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Review

Protein–lipid interactions and surface activity in the pulmonary surfactant system

Alicia G. Serrano, Jesús Pérez-Gil *

Departamento de Bioquímica y Biología Molecular I, Facultad de Biología, Universidad Complutense, Jose Antonio Novais 2, 28040 Madrid, Spain

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Abstract

Pulmonary surfactant is a lipid–protein complex, synthesized and secreted by the respiratory epithelium of lungs to the alveolar spaces, whose main function is to reduce the surface tension at the air–liquid interface to minimize the work of breathing. The activity of surfactant at the alveoli involves three main processes: (i) transfer of surface active molecules from the aqueous hypophase into the interface, (ii) surface tension reduction to values close to 0 mN/m during compression at expiration and (iii) re-extension of the surface active film upon expansion at inspiration. Phospholipids are the main surface active components of pulmonary surfactant, but the dynamic behaviour of phospholipids along the breathing cycle requires the necessary participation of some specific surfactant associated proteins. The present review summarizes the current knowledge on the structure, disposition and lipid–protein interactions of the hydrophobic surfactant proteins SP-B and SP-C, the two main actors participating in the surface properties of pulmonary surfactant. Some of the methodologies currently used to evaluate the surface activity of the proteins in lipid–protein surfactant preparations are also revised. Working models for the potential molecular mechanism of SP-B and SP-C are finally discussed. SP-B might act in surfactant as a sort of amphipathic tag, directing the lipid–protein complexes to insert and re-insert very efficiently into the air–liquid interface along successive breathing cycles. SP-C could be essential to maintain association of lipid–protein complexes with the interface at the highest compressed states, at the end of exhalation. The understanding of the mechanisms of action of these proteins is critical to approach the design and development of new clinical surfactant preparations for therapeutical applications.

Keywords: Lung surfactant; Protein–lipid interactions; Membrane proteins; Bilayers; Monolayers; Surface tension; Membrane fusion; Lipid polymorphism

Contents

Abbreviations: AFM, atomic force microscopy; ARDS, Acute Respiratory Distress Syndrome; DPPC, dipalmitoylphosphatidylcholine; ER, endoplasmic reticulum; LB, lamellar bodies; MVB, multivesicular bodies; NRDS, Neonatal Respiratory Distress Syndrome; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; RDS, Respiratory Distress Syndrome; SAPLIP, saposin-like proteins; SP-A, surfactant protein A; SP-B, surfactant protein B; SP-C, surfactant protein C; SP-D, surfactant protein D; TM, tubular myelin

* Corresponding author. Tel.: +34 91 3944994; fax: +34 91 3944672.
E-mail address: jpg@bbm1.ucm.es (J. Pérez-Gil).

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1. Introduction

Pulmonary surfactant is a complex mixture of lipids and proteins whose main function is to reduce the surface tension at the alveolar air–liquid interface of lungs in order to avoid alveolar collapse at the end of expiration and to facilitate the work of breathing. This proteolipidic material is synthesized by type II pneumocytes, one of the cell types forming the alveolar epithelium, and follows a regulated exocytic pathway leading to secretion into the thin aqueous layer covering the alveoli. Pulmonary surfactant is absolutely required for lung breathing. The lack, deficiency or inactivation of this essential material is the cause of severe respiratory disorders, sometimes lethal, such as the Neonatal Respiratory Distress Syndrome (NRDS) (Hallman et al., 2001), or the pulmonary dysfunction associated with the Acute Respiratory Distress Syndrome (ARDS), a disease related with different processes as injury, inflammation or sepsis (Seeger et al., 1993). Supplementation of the immature lungs of preterm babies with exogenous surfactants has dramatically improved clinical prognosis of these patients; however, the clinical management of ARDS is still under development (Gunther et al., 2001; Marraro, 2004; Medford, 2004). In this particular case it is of great importance to overcome the inhibitory effects on surfactant due to inflammation processes. Although animal-derived surfactants have been proven to improve clinical outcome of infants at risk for or having Respiratory Distress Syndrome (RDS), difficulties regarding large-scale production, suspension techniques, reproducibility and purity of natural surfactants limit their clinical usefulness. Therefore, a completely defined "humanized" lung surfactant mixture would be preferable. The development of efficient surfactant preparations for therapeutic practices is a main challenge within this area of investigation, but the design and production of this kind of preparations require an extensive knowledge of the molecular mechanisms governing the processes occurring at the respiratory surface.

2. Pulmonary surfactant: composition and function

Although the composition of pulmonary surfactant can vary from one species to another, or even from individuals within the same species, the major fraction of pulmonary surfactant in all organisms is constituted by the lipids. Thus, surfactant is formed by about 90% of lipids, most of them phospholipids, and 8–10% of proteins.

The lipid composition of pulmonary surfactant of different species was reviewed by Veldhuizen et al. (1998). In general terms, mammalian surfactants are composed of approximately 80% (weight of total material) of phosphatidylcholine (PC), about half of which is dipalmitoylphosphatydilcholine (DPPC). There are some exceptions, as the very recently discovered low DPPC content (∼15%) in surfactant from some heterothermic mammals, which spend a significant period of time in torpor (Lang et al., 2005). The remaining PC is composed primarily of molecular species containing monoenoic and dienoic fatty acids sterifying the *sn*-2 position of the glycerol backbone. The acidic phospholipids, phosphatidylglycerol (PG) and phosphatidylinositol (PI), account for 8–15% of the total surfactant phospholipid pool. In general, when PI levels are higher, PG content is lower. Surfactant from fetal lungs usually contains higher levels of PI but this is generally reverted in adult life. Except for the human, phosphatidylethanolamine (PE) is low (3–5%) and the other phospholipids are present at very low levels. Concerning neutral lipids, cholesterol is the major component, accounting for up to 8–10% of surfactant by weight, which means up to as much as 20% cholesterol/phospholipid ratio in a molar basis. Small amounts of mono-, di- and tri-acylglycerol have been also reported, as well as some free fatty acids, mainly palmitate. Overall, the most remarkable characteristic of surfactant composition when compared with other mammalian membranes is the unusual high content in DPPC and anionic phospholipids (PG plus PI). Nevertheless, as pointed out in a very recent study from Lang et al. (2005) where the phospholipid composition of surfactant from different mammals has been compared, a "consensus" well-defined surfactant phospholipid composition cannot be probably invoked. Rather, surfactant from each animal species is likely unique and has been evolutionarily optimized for particular conditions of development, respiratory rate, body temperature, etc., being probably further modified by individual pathophysiological situations such as dis-

eases, activity regime or other environmental or genetic factors.

