thus preventing 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 and 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. Immunochemistry studies indicated that VAMP-2 is co-localized with the lamellar body marker protein. This study characterized the expression and distribution of VAMPs in lung and type II cells and suggests that VAMP-2 may be the v-SNARE involved in regulated surfactant secretion.

Key Words: SNARE, VAMP, membrane fusion, lung surfactant, exocytosis

Introduction

Lung surfactant forms a monolayer at air-liquid interface in alveoli to reduce the surface tension, thus preventing alveoli from collapse. Surfactant is composed of phospholipids and surfactant proteins, and most of the components are synthesized in endoplasmic reticulum in alveolar epithelial type II cells and stored in the specified organelles, 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 alveolar lumen. Our previous studies have demonstrated the involvement of annexin A2, soluble N-ethylmelaimide-sensitive fusion protein attachment protein receptor (SNARE) and some other regulatory factors in the secretion of lung surfactant [190, 191, 214]. However, the precise underlying mechanism is still not clear and needs further investigation.

SNAREs are a protein family, which exist ubiquitously in eukaryotic cells and play a crucial role in membrane targeting, docking, and fusion [132, 137, 217-219]. SNARE proteins contain the characteristic coiled-coil domains, termed as SNARE motifs, which can form a trans-SNARE complex in two adjacent membranes to pull the membranes into close apposition, thus leading to membrane fusion [220]. There are two classes of SNARE proteins according their localizations in the cell. The vesicle SNARE (v-SNARE), vesicle associated membrane protein (VAMP), is located on the membrane of secretory vesicles, while the target SNAREs (t-SNARE), such as syntaxin and SNAP-25/SNAP-23, 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 [190]. Here, we attempted to identify the v-SNARE required in this process. We demonstrated the presence of VAMP-2, 3, and 8 in alveolar type II cells at mRNA and protein levels. Furthermore, VAMP-2 is localized on lamellar bodies. This study suggests that VAMP-2 may be the v-SNARE involved in the regulation of lung surfactant secretion.

Materials & Methods

Reagents and chemicals: Fetal bovine serum (FBS), trypsin-EDTA and DMEM were from Invitrogen (Carlsbad, CA). Enhanced chemilluminescence (ECL) reagent was from Amersham Pharmacia Biotech (Arlington Heights, IL). Mouse anti-VAMP-2 antibody was from Synaptic System (Goettingen, Germany). 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).

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. [237], as described previously [118].

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 were as shown in Table. III.1. 18S rRNA was amplified by using

classic 18S rRNA primer pairs. The conditions for PCR amplification were: 95°C for 2 min, 35 cycles of 95°C 30 sec, 55°C 30 sec, 72°C 1 min, followed by 72°C for 8 min. The PCR products were electrophoretically separated on 1% agarose gel.

Table. III.1 PCR primers for VAMP gene amplification

VAMP-1	5'-AGGGACCAGAAGTTGTCAGAGT-3'
GeneID: 25624	5'-GCCATCTCCATACCTCTGCA-3'
VAMP-2	5'-CTGGTGTGTAAGTGTCTTGGAG-3'
GeneID: 24803	5'-CAGAGATTTCAGGCAGGAATTA-3'
VAMP-3	5'-CTGATGTGGTTTCTTTCCTAGA-3'
GeneID: 29528	5'-AGGCATGTCTTCAACACTTG-3'
VAMP-4	5'-GAATCAGGTGGACGAAGTTAT-3'
GeneID: 364033	5'-TGTTATTGTCCCAGATCTTGTT-3'
VAMP-5	5'-CAGACCAAGTGACGGAAATC-3'
GeneID: 89818	5'-TCCACTCGGAAGAAAGATGA-3'
VAMP-7	5'-GGATTGTGTATCTTTGCATCA-3'
GeneID: 85491	5'-TCTATCAGCAATTCTAGCCTTT-3'
VAMP-8	5'-GAAATGACCGAGTCAGGAAC-3'
GeneID: 83730	5'-CGTAGCAAAGAGTATGATGAGG-3'

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. [239] and Chattopadhyay et al. [112]. A perfused rat lung was briefly homogenized in 1 M sucrose and 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 ×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 ×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.

Preparation of the plasma membrane: The preparation of the plasma membrane from rat lung tissue was performed as described previously [112]. 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 $MgCl_2$, 5 μM 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 ×*g* and resuspended in buffer B.

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, Stressgen, 1:1000; VAMP-2, Synaptic System, 1:5000; VAMP-3, 1:500; VAMP-8, 1:500) at 4 ^{o}C overnight, and then with secondary antibody (1:2500) at room temperature for 1 hour. Finally, the signal was developed with ECL reagents.

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 [238]. 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 (Stressgen) or VAMP-8 antibodies (1:100 dilution).

Subsequently, they were 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).

Immunohistochemistry: Immunohistochemistry was performed as described earlier [238]. The rat lungs were perfused with PBS and lavaged with normal saline. Lungs were then fixed with 4% paraformaldehyde. Paraffin-embedded lungs were then sectioned (2 μm) 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 permeablized with 1% Triton X-100 and blocked with 10% donkey serum in 50 mM PBS. They were then incubated overnight at 4 ^{o}C with anti-SP-C antibody (1:100 dilution) and anti-VAMP-2 (Stressgen) or VAMP-8 antibodies (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.

Results

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

There are seven members in VAMP family. We utilized 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 was used as a reference. As shown in Fig. III.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 expressions of VAMP-3 and VAMP-8 were also observed. They appear to be enriched in type II cells in comparison with lungs.

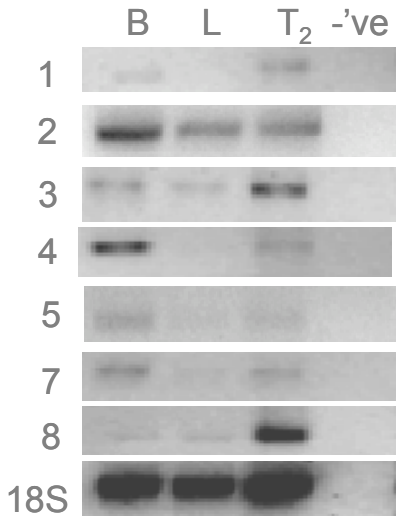


Fig. III.1 RT-PCR amplification of VAMP isoform genes in alveolar type II cells.

Total mRNA 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% agrose gels. A reaction without cDNA template was used as negative control (-'ve).

