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Molecular and Functional Properties of Surfactant Protein A

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I. Introduction

Surfactant protein A (SP-A) is a large oligomeric surfactant apolipoprotein primarily found in the alveolar fluid of mammalians. SP-A belongs to the "collectin" (collagen–lectin) family characterized by an N-terminal collagen-like domain

and a globular C-terminal domain that includes a C-type carbohydrate recognition domain (CRD). Collectins bind to a wide range of sugar residues that are rich in microbial surfaces in a Ca^{2+}-dependent manner. The collectin family has five well-characterized members: lung surfactant protein A (SP-A) and D (SP-D), serum mannose binding protein (MBP), serum bovine conglutinin, and collectin-43 (1). Recently, another novel human collectin from liver (CL-L1) has been cloned (2). Together with the first component of the complement (C1q), these proteins are also called defense collagens, and play important roles in innate immunity (1).

Substantial evidence indicates that SP-A is involved in innate host-defense and inflammatory immunomodulator processes of the lung (3–5). Unlike other collectins, SP-A is a lipid binding protein, a property that allows this collectin to position and concentrate along with the extracellular membranes that line the alveolar epithelium.Thus, SP-A is tightly associated with surfactant membranes and enriched in lattice-like arrays of intersecting membranes, characteristic of the alveolar fluid, called tubular myelin. In fact, SP-A is necessary for the formation of tubular myelin. These structures do not disrupt surface activity but optimize the surface properties of lung surfactant. This ability of SP-A to bind lipids is of relevance in several aspects of pulmonary surfactant biology (5,6).

The primary structure of mature SP-A is highly conserved among different mammalians with some important differences. It consists of four structural domains (Fig. 3.1): (1) an N-terminal segment (7–10 amino acids) involved in intermolecular disulfide bond formation; (2) a 79 residue collagen-like domain characterized by 23 Gly-X-Y repeats with an interruption near the midpoint of the domain; (3) a 35 aminoacid segment with high α-helical propensity, which constitutes the neck region between the collagen and the globular domain; and (4) a 115 residue C-terminal globular domain involved in lipid binding and also in Ca^{2+}-dependent binding of oligosaccharides. This domain contains two conserved tryptophan residues (located at positions 191 and 213) and a glycosylation site (located at residue Asn^{187}). SP-A is modified after translation (cleavage of the signal peptide, proline hydroxylation, and N-linked glycosylation) and assembled into a complex oligomeric structure that resembles a flower bouquet. In one of the initial steps of the assembly of SP-A, trimers of SP-A are built up by the association of three polypeptide chains, the collagen regions of which intertwine to form a collagen triple-helix that is stabilized by interchain disulfide bonds. In the final stage of the assembly, the octadecamers appear to be formed by lateral association of the N-terminal half of six triple-helical stems (7,8) (Fig. 3.2). Like SP-A, MBP and C1q are assembled into hexamers of trimers whereas SP-D and conglutinin form cruciform-shaped oligomers of four trimers (1).

In humans, there are two functional genes (*SP-A1* and *SP-A2*) (9) corresponding to two different SP-A cDNA sequences (10); however, in other mammalian species studied, except baboons (11), there is only one. The nucleotide sequence differences between the two human genes that result in amino acid

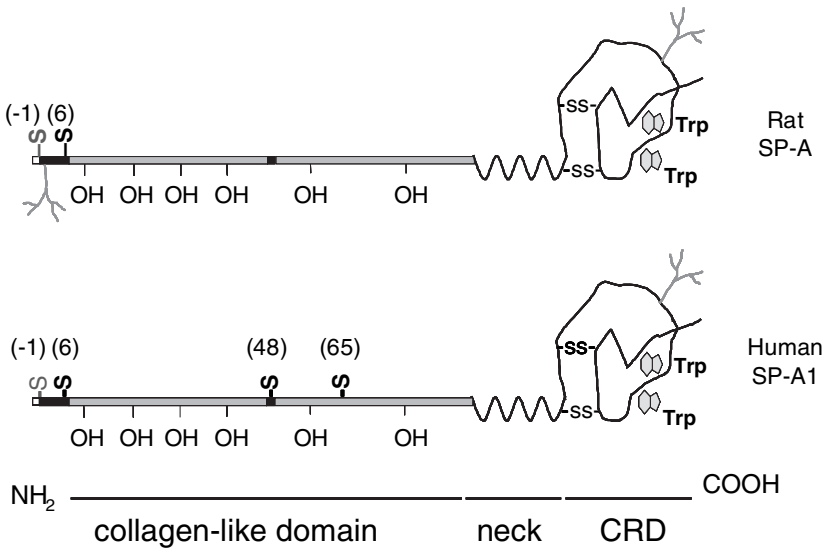

Figure 3.1 Domain structures of the polypeptide chain of human SP-A1 and rat SP-A. Branched structures represent N-linked carbohydrates. [-S] denotes cytein residues at different positions of mature hSP-A1 and rat SP-A. The collagen regions below and above the triple-helix interruption are shown. [OH] represents hydroxylation of praline residues at the Y position of G-X-Y tripeptide units.