The protein moiety of surfactant comprises four specific surfactant-associated proteins (Haagsman and Diemel, 2001). They can be classified in two groups, the hydrophilic surfactant proteins SP-A and SP-D, and the hydrophobic surfactant proteins SP-B and SP-C. SP-A is the major protein in surfactant representing about 5–6% of its dry weight and is isolated in strong association with surfactant phospholipids and hydrophobic proteins. SP-D, however, does not appear associated with the lipid-containing structures and it accounts only for ∼0.5% of the material obtained from lung lavage fluid. Both SP-A and SP-D belong to the collectin family of proteins. They are multimeric proteins with collagen-like domains and globular calcium-dependent carbohydrate-binding domains. These structural characteristics provide them the ability to bind many different ligands as calcium, sugars or lipid molecules in a concerted manner, and made them appropriate to play an important role in the innate immune system (Crouch and Wright, 2001). SP-A and SP-D have the ability to bind to a broad spectrum of pathogens including bacteria, viruses and fungi. The innate host defense activities in which these surfactant proteins participate at the alveolar airspaces are essential to maintain the sterile condition of the respiratory surface. Human lungs contain about 300 million alveoli of 75–300 μm in diameter, which means an area of approximately 70 m^2 in contact with the external medium and fully exposed to microorganisms. As a matter of fact, knock-out mice engineered to specifically deactivate the expression of SP-A or SP-D manifest significantly higher susceptibility to infections than wild-type animals, but no apparent respiratory dysfunction (LeVine et al., 1997; Wert et al., 2000; McCormack and Whitsett, 2002). In addition to its role in the innate immune response, SP-A has been somehow related to the surface activity of surfactant. The presence of SP-A is necessary for the formation of tubular myelin, considered one of the possible structural intermediates in surface film formation, which enhances surfactant adsorption but is not essential for normal breathing in in vivo models (Korfhagen et al., 1996). It has been also shown that SP-A has a synergistic effect in the enhancement of lipid interfacial adsorption promoted by SP-B and SP-C (Schürch et al., 1992; Bi et al., 2001; Possmayer et al., 2001), although a direct role of the hydrophilic surfactant collectins in interfacial film formation and/or surface tension reduction at the alveoli has not been proposed so far. Therefore, these proteins will not be further discussed in the present review. For more detailed information on SP-A and SP-D structure–function relationships, the reader is referred to other articles (for a review see Sano and Kuroki, 2005).

The other two proteins in surfactant are SP-B and SP-C. These are very small hydrophobic polypeptides resulting from the proteolytic processing of larger precursors along the exocytic pathway of pulmonary surfactant in type II pneumocytes. Each of them accounts for no more than 1–1.5% of total surfactant weight but in spite of their relatively low abundance, they play critical roles in formation and stabilization of pulmonary surfactant films (Weaver and Conkright, 2001). The importance of SP-B in surfactant is apparent from the lethal respiratory distress that is caused by SP-B deficiency in humans (Klein et al., 1998) and in SP-B knock-out mice (Clark et al., 1995; Tokieda et al., 1997). On the other hand, targeted deletion of the SP-C gene (SP-$C^{-/-}$) in mice leads to the development of severe progressive pneumonitis within a shorter or longer period of time after birth, depending on the genetic background (Glasser et al., 2001, 2003). It is important to note that the absence of SP-B in transgenic mice (SP-$B^{-/-}$) is accompanied by an incomplete processing of SP-C precursor protein leading to an additional deficiency of mature SP-C in alveolar airspaces (Clark et al., 1995; Vorbroker et al., 1995; Weaver and Conkright, 2001), so that the independent roles of each protein cannot be unequivocally established. With regard to this, a conditional model where the temporary loss of SP-B in adult mice resulted in surfactant dysfunction and respiratory failure in spite of maintenance of SP-C and surfactant lipid composition has been recently developed (Ikegami et al., 2005). Thus, this model confirmed a critical role of SP-B in adult lung function.

The activity of pulmonary surfactant at the alveolar spaces involves three main processes that have to be optimized for a proper surface behaviour (Fig. 1): (i) transference of surface active material from the aqueous

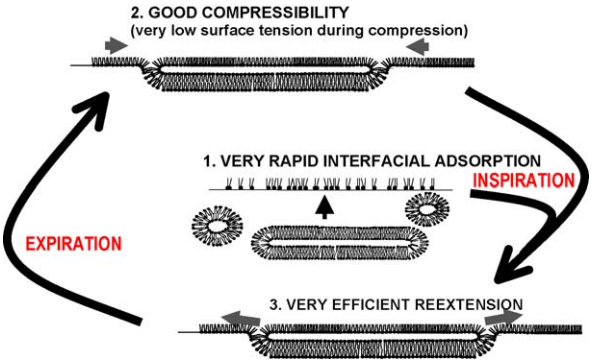

Fig. 1. Main activities of pulmonary surfactant complexes during the compression–expansion breathing cycling at the respiratory air–liquid interface.

hypophase close to the interface followed by interfacial adsorption of the active molecules to form the surface active film, (ii) surface tension reduction to values close to 0 mN/m during the compression of the respiratory surface that occurs at expiration and (iii) re-spreading of the surface film during expansion at inspiration.