VAMP proteins are expressed in lamellar body fraction:

To explore the distribution of VAMP proteins in alveolar type II cells, we utilized Western Blotting method to examine the different fractions of type II cells. When polyclonal anti-VAMP-2 antibodies (Stressgen) were used, in addition to the band expressed in brain (18 kDa), there is another band for VAMP-2 with a lower molecular weight [Fig. III.2 VAMP-2 (1)]. In lung tissue, type II cells, lamellar body fraction and the plasma membrane, this lower band was dramatically enriched in lamellar body fraction. When monoclonal anti-VAMP-2 antibodies (Synaptic System) were used, only the 18 kDa band was observed [Fig. III.2 VAMP-2 (2)]. VAMP-8 was also detected in both lamellar body and the plasma membrane factions. VAMP-3 was mainly present in the plasma membrane fraction and was not detected in lamellar body fraction.

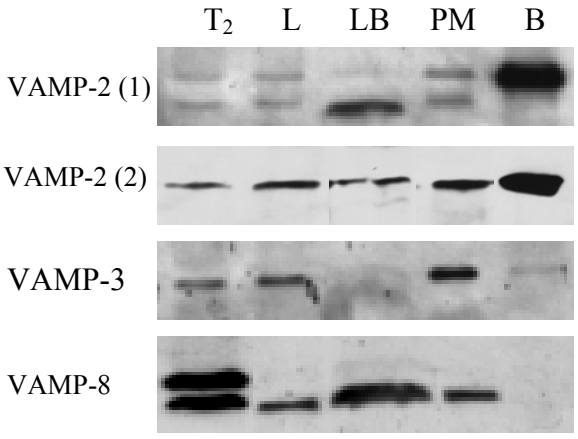


Fig. III.2 The expression of VAMP proteins in alveolar type II cells.

Freshly isolated type II cells (T_2), lung tissue homogenate (L), lamellar body fraction (LB) and the plasma membrane fraction (PM) isolated from lungs were lysed and same

amounts of total protein were separated by 12% SDS-PAGE and probed with two anti-VAMP-2 antibodies [(1) Stressgen Inc. and (2) Synaptic System GmbH], anti-VAMP-3 and -8 antibodies, respectively. Brain (B) was used as a reference.

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

To examine the cellular localization of VAMP-2 in lung tissue, we performed immunohistochemistry by 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, indicating that VAMP-2 is localized in type II cells (Fig. III.3). 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 overlapped with the staining of LB-180 (Fig. III.4A). However, VAMP-8 showed a diffusible staining pattern and was partially overlapped with LB-180 (Fig. III.4B).

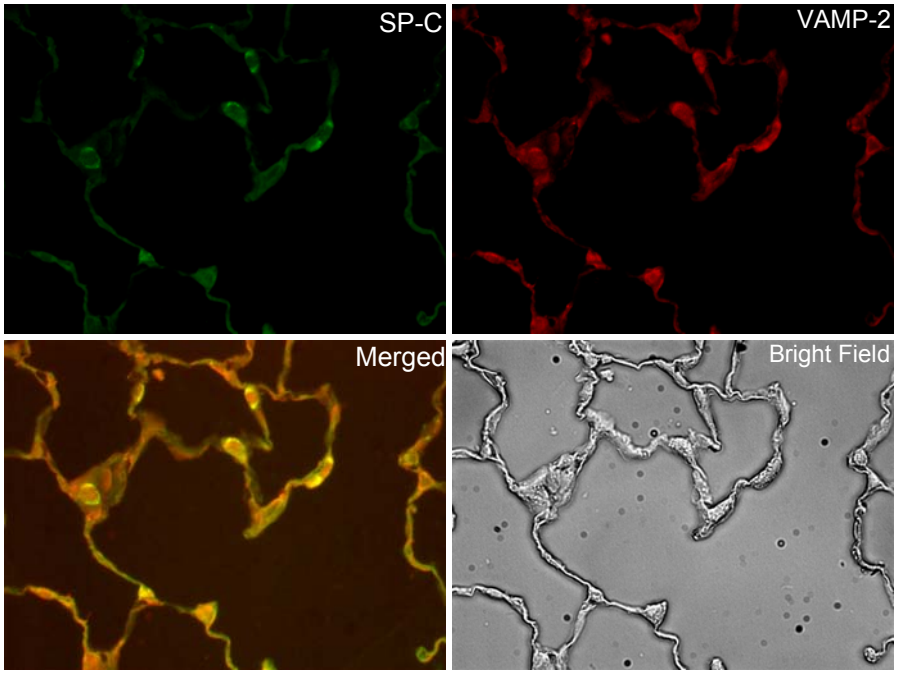
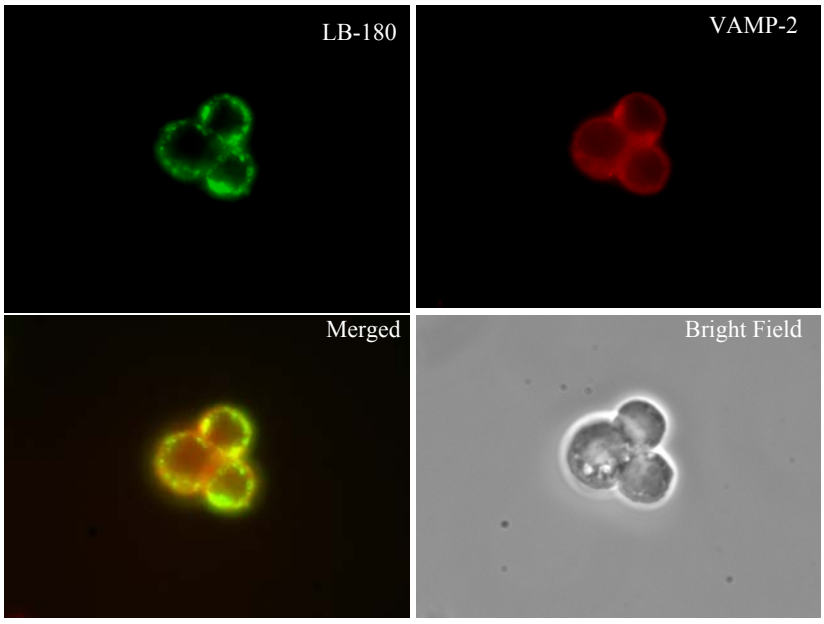


Fig. III.3 VAMP-2 is localized in alveolar type II cells.

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. Fluorescence was observed at 200x magnification.