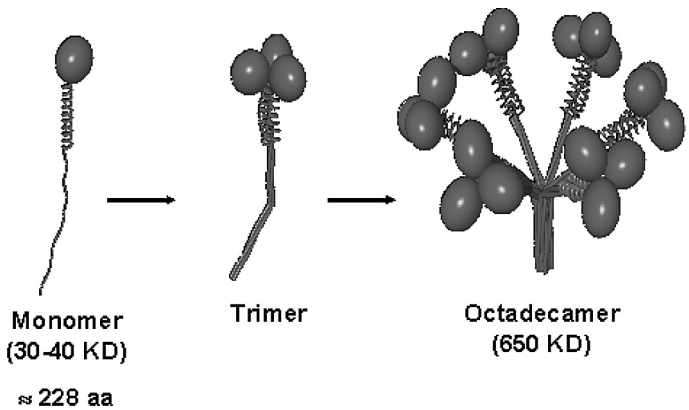

Figure 3.2 Three-dimensional model of SP-A monomer, trimer, and octadecamer. Oligomerization is an intracellular process that ocurrs in a zipper-like fashion along the C-terminal to N-terminal axis. Triple-helix formation from separated polypeptide chains requires previous trimerization of C-terminal globular domains, likely by atrimeric α-helical coiled-coil structure. Octadecamers appear to be formed by lateral association of the N-terminal half of six triple-helical stems, forming a micorfibrillar end piece stabilized by disulfide bonds. Q1

changes are located in the signal peptide, collagen-like, and globular domains of the resulting proteins (12,13). Interestingly, although both genes are expressed in lung alveolar type II cells, the *SP-A2* gene is expressed primarily (if not exclusively) in tracheal and bronchial submucosal gland cells (14–16). Octadecameric oligomers of human SP-A isolated from the bronchoalveolar lavage may be hetero-oligomers of both SP-A1 and SP-A2 homotrimers; alternatively, Voss et al. (17) have postulated that human SP-A may consist of homo-oligomers of heterotrimers composed of two SP-A1 molecules and one SP-A2 molecule. Whether the two gene products are expressed in a 2:1 (SP-A1/SP-A2) ratio and actually form heterotrimeric structures remains to be defined. The functional importance of having two distinct chain types in human SP-A is also unknown.

The present chapter will focus on the structural aspects of SP-A from human and experimental animals and the role of structural domains of SP-A in the binding of this protein to surfactant membranes, microbes, and alveolar and inflammatory cells present in the alveolar fluid. The binding capabilities of SP-A are involved in its putative biological functions: (a) improvement of surfactant biophysical function and integrity, (b) defense against alveolar pathogens, and (c) immunomodulation of the inflammatory response (Fig. 3.3). F3

II. Structure/Function Relationship

A. Domains Required for Oligomeric Assembly

The domains of SP-A that are essential for trimerization are the collagen-like region and the neck domain, which likely forms a rigid α-helical coiled-coil

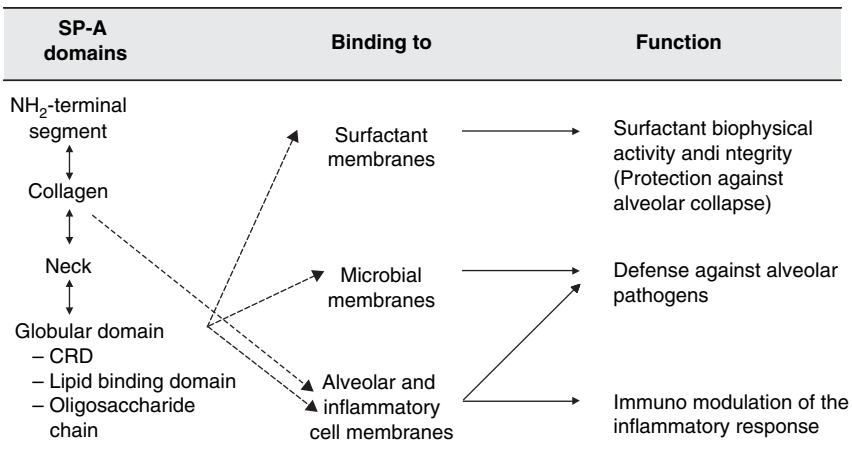

Figure 3.3 Relationships among structural domains, binding capabilities, and potential biological functions of SP-A.