It is widely assumed that the surfactant components directly implicated in the surface tension reduction are the phospholipids. However, none of the phospholipid components can behave as a good surfactant by itself. For instance, a pure DPPC monolayer has been shown to attain surface tensions values close to 0 mN/m upon compression (Nag et al., 1999; Cruz et al., 2000; Wustneck et al., 2005), but the rate of adsorption of this phospholipid from the hypophase to the air–liquid interface is extremely slow (Cruz et al., 1997, 2000). Conversely, phospholipids with good adsorption and spreading properties do not produce low enough surface tension when their monolayers are subjected to compression (Takamoto et al., 2001). There is no question that the presence of the proteins, particularly hydrophobic proteins, is absolutely required for suitable efficiency of a given material as a surface tension reducing agent under conditions mimicking compression–expansion cycles of breathing. In addition to the in vivo evidences for SP-B and SP-C requirement, in vitro studies have clearly demonstrated that interfacial adsorption, film stability and re-spreading capacities are three critical properties that cannot be fully achieved by a strictly lipidic preparation (Halliday, 1996). The conclusion is, therefore, that the mixture of lipids and proteins has been evolutionary optimized in pulmonary surfactant to exhibit simultaneously these three properties. The experimental data collected to date suggest that the proteins would be mainly involved in promoting adsorption, stabilization and re-spreading of the surface film, while the lipids would be essentially responsible for the maximal surface tension reduction upon compression.

In the present review, the structure, lipid–protein interactions and surface activity properties of SP-B and SP-C will be discussed in detail and related to the current models on the molecular mechanisms of their participation in the respiratory function.

3. From the secretory pathway to the air–liquid interface: technical approaches

Surfactant lipids and proteins are synthesized and secreted by type II pneumocytes. The exocytic pathway goes from the endoplasmic reticulum (ER) to the lamellar bodies (LB), the specialized organelles where all lipids and proteins are assembled and stored in densely packed concentric lamellar structures. Secretion takes place via fusion of these surfactant containing vesicles with the plasma membrane and leads to the formation of a new highly ordered lipid–protein structure, specific of this membranous system, called tubular myelin (TM) (Fig. 2). Under transmission electron microscopy TM has a unique architecture consisting of crossing bilayers forming square lattices. This structure can be reproduced in vitro in the presence of SP-A, SP-B, DPPC, PG and calcium (Suzuki et al., 1989; Williams et al., 1991; Poulain et al., 1992). SP-A seems to be placed at the corners of the lattices (Palaniyar et al., 1999). Although not strictly required for normal lung function, TM is considered a precursor form of the interfacial film. It has been proposed that it is a highly adsorptive intermediate of surfactant material because of the very efficient interfacial adsorption of surfactant preparations containing this structure (Williams, 1992). However, the absence of TM in SP-A knock-out mice does not alter breathing function (Korfhagen et al., 1996), suggesting that there might be alternative mechanisms for surfactant transfer to the interface, in such a way that tubular myelin might be an enhancer rather than an essential element in this process. Thus, in vivo, the formation of the interfacial film comes from the adsorption of lipid–protein complexes from highly organized macromolecular structures and trough mechanisms that are still not well understood. Recent studies have shown that LB-like particles maintain after secretion much of their packed structure, which is able to transfer the lipid/protein material directly into the interface in a very efficient and cooperative manner (Haller et al., 2004). Such almost instantaneous interfacial conversion is dependent on surface tension, indicating that the architecture of lipid–protein complexes in LBs may be particularly well suited to respond to surface forces. Once the interfacial surfactant film is formed at the first

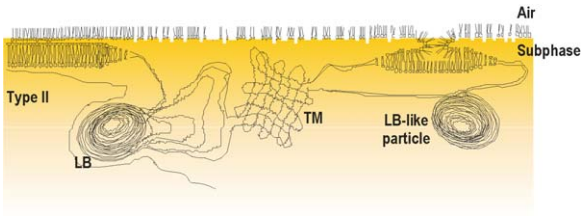

Fig. 2. Structure of pulmonary surfactant at the alveolar airspaces. Epithelial type II pneumocytes synthesize and store pulmonary surfactant in the form of compact arrays of bilayers in the lamellar bodies (LB). Upon secretion, some of these LBs maintain their packed structure in the form of LB-like particles, and some of them are unpacked to form tubular myelin (TM), an ordered structure of crossing bilayers. LB-like particles and TM are highly surface active intermediates able to insert very efficiently surface active phospholipids into the air–liquid interface.

air inspiration of the newborn, it will be stable under the dynamic compression–expansion cycles of breathing along the entire life. At present, it is widely accepted that there is a reservoir of surfactant attached to the interface, which plays an important role in the maintenance of the required stability of such a film. This reservoir, likely composed of multiple tightly packed membranes, keeps enough new and recycled material in close proximity to the interface in order to facilitate the squeeze-out and re-insertion of surface active components under cycling, that is, ensuring the proper renewal of the surface film. In fact, multilayered structures consistent with this model have been already visualized by transmission electron microscopy and can be also detected by fluorescence and scanning force microscopies (von Nahmen et al., 1997; Schurch et al., 1998; Piknova et al., 2001). So, after secretion, alveolar forms of surfactant include LBs and LB-like particles, TM and the interfacial film itself consisting of the surface monolayer plus the additional multilayered reservoir located underneath. These three-dimensional structures constitute a highly complex network of membranes within the small space comprised between the surface of the epithelial cells and the air–liquid interface (represented in Fig. 2).