A



B

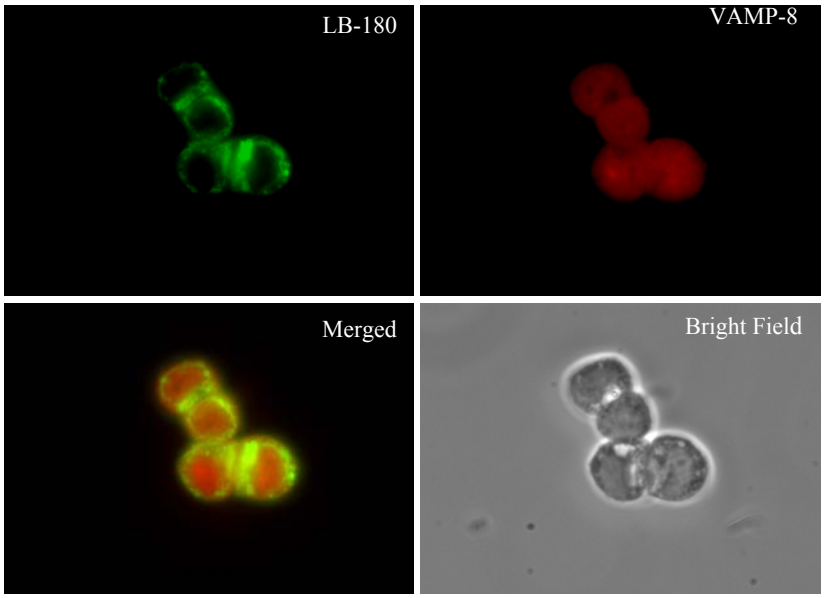


Fig. III.4 VAMP-2 is localized on lamellar bodies in alveolar type II cells.

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. A: Dual labeling of VAMP-2 and LB-180; B: Dual labeling of VAMP-8 and LB-180. Fluorescence was observed at 400x magnification.

Discussion

SNARE proteins play a central role in eukaryotic membrane trafficking events and the underlying mechanism is conserved among different spices. 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.

From the RT-PCR results, we found that various isoforms of VAMP genes are expressed in type II cells. There are seven members in 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 [175-177]. VAMP-1 is also reported to be expressed in non-neuronal tissues [178], 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 [179], antibody [180]; and the cytoplasmic domain [181]. 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 [182]. VAMP-7 is resistant to tetanus neurotoxin cleavage [183]. It is associated with the late endosome and involved in endocytosis and intracellular trafficking between ER and Golgi [184, 185]. VAMP-8 has been reported as being involved in the endocytic pathway as an intermediate in the fusion between early and late endosomes [186, 187]. It has recently been reported that VAMP-8 is required in regulated exocytosis in pancreatic acinar cells and platelets [188, 189]. VAMP-2 was initially identified as a v-SNARE of synaptic vesicles in neurons, playing an important role in synaptic vesicle exocytosis [165].

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 [170-174]. 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. The lower band was highly enriched in lamellar body fraction. However, we only detected the 18 kDa band by using a monoclonal anti-VAMP-2 antibody. There are two possibilities: (1) the lower band is the degradation product of VAMP-2; (2) it is another VAMP isoform. The identity of the lower band is not known and needs further investigation. VAMP-2 was also detectable in a plasma membrane fraction. Immuno-staining study showed that VAMP-2 was co-localized with lamellar body marker, LB-180, indicating that VAMP-2 is localized on lamellar bodies. This association 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, VAMP-8 was also expressed in type II cells but not restricted only on lamellar bodies. The presence of more than one VAMP isoform and their distinctive distribution patterns suggest that different VAMP isoforms are involved into different processes in type II cells, rather than simply functional redundancy.

Acknowledgements:

Contributions of co-authors: Jin NL. and Howard DM. performed the RT-PCR; Howard DM. performed part of the Western Blotting; Liu L. is the PI.

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CHAPTER IV

Proteomic Analysis of Lamellar Bodies Isolated from Rat Lung

Abstract

Lung surfactant is a surface active material existing on the alveolar walls. It reduces the surface tension of alveoli and prevents alveoli from collapse. Lung surfactant is synthesized in alveolar epithelial type II cells and stored in specified organelles, lamellar bodies. Lamellar bodies are lysosome-related organelles and surfactant is released into alveolar space through the fusion of lamellar bodies with the plasma membrane. To better understand the mechanisms of surfactant secretion, we carried out proteomic analyses of lamellar bodies isolated from rat lungs. With the peptide mass fingerprinting by MALDI-TOF mass spectrometry, 44 proteins were identified with high possibility data matches. These proteins fell into diverse functional categories: surfactant related proteins, membrane trafficking, calcium binding, signal transduction, structural proteins, ion channels, protein processing and miscellaneous proteins. This proteomic profiling of lamellar bodies provides a new perspective on lamellar body biogenesis and surfactant secretion.

Key Words: Lung Surfactant, Lamellar body, Proteomics, MALDI-TOF

Introduction

Lung surfactant is a surface active material. It forms a monolayer at the air-liquid interface and reduces the surface tension of alveoli, thus preventing alveoli from collapse. Deficiency of surfactant at the alveolar surface results in respiratory distress syndromes (RDS) in both new-borns and adults. Lung surfactant is synthesized and secreted by alveolar type II cells. It is mainly composed of phospholipids and surfactant proteins A, B, C, and D. Most components of surfactant are synthesized in endoplasmic reticulum and stored in the specified organelles, lamellar bodies.

Lamellar bodies are lysosome-related, large secretory organelles with 1 to 2 μm in size. Similar to lysosomes, lamellar bodies contain soluble lysosomal enzymes, like acid phosphatase, and lysosome associated membrane proteins (LAMP) [73-75]. Lamellar bodies also have an acidic interior of pH about 6.1 or below [76, 77]. However, lamellar bodies are different from lysosomes in that they are specialized for storage and secretion of surfactant rather than for degradation. The principle components in lamellar bodies are phospholipids, which are tightly packed as concentric arrangements of bi-layer membranes. Secretion of surfactant involves the translocation, docking and fusion of lamellar bodies with the apical plasma membrane.

The molecular mechanisms that control the exocytosis of lamellar bodies are still poorly understood [95, 96, 215, 216]. The recent emergence of powerful proteomic techniques has made it possible to profile the protein components in a specific tissue or subcellular organelle. To better understand the regulation of lamellar body biogenesis and exocytosis, we performed proteomic analysis of lamellar bodies isolated from rat lungs. We carried out both one-dimensional and two-dimensional gel electrophoresis, followed

by MALDI-TOF mass spectrometry. Here, we report the first proteomic profiling of lamellar bodies, which provides the bases to define the mechanisms of lamellar body exocytosis.