In vitro, different biophysical techniques (Fig. 3) try to mimic the situation occurring in the alveoli in order to elucidate the molecular mechanisms governing the three principal processes involved in surfactant activity:

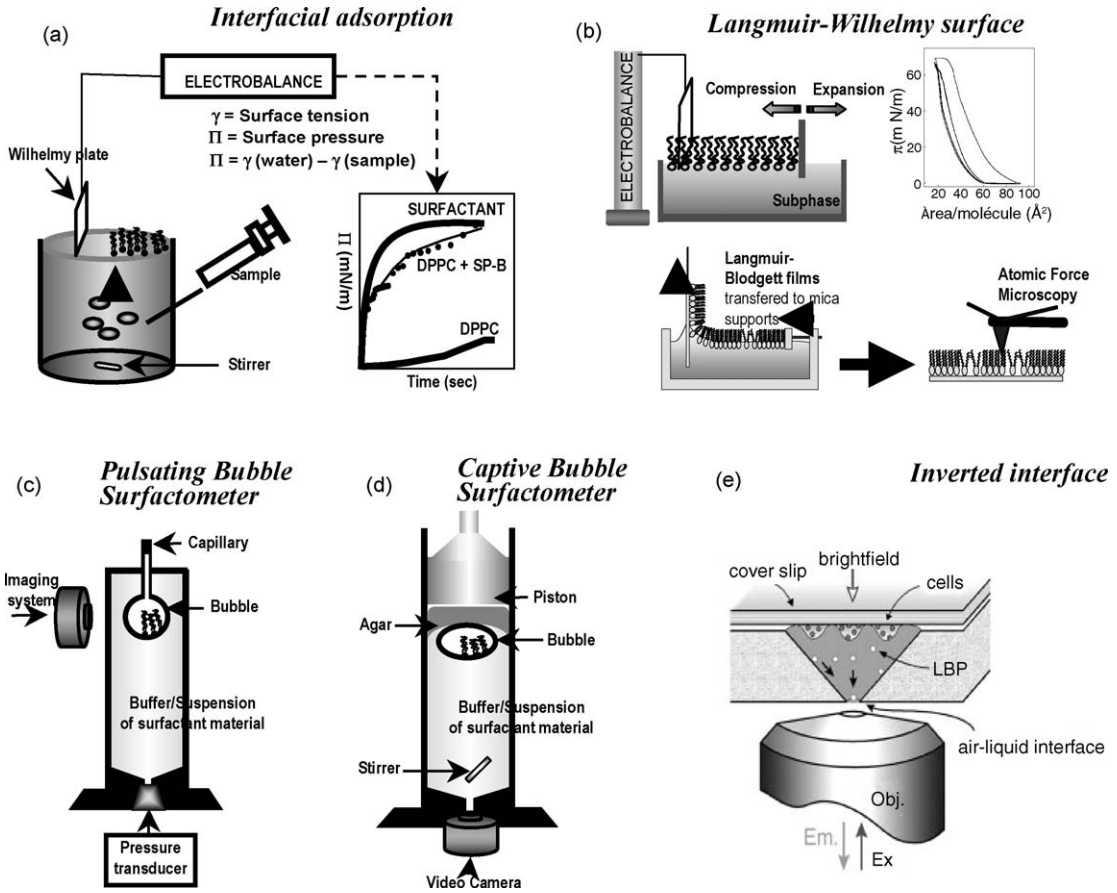

Fig. 3. Biophysical techniques used to evaluate surface activity of lipid–protein surfactant preparations in vitro. (a) Interfacial adsorption experiments in modified surface balances allows evaluation of adsorption *Π–t* kinetics, showing the effect of surfactant proteins to promote transfer of phospholipids from bilayers into the air–liquid interface. (b) Experiments in Langmuir–Wilhelmy surface balances allow determination of compression and expansion *Π–A* isotherms. These balances can be also used to transfer interfacial lipid and lipid–protein films into solid supports, and the study of these Langmuir–Blodgett films by epifluorescence or atomic force microscopy (AFM). (c) The pulsating bubble surfactometer monitorizes surface tension changes under compression–expansion cycling at quasi-physiological conditions. (d) The captive bubble surfactometer provides the most reliable compression–expansion isotherms of pulmonary surfactant complexes, as they are adsorbed at the interface of a closed air bubble that can be also cycled at breathing rates. (e) The recent development of the so-called inverted interface microscope allows correlation of changes in surface tension with the structural transformations produced by surfactant complexes at the interface, as observed by fluorescence microscopy (taken from Bertocci et al., 2005, with permission).

interfacial adsorption, surface tension reduction and film re-spreading.

Interfacial adsorption kinetics can be followed using a Whilhelmy dipping plate attached to an electro-balance suited to monitor changes in surface tension (Fig. 3a). The hypophase is usually contained in a small trough with a fixed area and a lateral perforation permitting the injection of surface material into the subphase without perturbing the interface. The progressive incorporation of the injected material into the air–liquid interface leads to changes in surface tension that can be registered as a function of time. One main limitation is that, in vitro, the rate of interfacial adsorption is usually measured at much lower concentration of surface active material in the hypophase than that probably occurring in vivo.

For the study of spread films of different compositions, one of the oldest but still most used devices is the *Langmuir–Wilhelmy surface balance* (Fig. 3b). As designed by Clements (1957) for his initial seminal studies on surfactant, it consists of a trough containing the aqueous hypophase whose surface area can be varied, and a Wilhelmy dipping plate attached to an electrobalance. This balance is particularly suited for studying spread films because precise amounts of surface active material can be deposited at the interface. Changes in the surface area permit to mimic the compression–expansion cycles of breathing but in a relatively static way that does not reflect the dynamic process of breathing. One of the most important advantages of this technique is that radiography and epifluorescence microscopy of the surface film can be easily performed using labeled proteins or lipids (Nag et al., 1997; Plasencia et al., 2005; Yan et al., 2005). This enables visualization of lipid and lipid–protein domains as well as the study of the lateral distribution of the membrane-associated proteins at the air–liquid interface. In addition, monolayers prepared with this kind of balances can be transferred onto mica supports for scanning force microscopy analysis (von Nahmen et al., 1997; Diemel et al., 2002; Bernardino de la Serna et al., 2004; Cruz et al., 2004). This technique is providing in the recent years important complementary information on film topology avoiding the use of extrinsic probes. This matter is of interest, as recent investigations have shown that even small traces of fluorescently labeled lipids such as NBD-PC could produce significant perturbations, especially at the nanometer scale, that are analyzable only by comparing scanning force and epifluorescence microscopy images (Cruz et al., 2005).

A pioneer technique developed by Enhorning in 1977 to analyze pulmonary surfactant dynamics is the *pulsating bubble surfactometer* (Fig. 3c). It consists of a sample microchamber connected to the atmosphere by a small capillary. An air bubble attached to this capillary is pulsated by varying the pressure inside the sample cuvette as a way to mimic the compression–expansion breathing cycles of an alveolus. Surface tension is calculated from the Law of Young and Laplace ($P = 2 \times \gamma/r$, where P is the pressure across the bubble, γ the surface tension and r is the radius of the bubble or "artificial alveolus"). The surface tension at maximum bubble radius is indicative of surfactant adsorption during surface expansion, while γ at minimum radius indicates the film's ability to reduce surface tension during compression. The pulsating bubble surfactometer has been extremely useful for monitoring the quality of pulmonary surfactant samples relative to their biological activity (Gunther et al., 1999; Brackenbury et al., 2002; Merrill et al., 2004), and has become very popular in clinical research, but it is not very accurate for mechanistic studies because of leakage of material through the capillary. At present this technique is falling in disuse in favor of the *captive bubble surfactometer* (Fig. 3d), which avoids the problem of leakage and mimics dynamic natural breathing frequencies. This model system was introduced by Schurch et al. in 1989 and later modified by Putz et al. (1994). It consists of an air bubble floating against an agar gel in an airtight chamber. The surface tension at the bubble surface is calculated from the shape of the bubble that can be monitored by a video camera (Schoel et al., 1994). Unlike the pulsating bubble surfactometer, the subphase in a captive bubble experiment can be stirred and spread films can be prepared only requiring few nanograms of proteins. However, the main disadvantage of this technique is that it is time-consuming. The difficulty of calculations from the video images was an important inconvenient at the beginning, but today this problem has been fully overcome by a proper software development.

More recently a new noninvasive optical technique has been developed to measure surface tension in a miniaturized air–liquid interface (Bertocchi et al., 2005). The designers of the method have called it "*inverted interface*" (Fig. 3e). It is based on a sapphire cone with 300 μm aperture, which relies on surface coherence to keep the liquid sample in a fixed and almost planar position above the objective of an inverted microscope. The radius of curvature of the fluid meniscus is related to the surface tension and produces a characteristic pattern of back-reflection upon illumination. This novel method allows observation of pulmonary surfactant transformations as it comes into contact with the air–liquid interface, and their correlation with the decrease on surface tension. However, changes in surface tension can only be followed at this inverted interface down to a certain value, therefore preventing the analy-

sis of structure–function correlations in surfactant films at tension values approaching equilibrium, the most relevant ones in terms of the dynamics of the surfactant reservoir.

4. Pulmonary hydrophobic surfactant proteins: the keys of surface activity in the lungs

4.1. SP-B: an amphipathic tag to the interface

The only indispensable protein, among pulmonary surfactant proteins, to initiate air breathing, SP-B, is a small hydrophobic polypeptide of 79 amino acids. It is synthesized as a longer precursor (381 amino acids), which undergoes proteolytic cleavage of N- and C-terminal propeptides in at least three different steps, to give rise to the mature sequence (Brasch et al., 2003; Ueno et al., 2004). In type II pneumocytes SP-B processing occurs along the exocytic pathway of surfactant, and it is not completed until assembled in LB together with the other lipids and proteins (Voorhout et al., 1992; Korimilli et al., 2000).

Mature SP-B is isolated from animal lung lavages as a homodimer containing three intramolecular disulphide bonds per monomer and an additional disulphide stabilizing the dimer. These covalent linkages have been shown to constrain the protein flexibility without significantly perturbing its activity in in vitro studies (Serrano et al., 2005). The exact three-dimensional structure of SP-B is still unknown. There are abundant experimental data obtained from indirect techniques such us circular dichroism (Andersson et al., 1995), infrared spectroscopy (Vandenbussche et al., 1992; Pastrana-Rios et al., 1995), spin resonance (Perez-Gil et al., 1995) or fluorescence anisotropy (Baatz et al., 1990), that provide a good picture of the conformation and disposition of the polypeptide, either in solvents or in membrane environments. Also, sequence alignments reveal that the location of the Cys residues in SP-B is a common feature among a group of proteins referred to as the saposin-like proteins (SAPLIP) (Munford et al., 1995). All members of this family are membrane-interacting proteins and they are supposed to share a similar 3D-structure, which has been already revealed for some of them as NK-lysin (Liepinsh et al., 1997), saposin B (Ahn et al., 2003) or amoebapore (Vorbroker et al., 1995; Hecht et al., 2004). Thus, SP-B is known as a mainly α-helical protein (40–45%), containing four to five amphipathic helices that allow it to interact with and perturb lipid membranes (Fig. 4). The disposition of SP-B in lipid bilayers and monolayers as