Materials & Methods

Reagents and chemicals: The ReadyPrep 2-D cleanup kit, ReadyPrep reduction-alkylation kit, ReadyPrep protein extraction kit and non-linear pH 3-10 ReadyStrip IPG strips were from Bio-Rad Laboratories (Hercules, CA).

Isolation of Lamellar Bodies from rat lung: Lamellar bodies were isolated from rat lungs by upward flotation on a discontinuous sucrose gradient, as described by Chander et al. [239] and Chattopadhyay et al. [112]. A perfused rat lung was homogenized in 1 M sucrose and 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.

One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): Lamellar bodies were directly lysed in 1x SDS sample buffer and fractionated on 10% SDS-PAGE. The gel was subjected to colloidal coomassie brilliant blue staining to visualize protein bands described as below.

Two-dimensional gel electrophoresis: Lamellar bodies were pretreated by using ReadyPrep 2-D cleanup kit before being applied to electrophoresis. In brief, 500 μg of LB in a final volume of 100 μl was mixed with 300 μl of precipitation agent 1 and incubated on ice for 15 min, followed by adding 300 μl of precipitation agent 2. The mixture was centrifuged at 12,000 x g for 5 min and supernatant was removed. After being washed with washing reagent, the pellet was air-dried at room temperature and

resuspended in appropriate volume of 2-D rehydration/sample buffer. Supernatant was stored at -80 ^{o}C or used directly for isoelectric focusing (IEF). Sample in 300 µl of rehydration buffer was loaded into rehydration/equilibrium tray and a 17-cm non-linear pH 3-10 IPG strip was placed onto the sample. The strip was overlayed with 2-3 ml of mineral oil and left overnight to rehydrate. The strip was transferred to focusing tray and subjected to IEF at 50,000V-hr in a PROTEAN IEF cell. After IEF, the strip was transferred and incubated in 6 ml of equilibrium buffer I and II sequentially for 10 min each at room temperature. The strip was then applied to SDS-PAGE at 48 mA for 4-5 hrs.

Colloidal Coomassie Brilliant Blue Staining: After SDS-PAGE, the gel was fixed with 40% ethanol, 10% acetic acid overnight, followed by washing with water twice for 10 min. The colloidal coomassie brilliant staining working solution was made freshly by mixing 80% stock solution (0.1% coomassie brilliant blue G-250, 2% ortho-phosphoric acid, 10% ammonium sulfate) with 20 % methanol. The gel was stained at least overnight and destained with 1% acetic acid till satisfactory staining was achieved.

Matrix Assisted Laser Desorption/Ionization - Time Of Flight Mass Spectrometry (MALDI-TOF MS): Acrylamide bands or spots were harvested and washed 3 times with 400 µl of 50% acetonitrile (ACN)/25 mM ammonium bicarbonate (pH 8.0) on a rocker shaker for 1 hour each time. The gel pieces were then incubated with 400 µl of 100% ACN at room temperature for 5 min to dehydrate. The gel pieces were digested by trypsin and equal volume of digest and saturated matrix were mixed and analyzed by MALDI-TOF MS using a Voyager-DE Pro MALDI-TOF MS Workstation (Applied Biosystems).

Protein Identification Analysis: Protein identification was carried out by searching the peptide spectra against the Mass Spectrometry protein sequence database (MSDB) using

the Mascot web based search engine. The search parameters used were: taxonomy, *Rattus*; allow up to 1 missed cleavage; variable modifications, carbamidomethyl (C), oxidation (M), propionamide (C), and pyro-glu (N-term Q); mass value, MH+; allowed error, 100 ppm.

Results and Discussion

The lamellar bodies were isolated from rat lung based on the principle of upward flotation of lamellar bodies during centrifugation on a discontinuous sucrose gradient. This protocol was first introduced by Duck-Chong [256], and well established as described by Chander et al. [239] and Chattopadhyay et al. [112]. The isolated lamellar body fraction consists of intact lamellar bodies and large amounts of concentric multilamellated membrane structures [256], and contain no other organelles except for very low amounts of microsomes [257].

After electrophoresis, the gels were stained with colloidal Coomassie Brilliant Blue dye. One-dimensional SDS-PAGE revealed more than 50 protein bands (Fig. IV.1). The diversity of protein components suggests that storage and secretion of surfactant may not be the sole function of lamellar bodies. The well-separated protein bands were harvested for peptide fingerprint analysis.

Due to the complexity of protein components of lamellar bodies, we also performed 2-dimensional PAGE to get better separation. Since lamellar bodies contain large amounts of lipids which could affect the electrophoresis, we treated the sample with acetone to remove lipid components. However, the treated sample was not able to dissolve. We then utilized the ReadyPrep kit from BioRad to prepare the samples. After IEF, proteins were separated on polyacrylamide gels. As shown in Fig. IV.2, more than 100 protein spots were revealed. These well-separated spots were harvested for peptide fingerprint analysis.

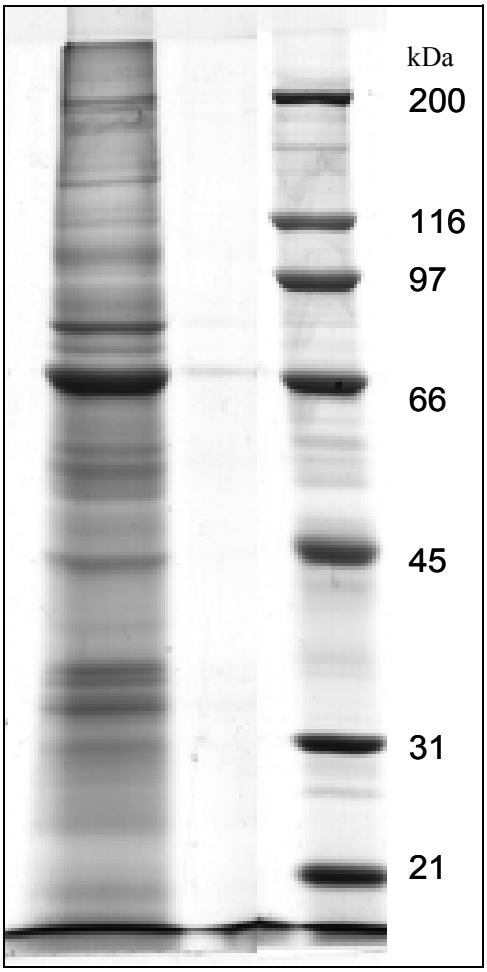

Fig. IV.1. One-dimensional SDS-PAGE of proteins of lamellar bodies.