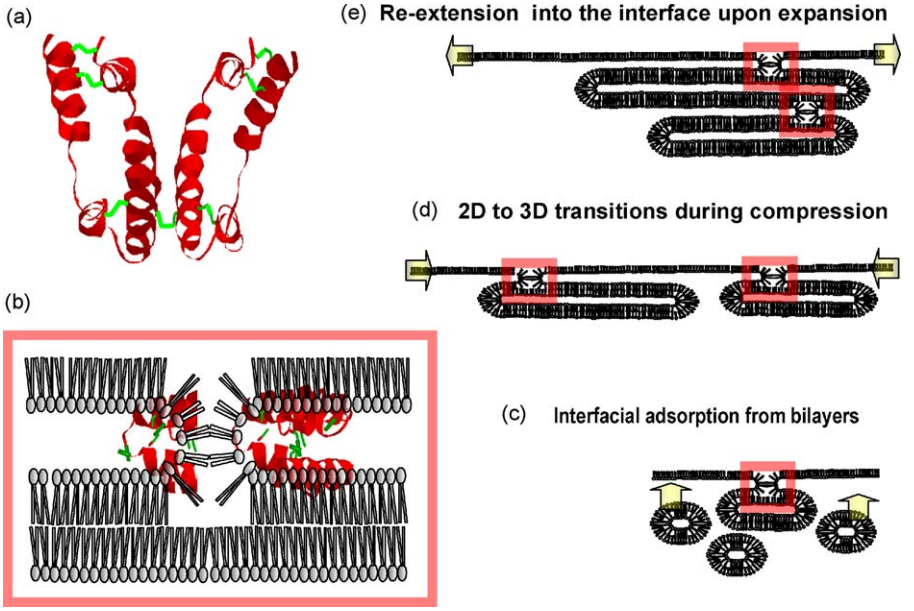

Fig. 4. Structure, disposition and activities of pulmonary surfactant protein SP-B. (a) Model of the structure of dimeric surfactant protein SP-B according to the saposin-like folding. Potential amphipathic α-helical segments have been represented in the model of the protein as well as the location of the three intramolecular and the one intermolecular disulphide linkages (green). (b) Probable disposition of SP-B associated with surfactant bilayers and monolayers. The amphipathic helical motifs of the protein are probably located at the surface of the membranes, parallel to the plane of bilayers and monolayers. The folding of SP-B dimer probably posseses hydrophobic cavities able to stabilize lipid intermediates in the transit from bilayers to the interface. (c–e) Different processes in the surfactant cycle at the interface where SP-B (red boxes) could facilitate bilayer–bilayer and bilayer–monolayer transitions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

well as the effect produced on the lipids has been also analyzed by many different techniques such as ^{2}H NMR (Morrow et al., 1993, 2004), spin resonance (Perez-Gil et al., 1995), infrared spectroscopy (Brockman et al., 2003), atomic force microscopy (Cruz et al., 2004) or epifluorescence microscopy (Cruz et al., 2000).

Phospholipid monolayers compressed to pressures into the liquid-expanded to liquid-condensed transition plateau show coexistence of highly condensed domains floating in a background of less packed (liquid-expanded) lipids. SP-B has been observed by epifluorescence microscopy to partition strictly into the liquid-expanded areas of these films (Nag et al., 1997). Its presence increases the number and reduces the size of condensed regions, both at the micro- and the nanoscopic structural levels (Cruz et al., 2000, 2004), leading to a structural stabilization of the films at all ranges of the compression scale, including films compressed to the highest pressures (Cruz et al., 2000). In phospholipid bilayers, SP-B is placed with the axes of the helices nearly parallel to the interface of the membranes (Vandenbussche et al., 1992). A model has been then proposed in which the more hydrophobic face of each helix would interact with deeper regions of the bilayers interface, while the nine positive charged residues of the protein would interact with the polar headgroup of anionic phospholipids (see Fig. 4). It has been reported that SP-B interacts with surfactant lipids showing certain selectivity towards PG (Baatz et al., 1990; Perez-Gil et al., 1995) and that it has the ability to produce aggregation, fusion and lysis of phospholipid vesicles (Shiffer et al., 1988; Oosterlaken-Dijksterhuis et al., 1992; Poulain et al., 1992; Chang et al., 1998; Ryan et al., 2005). The electrostatic interactions between cationic SP-B and anionic phospholipids could be on the basis of such effects. All these properties have been proposed to be critical for the surface activity of the protein as well as for its role in LB or TM formation. Lung morphogenesis and surfactant phospholipid synthesis in SP-B$^{-/-}$ mice proceed normally prior to birth (Clark et al., 1995). However, LB formation is disrupted, resulting in abnormal inclusions consisting of multivesicular bodies (MVB) and disorganized lamellae. Neither mature LBs nor TM were detected in SP-B$^{-/-}$ mice. The fusogenic properties of SP-B suggest a mechanistic basis for its involvement in the lipid rearrangements required for the transition of surfactant from the intracellular storage form (LB) to the functional extracellular structures (TM), as well as for the conversion of bilayers into interfacial monolayers. In addition, SP-B deficiency results in aberrant processing of the SP-C proprotein leading to a significant decrease in mature SP-C levels. It has been proposed that the membranolytic properties of SP-B might be necessary for the complete processing of proSP-C in late endosomes/multivesicular bodies MVB, prior to LB assembly (Weaver and Conkright, 2001). Weaver and Conkright suggested the possibility that SP-B could be required for membrane lysis of the internal vesicles of MVB, where proSP-C would be anchored as a transmembrane protein, in order to permit the access of the appropriate processing proteases.