Lamellar bodies were isolated from perfused rat lungs as described in materials and method. Approximately 100 μg of total protein were loaded for electrophoresis on 10% Bis-Tris polyacrylamide gels. The protein bands were visualized with Coomassie Brilliant Blue G-250 dye. The molecular weight markers (kDa) are shown to the right of the panel.

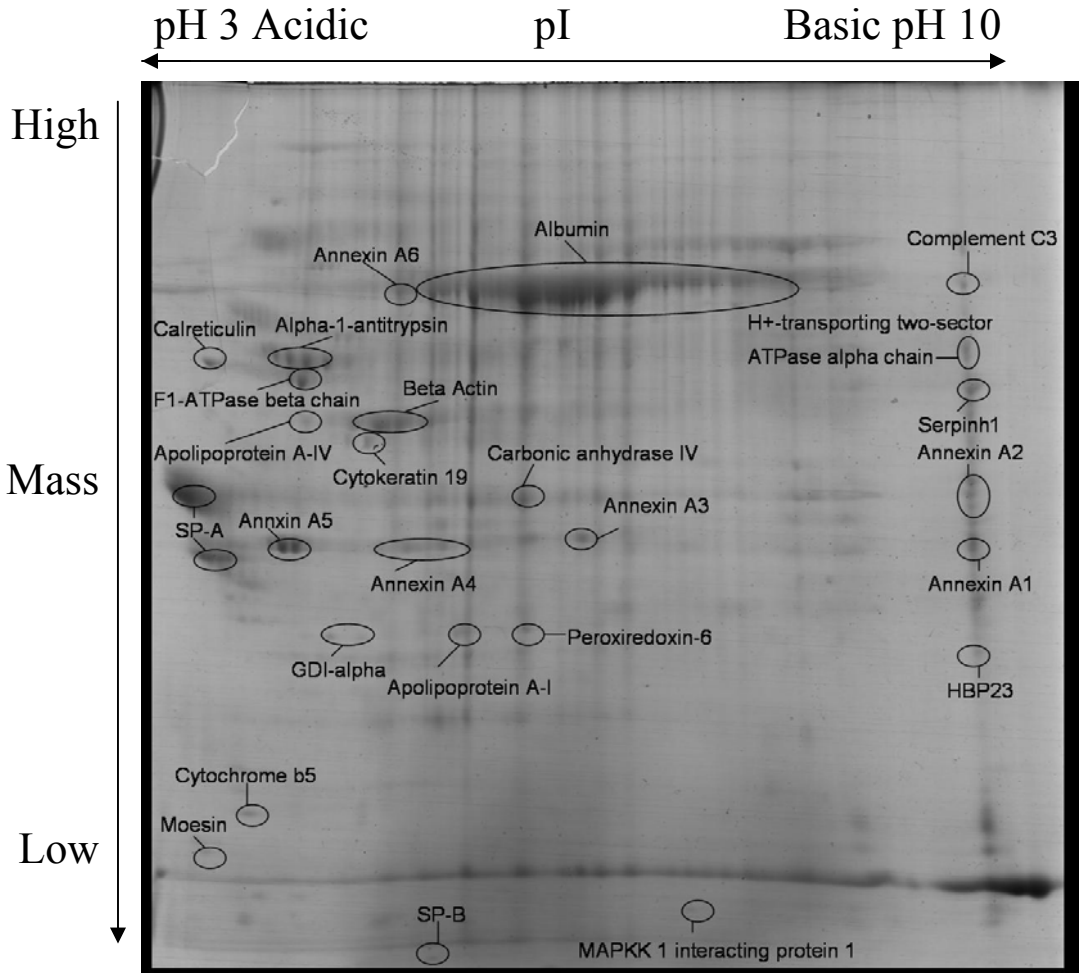

Fig. IV.2. Two-dimensional SDS-PAGE of proteins of lamellar bodies.

Approximately 500 μg of total lamellar body protein was cleaned up and applied to IEF. The protein samples in the IPG strip were then separated by polyacrylamide gel electrophoresis. The protein spots were visualized with Coomassie Brilliant Blue G-250 dye. The arrows on the top indicate the isoelectric points (pI) of the proteins. The molecular weight is shown to the left of the panel.

To identify protein components in lamellar bodies, we utilized the powerful technique Matrix Assisted Laser Desorption/Ionization – Time Of Flight Mass Spectrometry (MALDI-TOF MS). By trypsin digestion, each protein produces a unique set of peptides, termed as peptide fingerprints. A MALDI-TOF instrument generates the unique cutting profile of a target protein, expressing it as a mass spectrum. Through comparing the mass spectra to databases, a target protein can be identified. Fig. IV.3. is a mass spectrum of one protein sample from the lamellar bodies. It matches rat annexin A2.

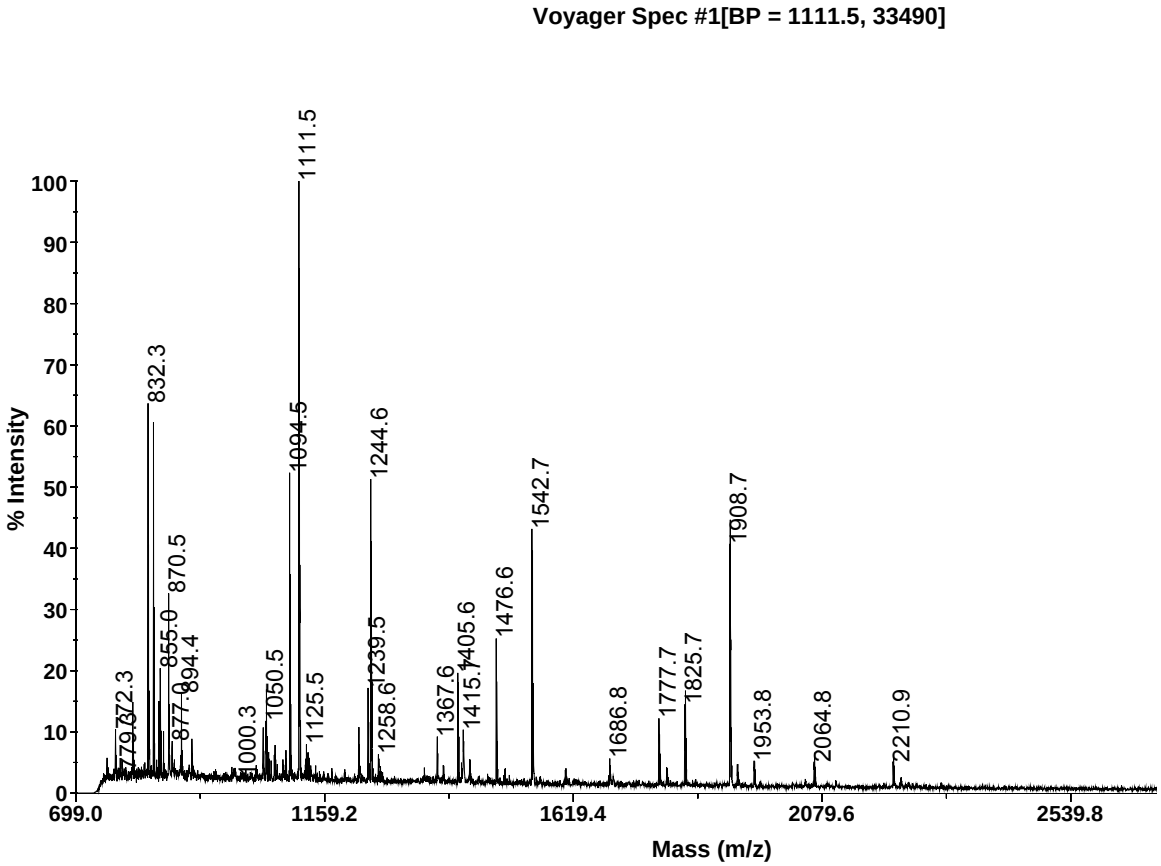

Fig. IV.3. The mass spectrum of annexin A2 by MALDI-TOF mass spectrometry.

Peaks represent peptides detected by the instrument. X-axis is mass to charge ratio for a defined range of masses, representing the mass of ionized peptides. Y-axis represents the number of ionized peptides detected, as a percentage to the maximum value (100%).

The peptide spectra generated from lamellar bodies proteins were searched against the Mass Spectrometry protein sequence database (MSDB) using the Mascot web based search engine. According to the Probability Based Mowse Score, the higher score a protein identification has, the more likely the match is true. An example is shown in Fig. IV.4. protein scores greater than 56 are significant ($p<0.05$). The score of matched identification JH0819, represented by the red column, is 119. It is very likely that the protein sample examined is JH0819 (rat calreticulin precursor).

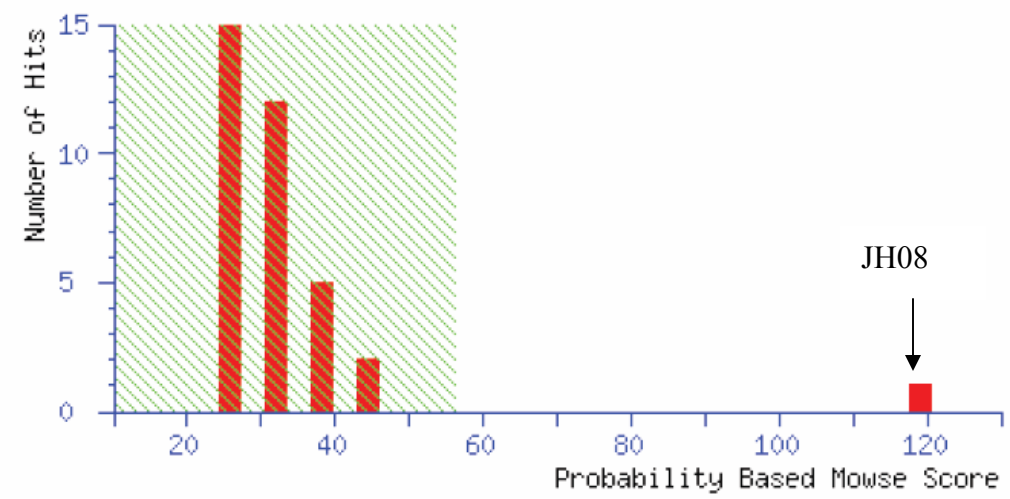

Fig. IV.4. An example of Mascot search result: Probability Based Mowse Score.
The score is $-10*\text{Log}_{10}(P)$, where P is the probability that the observed match is a random event. Protein scores greater than 56 are significant ($p<0.05$).

Table IV.1. lists the 44 proteins identified from the highly purified lamellar bodies using peptide mass fingerprinting. Listed for each protein include NCBI accession number, the number of peptides matched and percent of sequence covered, the Mowse scores and molecular mass. The functional classification was performed by a literature search in the Pubmed database.

Table IV. 1. Proteins identified in lamellar bodies with MALDI-TOF MS

Protein identified	NCBI accession #	Peptide matched	Sequence covered %	Mascot Score	Molecular mass
Calcium binding proteins					
Annexin A1	P07150	16	42	137	38674
Annexin A2	Q07936	16	49	129	38523
Annexin A3	LURT3	15	45	148	36341
Annexin A4	Q5U362	14	42	140	35871
Annexin A5	P14668	18	52	156	35591
Annexin A6	P48037	14	20	66	75575
Annexin A7	Q6IRJ7	8	17	57	49987
Calreticulin precursor	AAH62395	12	24	119	47966
Structural proteins					
Actin-beta	ATRTC	10	31	97	41724
Vimentin	P31000	20	38	119	53569
Myosin-9 (Nonmuscle myosin heavy chain IIa)	Q62812	47	24	143	226066
Tropomyosin 1	AAA42289	10	32	60	32938
Moesin (Membrane-organizing extension spike protein)	O35763	16	20	64	67566
Cytokeratin 19	AAR36876	15	38	108	44609
Surfactant related proteins					
Surfactant protein A precursor	P08427	10	43	63	26272
Surfactant protein B	Q6IN44	7	19	62	41590
ABCA3	AAH88202	7		160	240735
Peroxiredoxin-6	NP_446028	8	33	78	24672
Apolipoprotein A-I precursor	AAH89820	10	35	84	30043
Apolipoprotein A-IV	AAH91159	16	39	122	44429
Lysosome membrane protein II	P27615	8	16	52	53925
Ion channels					
H^+-transporting two-sector ATPase alpha chain precursor	J05266	12	29	57	58790
F1-ATPase beta chain	1MABB	10	32	83	49043
Unnamed protein product	CAF05438	20	14	113	146309