Whether the fusogenic and lytic properties of SP-B are critical for its activity at the interface is still not completely clear, but the essential role of the protein to promote interfacial adsorption of surfactant has been demonstrated in many different in vitro assays. Some authors have suggested that SP-B (and SP-C) accelerates adsorption by acting as a sort of catalysts that stabilize the high-energy intermediates required for the phospholipids to pass from the bilayers to the interface (Schram and Hall, 2001, 2004). Whatever the molecular mechanism, SP-B is the most active polypeptide, among surfactant proteins, in promoting phospholipid adsorption from the hypophase to the air–liquid interface (Oosterlaken-Dijksterhuis et al., 1991a,b; Nag et al., 1999; Cruz et al., 2000), although additive and synergistic effects have also been reported for the simultaneous presence of SP-C (Oosterlaken-Dijksterhuis et al., 1991a,b; Wang et al., 1996) or SP-A (Venkitaraman et al., 1990; Schürch et al., 1992; Bi et al., 2001; Possmayer et al., 2001), respectively. On the other hand, SP-B has also a potential role in maintaining the stability of highly compressed films (Nag et al., 1999; Cruz et al., 2000; Krol et al., 2000; Serrano et al., 2005) as well as in facilitating the respreading of surfactant material during expansion, so that minimum surface tension can still be attained after successive compression–expansion cycles (Oosterlaken-Dijksterhuis et al., 1991a,b; Taneva and Keough, 1994; Krol et al., 2000; Veldhuizen et al., 2000; Zaltash et al., 2000). It has been proposed that SP-B could act as a bridge between bilayers or bilayers and monolayers (Fig. 4b–e) (Zaltash et al., 2000). In this way SP-B could keep both the components squeezed-out from the interface during compression and the new material reaching the interface "de novo", in close proximity to the interface, during the time the monolayer has to support maximal pressures, just before expansion and re-spreading. That is to say that SP-B would have some participation in sustaining the surfactant reservoir, keeping attached to the interface the material needed for surface film renewal while dynamic compression–expansion cycling continues uninterruptedly. This has been a common conclusion of many different studies during the last years (Lipp et al., 1996, 1997; Nag et al., 1999; Krol et al., 2000; Perez-Gil,

2002). Actually both phenomena, the enhancement of adsorption and the facilitation of re-spreading, might be connected by a similar mechanism with regard to the SP-B action. Due to its intrinsic amphipathic structure, SP-B is rapidly adsorbed into an air–liquid interface, either open or phospholipid replenished. As well, when SP-B–phospholipid complexes are injected into an aqueous subphase, formation of lipid–protein mixed-monolayers can be detected at the air–water interface shortly after injection. The protein seems therefore to be somehow tagged to be directed to the interface, and could carry to the interface any associated structure such as surfactant membranes (Fig. 4c). Once there, SP-B would induce lipid packing perturbations in both membranes and monolayer, leading to the transfer of surface active species to the interface. The high tendency of SP-B to remain in close association with the surface film and its strong affinity to interact with surfactant lipids, will favor SP-B-promoted formation of buckled structures (reservoir) under compression, so that the squeezed-out components will not be lost in the subphase but remain in close proximity to their place of action (Fig. 4d).

Latest investigations have tried to find critical structural determinants for SP-B surface activity in order to define the minimum requirements a simple synthetic peptide should fulfill to efficiently mimic SP-B activity at the interface. A recent study designed to map essential functional motifs in the sequence of SP-B revealed that the N-terminal half of the protein, including residues 1–37, contains all the determinants necessary to promote fusion and lysis of membranes as well as to produce and sustain very low surface tensions (Ryan et al., 2005). The importance of the N-terminal region for the function of SP-B has also been suggested by other authors studying lipid–protein interactions and surface activity of a peptide comprising amino acids 1–25 of the protein, $SP\text{-}B_{1\text{–}25}$. This region mimics many of the biophysical activities of mature SP-B both in vitro (Bruni et al., 1991; Longo et al., 1993; Gordon et al., 1996; Lipp et al., 1996) and in vivo (Gupta et al., 2001). Furthermore, the study by Ryan et al. (2005) showed that the N-terminal tail (residues 1–9) of SP-B is particularly critical for the surface tension reducing properties of the protein. For instance, substitution of W9 or Pro 2, 4 and 6 resulted in significant reduction of surface activity without affecting fusogenic or lytic properties. Other results from our laboratory (Serrano et al., 2006) show that the absence of Trp 9 affects negatively the affinity of the N-terminal region for the interface. On the other hand, the substitution of Pro 2, 4 and 6 affects the secondary structure and probably the local conformation of the N-terminal region of the peptide. These results suggest that the N-terminal tail of SP-B has been evolutionarily optimized to insert rapidly into and maintain association with highly packed surface films.

An important point of discussion within the mechanisms governing the activity of SP-B at the interface is whether this protein (as well as SP-C) is involved in a possible DPPC enrichment of the surface active film under compression, and/or a supposed selective squeeze-out of non-DPPC components. Actually, the discussion is first focused on whether a DPPC refining, by whatever mechanism, does really take place during the cycling of surfactant film in vivo. According to the classical model, accepted over the years, both processes necessarily occur, assuming that the presence of a highly DPPC-enriched monolayer is absolutely required to form tightly packed films, able to achieve surface tensions close to 0 mN/m upon compression during dynamic cycling (Bangham et al., 1979; Schurch et al., 1995; Nag et al., 1999). However, recent results suggested an alternative model where none of both processes is really required (Piknova et al., 2001). This new model is based on epifluorescence studies of monolayers containing the full set of purified phospholipids (PPL) from pulmonary surfactant. These experiments gave evidence of lateral phase separation occurring in surfactant films under compression, as previously observed for single-component monolayers. However, compression of these particular surfactant films (made of PPL) to high pressures (low surface tensions) did not yield more than 30–40% of condensed phase, which corresponds approximately to the fraction of DPPC in pulmonary surfactant. So, no DPPC-enrichment seems to be necessary to get the lowest tensions upon compression of these films. The extraordinary stability required for the films of pulmonary surfactant to reach the lowest tensions would be provided by the formation of particular compression-promoted structures, probably including 2D- to 3D-structural transitions, that would be optimized by the particular lipid and protein composition of this system.

In the classical model, SP-B would enhance the formation of the surfactant reservoir, mainly composed of non-DPPC components that would be excluded together with the protein during compression. SP-B/PG interactions, for instance, have been classically considered critical for this mechanism, a sort of *selective molecular squeeze-out*. According to the revised model, SP-B would contribute to the stability of compressed surfactant states by promoting the organized formation of buckled layers of the whole film, whose association with the surface would be also maintained by lipid–protein interactions. SP-B would therefore ensure an *organized reversible structural squeeze-out*. SP-B/PG interactions

would be also important, but in a more diffused way, to provide an electrostatic component to the role of SP-B as a "connector" between surface layers.