Membrane traffic					
Ehd1	AAH82030	11	20	65	60565
Annexin A1	P07150	16	42	137	38674
Annexin A2	Q07936	16	49	129	38523
Annexin A3	LURT3	15	45	148	36341
Annexin A4	Q5U362	14	42	140	35871
Annexin A5	P14668	18	52	156	35591
Annexin A6	P48037	14	20	66	75575
Annexin A7	Q6IRJ7	8	17	57	49987
Actin-beta	ATRTC	10	31	97	41724
Protein processing					
Serpinh1 protein	Q5RJR9	7	18	56	46532
Dipeptidyl peptidase IV membrane-bound form precursor	A39914	15	20	85	90869
Alpha-1-antitrypsin precursor	AAA40788	10	24	87	45807
Signal transduction					
Mitogen-activated protein kinase kinase 1 interacting protein 1	AAH86353	6	57	65	13571
Rho GDP dissociation inhibitor (GDI) alpha	Q5XI73	7	36	90	23393
Rho-associated kinase beta	AAB37571	24	18	71	159527
Miscellaneous proteins					
Albumin precursor	P02770	17	30	128	68674
Poly-ubiquitin	Q63654	5	44	82	11234
Complement C3 precursor	625256	13	7	61	186342
Heme-binding 23K protein (HBP23)	1QQ2A	7	28	58	22095
Putative alpha (1,3) fucosyltransferase	CAC81972	10	19	59	42931
Periaxin	I58157	19	13	102	146948
Carbonic anhydrase IV	AAH97329	10	25	72	35054
Heat shock 70kDa protein 5	AAH62017	19	33	95	72302
Protein disulfide isomerase associated 3	AAH62393	16	32	95	56588
Transferrin	AAP97736	14	19	80	76346
Ferroxidase precursor	A35210	11	9	122	120588

RSB-11-77 protein	NP_872610	12	28	75	42058
Cytochrome b5	AAB67609	13	27	78	11400

Calreticulin is a Ca^{2+}-binding protein for the calcium storage in ER [258]. It also has been reported to act as an important modulator of the regulation of gene transcription by nuclear hormone receptors [259]. Lamellar bodies contain a high level of calcium, especially lamellar bodies in apical area and the exocytotic lamellar bodies contain significantly higher calcium compared to those in perinuclear area [260]. This suggests that this high content of calcium in lamellar bodies may be a supply for the increase in local calcium concentration during the fusion events. Calreticulin may be the main calcium holder in lamellar bodies and may control the release of the calcium ions.

Annexins are important Ca^{2+}-binding proteins. Their functions include membrane organization, membrane-cytoskeleton linkage, membrane transportation and ion conductance across membranes. Annexin A1-7 were identified in lamellar bodies in this study. Annexin A1, 2, 3, 6 have been reported in type II cells with immunobolting and annexin A4 and A5 were not detected in this study [118]. Mayran et al. reported the presence of annexin A1, 4 and 6 in type II cells by using immunohistochemistry [261]. Annexin A5 was reported to be secreted from type II cells and its secretion was stimulated by TPA but inhibited by SP-A, showing the same pattern as that of surfactant secretion [262]. This suggests that annexin A5 may be released through lamellar bodies. Annexin A2 is important in various aspects of membrane trafficking [192, 193]. It mediates the fusion between lamellar bodies and the plasma membrane [112] and has been shown to be required in regulated lung surfactant secretion [214]. In addition to annexin A2, annexin A7 also has been proposed to be involved in regulating lung

surfactant secretion [119, 263, 264]. Annexin A1 and A6 have been reported to play roles in endocytosis [265, 266]. The presence of annexin A2 in lamellar bodies is in line with its role in surfactant secretion. The identification of other annexins may indicate their functions at other aspects, such as intracellular translocation.

It has been reported that actin [114] and microtubules [115] play a role in the transportation of lamellar bodies. In stimulated type II cells, annexin A2 may be involved in the disassembly and assembly of the cytoskeleton [117]. The identification of several cytoskeletal protins and actin-binding proteins (Beta-actin, Vimentin, Myosin-9, Moesin etc.) in lamellar bodies may suggest these proteins are involved in the movement of lamellar bodies.

An interesting protein identified is Ehd1, which stands for the EH domain-containing 1 protein. The EH domain includes an EF-Ca^{2+}-binding motif, a highly conserved ATP/GTP-binding domain, and a central coiled-coil structure [267]. It has been reported that Ehd1 interacts with SNAP-29 and plays a role in the endocytosis of Insulin-like Growth Factor 1 (IGF1) receptors [268]. Its role in the regulation of endocytic recycling has been further confirmed in other systems [269].

Rab proteins are small GTP-binding proteins that play roles in vesicular trafficking of molecules between cellular organelles. They serve as functional switches for the GTP-GDP exchange reaction.Rab GDP dissociation inhibitors (GDIs) can reduce the rate of GDP dissociation from Rab proteins [270]. Rab GDI alpha has been reported to bind Rab3A and modulates their activity and vesicle-mediated transport [271]. The identification of GDI alpha in lamellar bodies suggests that it may be involved in the docking of lamellar bodies at the plasma membrane.

Lamellar bodies are lysosome-related organelles and retain some lysosoma-like features. Along with lysosome membrane protein II, some proteases (Dipeptidyl peptidase IV) and protease inhibitors (Serpinh1 and alpha-1-antitrypsin precursor) were identified, which suggests that lamellar bodies may not only store surfactant, they may also play a role in surfactant processing or remodeling. We also identified the proton transporting ATPases (H^+-transporting two-sector ATPase alpha chain precursor and F1-ATPase beta chain). These ATPases can pump protons into lamellar bodies to maintain the low pH inside lamellar bodies[77, 272], which is essential for surfactant processing [75, 273, 274] and Ca^{2+} uptake [275].

Some other proteins involved in lamellar body biogenesis were also identified. ABCA3 is a unique type II cell marker that is thought to transport lipids to lamellar bodies [79, 82]. Peroxiredoxin-6, which possesses both phospholipase A_2 and peroxidase activity [276], is responsible for the degradation of recycled DPPC in type II cell [277]. Apolipoprotein A-I is the major apoprotein of high-density-lipoprotein (HDL). The identification of apolipoproteins in lamellar bodies suggests that the cholesterol of surfactant may originate from HDL.

There are some limitations of the current techniques. For example, many membrane proteins are difficult to dissolve or precipitate during IEF. Proteins with extreme large or small sizes or pI values are also very difficult to identify. Additionally, a large amount of peptides are required to yield a satisfactory mass spectrum. Many proteins in lamellar bodies may be functionally important, but are not abundant enough to be detected. We had many spots yield good mass spectra, but no high data matches were found. This is possibly due to the limitation of *Rattus* database.