4.2. SP-C: a sticking handle in highly compressed films

SP-C is the only really specific surfactant protein since it is the unique protein to be present only in pulmonary surfactant. It consists of a short lipopeptide with 35 amino acids including 2 palmitoylated Cys at positions 5 and 6, in most species (Haagsman and Diemel, 2001). Human SP-C is synthesized as a precursor of 197 amino acids in which the mature sequence is flanked by N- and C-terminal propeptides (Wang et al., 2002). As stated above, routing and processing of proSP-C seems to be dependent on the presence of proSPB since SP-B knock-out mice accumulate an unprocessed intermediate of proSP-C and abnormal low levels of the mature protein in lung homogenates (Clark et al., 1995; Weaver and Conkright, 2001). Unlike SP-B, SP-C is an integral membrane protein, which consists of an extremely hydrophobic transmembrane domain and a 10–12 amino acid extramembrane segment located at the N-terminus of the mature peptide (Fig. 5a). The three-dimensional structure of SP-C was determined by NMR in apolar solvents (Johansson et al., 1994), but at that time the N-terminus tail appeared as a "flexible disordered" segment. More recently the study of the N-terminal segment of the protein has been addressed using synthetic peptides based in the sequence of this region of the lipopeptide (Plasencia et al., 2001, 2004, 2005; Bi et al., 2002). These peptides from the N-terminal tail of SP-C seem to adopt an amphipathic conformation, with strong tendency to partition into the interface of phospholipid membranes and monolayers, even in the absence of palmitoylated cysteines (Plasencia et al., 2004, 2005). The interaction of this region with membranes produces perturbations of the lipid packing, with consequences in membrane permeability. It is therefore proposed that the N-terminal segment of SP-C would be the most dynamic part of the molecule and the main motif responsible for the activity of SP-C to promote interfacial adsorption of phospholipids from bilayers into the air–liquid interface (Fig. 5b and c). In this regard, SP-C seems to have an activity which is partly redundant, although with lower efficiency, with that of SP-B. The role of palmitoylation for the activity of SP-C has been outlined by studying the behaviour of palmitoylated and non-palmitoylated versions of peptides from the N-terminal segment of SP-C in lipid/protein monolayers subjected to compression/expansion dynamics, by infrared spectroscopy (Bi et al., 2002). It was determined that palmitoylation was required for the peptides to maintain association with phospholipid films compressed to the highest pressures (the lowest surface tensions). These results are the basis for a model that proposes that SP-C, principally via its palmitoylated N-terminal segment, would be strictly required to maintain the association of surfactant complexes (the reservoir) with the interfacial film at the most compressed states, those reached at the end of expiration (Fig. 5d and e). Structural studies of compressed

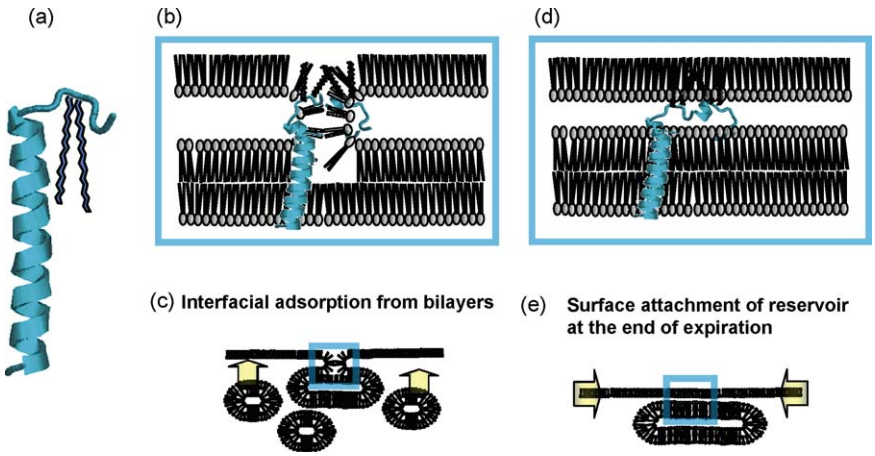

Fig. 5. Structure, disposition and activities of pulmonary surfactant protein SP-C. (a) Model of the structure of surfactant protein SP-C according to the three-dimensional structure of the protein determined in organic solvents. (b) Possible disposition of SP-C in bilayer–monolayer complexes, showing how the N-terminal palmitoylated segment of the protein could be responsible for inducing lipid packing perturbations associated with (c) promoting interfacial phospholipid adsorption from surfactant suspensions. (d) The palmitoylated segment of SP-C would maintain association of lipid–protein complexes with highly compressed interfacial films and (e) stabilizing the interface at the end of expiration during successive breathing cycles.

phospholipid/protein films containing SP-C revealed that the protein really promotes formation of attached membrane patches during compression (von Nahmen et al., 1997; Kramer et al., 2000; Malcharek et al., 2005; Wang et al., 2005). SP-C-promoted attachment would then facilitate the ulterior reinsertion of surface active molecules from the reservoirs, with the probable critical participation of SP-B, during re-expansion. According with this model, animal models in which the expression of the SP-C gene has been knocked-out develop chronic respiratory diseases as a result of intrinsic instability of the alveolar spaces (Glasser et al., 2001, 2003). SP-C is a relatively modern protein in evolution. The presence of SP-B-related proteins has been reported in the surface active material of most air-breathing animals including nonmammalian vertebrates such as amphibians (Miller et al., 2001), reptiles (Johnston et al., 2002) or birds (Zeng et al., 1998; Bernhard et al., 2001). In contrast, SP-C has been only detected in mammals, and the expression of the SP-C gene can be strictly correlated with the ontogenetic differentiation of the mammalian lung tissue (Wert et al., 1993; Besnard et al., 2005). The presence of SP-C may therefore have come up as a solution to sustain the extremely low surface tensions required to stabilize the very efficient but delicate structure of the mammalian alveoli.

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