In summary, the proteomic profiling of lamellar bodies generated from this study provides a new perspective for the study on lamellar body biogenesis and surfactant secretion.

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V. SUMMARIES AND CONCLUSIONS

Lung surfactant secretion is a slow but highly regulated process and the secreted contents are unique lipo-protein complexes. Thus, it is not surprising that surfactant secretion has its different aspects from the neurotransmitter release in synapses or hormone secretion in endocrine systems. So far, the underlying principles of surfactant secretion are still not clear. In this dissertation, an attempt to elucidate the molecular mechanisms of the regulated lung surfactant has been made from three aspects.

Based on our previous studies, SNARE proteins are present in type II cells and required for regulated lung surfactant secretion. However, the SNARE proteins have not been shown to have the capability to sense the elevation of cytoplasmic Ca^{2+}, nor mediate the membrane fusion directly. On the other hand, the Ca^{2+}-binding protein, annexin A2, has been reported to mediate the fusion of lamellar bodies and the plasma membrane and promote surfactant secretion in type II cells. The aim of the first aspect of this project was to investigate whether annexin A2 works with SNARE proteins in the same pathway, acting as a Ca^{2+} sensor and fusogen (Chapter II). To test the interaction between annexin A2 and SNARE proteins, a GST fusion protein pull-down assay was utilized. SNAP-23 showed a specific binding with annexin A2 in a Ca^{2+}-dependent manner, while syntaxin and VAMP families had no significant binding. The interaction between SNAP-23 and annexin A2 was confirmed with a mammalian two-hybrid assay. These two proteins were co-localized in type II cells by immunocytochemistry. To identify the binding sites of

SNAP-23, a series of deletion mutants were constructed and applied to GST pull-down assay. The deletion of the cysteine-rich region (CRR) of SNAP-23 abolished its binding with annexin A2. The mutations of the cysteine residues rendered SNAP-23 to lose its binding with annexin A2. Furthermore, annexin A2 was able to co-immunoprecipitated with wild type SNAP-23 but not with CRR deleted SNAP-23 mutant. These results indicated that CRR is required for the interaction between SNAP-23 and annexin A2. To study their functional interaction, an *in vitro* membrane fusion model was utilized. Anti-SNAP-23 antibodies inhibited the annexin A2-mediated fusion between isolated lamellar bodies and the plasma membrane. All these results supported that annexin A2 functions together with SNARE proteins in regulated surfactant secretion through its interaction with SNAP-23.

We have previously demonstrated the involvement of t-SNAREs, syntaxin 2 and SNAP-23, in regulated surfactant secretion. The second aspect of my work was to characterize the v-SNARE proteins involved (Chapter III). The gene expression of VAMP isoforms was detected by using RT-PCR, and VAMP-2, -3 and -8 had strong expression in lung and type II cells. The protein expression patterns of VAMP-2, -3 and -8 at various fractions of lung and type II cells were detected with Western blotting. VAMP-2 and VAMP-8 were present in type II cells and enriched in lamellar body fraction, whereas VAMP-3 was mainly present at plasma membrane fraction. Furthermore, immunostaining studies showed that VAMP-2 was localized on lamellar bodies, while VAMP-8 had more diffusible distribution with a partial localization on lamellar bodies. There studies suggest that VAMP-2 may be the v-SNARE protein involved in surfactant secretion.

Lamellar bodies are lysosome-related organelles and have unique features. Taking the advantage of the powerful sub-cellular proteomic techniques, a proteomic analysis of lamellar body proteins was carried out. Highly-purified lamellar bodies were applied to one- or two-dimensional polyacrylamide gel electrophoresis and MALDI-TOF mass spectrometry was performed. 44 proteins were identified and categorized according to their possible functions, including membrane trafficking, calcium binding and lamellar body related functions. This proteomic profiling of lamellar body proteins provides a base to better understand the biogenesis and function of lamellar bodies.

In summary, we demonstrated that annexin A2 physically and functionally interacted with SNAP-23. We also characterized VAMP proteins in lung and type II cells. Finally, a proteomic profiling of lamellar body proteins was achieved.

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Thesis: MECHANISMS OF REGULATED LUNG SURFACTANT SECRETION

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Scope and Method of Study:

This project was to study the molecular mechanisms of lung surfactant secretion from three aspects. The first part was to investigate the interaction between SNARE proteins and annexin A2. GST-tagged protein pull-down assay was the major technique utilized. The physical interaction of recombinant GST-tagged SNARE proteins with annexin A2 were tested. To confirm the interaction between annexin A2 and SNAP-23, additional methods were used, including co-immunoprecipitation, mammalian two-hybrid assays. Immunocytochemistry was used to study the co-localization of annexin A2 and SNAP-23. Deletion and site-directed mutagenesis were used to identify the binding sites for annexin A2 on SNAP-23. Furthermore, an *in vitro* bio-membrane fusion assay was utilized to study the functional interaction between annexin A2 and SNAP-23. The second part was to identify the v-SNARE protein involved in regulated surfactant secretion. Various VAMP genes were amplified from alveolar type II cells by using RT-PCR. The expression of VAMP proteins were detected with Western blotting. Immunohistochemistry and immunocytochemistry were utilized to study the localization of VAMP in lung and type II cells. In the third part, the proteomic profile of lamellar bodies was analyzed. Lamellar bodies were isolated from rat lungs and the proteins were separated with 1-D and 2-D SDS-PAGE. The proteins were applied to MALDI-TOF MS, and identified by searching the peptide spectra against the Mass Spectrometry protein sequence Database (MSDB) using the Mascot web based search engine.

Findings and Conclusions:

I.

1. SNAP-23 specifically binds with annexin A2 in a Ca^{2+}-dependent manner.
2. The cysteine rich domain of SNAP-23 is required for its binding with annexin A2.
3. SNAP-23 is required in annexin A2 tetramer mediated membrane fusion between isolated lamellar bodies and the plasma membrane.

II.

1. VAMP-2 and -8 are expressed in type II cells and lamellar bodies.
2. VAMP-2 is localized on lamellar bodies and VAMP-8 is partially localized on lamellar bodies.

III.

1. Forty-four proteins in lamellar bodies were identified.
2. Proteins involved in membrane trafficking and Ca^{2+}-binding may play roles in lamellar body biogenesis and fusion with the plasma membrane.

ADVISER'S APPROVAL: Dr. Lin Liu