(Figs. 3.1 and 3.2). Interchain disulfide cross-linkage at the N-terminal region stabilizes this structure. SP-A is assembled as multimers of trimeric subunits (Fig. 3.2). The N-terminal region is involved in covalent interactions between triple-helix stems to form higher oligomers. Researchers believe that SP-A oligomerization occurs in a zipper-like fashion along the C-terminal to N-terminal axis (18) as occurs with other collectins (SP-D and MBP-C) (19,20). Thus, the triple-helix formation from separate polypeptide chains requires previous trimerization of C-terminal globular domains, likely by a trimeric α-helical coiled-coil. In turn, triple-helix formation aligns the polypeptide chains for disulfide bond formation at the N-terminal segment, and, in the case of human SP-A, at the collagen interruption (Fig. 3.1).

Scientists assume but have not yet shown that the linking region between the collagen-like domain and the globular domain form a rigid coiled-coil structure. An α-helical coiled-coil can be predicted from the amino acid sequence by its characteristic heptad repeat pattern a-b-c-d-e-f-g-d, where residues "a" and "d" are hydrophobic aminoacids. Figure 3.4 shows alignment of the potential coiled-coil region of human SP-A from different species. For comparison, human SP-D is also shown. The X-ray crystallographic data for SP-D (21) demonstrated the existence of a coiled-coil organization in the neck domain as it was found for MBP (22). In the SP-A neck fragment, most of the residues in "a" and "d" of the four heptad repeat are hydrophobic (Leu, Val, Ile, Met, Ala, and Phe) or amphipathic (Gln, Tyr), although there are some departures from this role (i.e., hydrophilic residues such as Thr, Ser, and His) as occurs in MBP (22). There are two highly conserved positively charged residues (His^{95} and Lys^{96} at positions "e" and "f", respectively) and one negatively charged residue (Glu^{93} at position "c") in this otherwise very hydrophobic region. Hydrophobic amino acids at every turn of the helix form the interior of the coiled-coil and stabilize this rigid structure. Between the staggered

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AHLDEE	LQSA LHE	IRHQ ILQ	SMGV LSF	QEFM LAV	G	Pig
ASLDEE	LQTT LHD	LRHQ ILQ	TMGV LSL	HESL LVV	G	Canine
AYLDEE	LQAT LHE	LRHH ALQ	SIGV LSL	QGSM KAV	G	Rabbit
AYLDEE	LQTE LYE	IKHQ ILQ	TMGV LSL	QGSM LSV	G	Rat
AYLDEE	LQTA SYE	IKHQ ILQ	TMGV LSL	QGSM LSV	G	Mouse
81	87	94	101	108	115	
AHLDEE	LQAT LHD	FRHQ ILQ	TRGA LSL	QGSI MTV	G	hSP-A
	d efg a bc	d efg a bc	d efg a bc	d efg a bc		
	204					
	VASLRQQ	VEALQGQ	VQHLQAA	FSQYKKV	ELFP	hSP-D
	a d	a d	a d	a d		

Figure 3.4 Sequences of the neck region of SP-A from different species. Human SP-D sequence is also shown. Most of the residues in "a" and "d" position of the heptad repeat are hydrophobic.

triple-helix of the collagen portion and the coiled-coil structure, in which the three polypeptide chains are in register, there is a highly conserved sequence with three contiguous negatively charged residues (Asp^{84}-Glu^{85}-Glu^{86}). This short region between the collagen and coiled-coil regions cannot be aligned in perfect register. The potential function of this very acidic region, besides serving as an adapter between the aligned and the nonaligned regions of SP-A trimer, has not been examined.

Oligomerization in SP-A and other collectins seems to be needed for many of their functions. Although most SP-A interactions with ligands occur in the globular domain, the binding affinity depends on the oligomeric status of SP-A. The binding affinity of a single SP-A lectin domain for carbohydrates is very low. However, the greater multiplicity of lectin domains found in higher-order oligomers and self-aggregated forms of SP-A is required to give high-affinity binding to carbohydrate-bearing surfaces (1,3,4). In addition, the degree of SP-A oligomerization and stability of the collagen domain is correlated with lipid-related functional capabilities of SP-A (23,24).

In relation to the functions of the collagen domain of SP-A, it is clear that its high tensile strength, stability, and relative resistance to proteolysis make this domain perfect as a cross-linker between globular domains and the N-terminal segment. However, the collagen-like domain functions as scaffolding that amplifies the ligand binding activities of globular domains. Table 3.1 shows structural and functional properties of SP-A related to a structurally intact collagen-like domain (23–36). T1

No mutation in SP-A associated with a respiratory pathology has yet been identified. Interestingly, an association has been found between a mutation in the collagen-like region of MBP and low levels of MBP in serum, which results in an infantile illness characterized by recurrent infections and failure to thrive (37,38). In the bronchoalveolar lavage from patients with birch pollen allergy, SP-A exists not only in fully assembled complexes of octadecamers as in healthy individuals, but also in smaller oligomeric forms (e.g., dodecameric, nonameric, or hexameric) (39).

B. The Globular Domain

The C-terminal globular domain is involved in the binding of SP-A to calcium, carbohydrates, and phospholipids, and is critical for host defense, inmunomodulation of the inflammatory response, and surfactant-related functions. This region contains $\sim$115 amino acids, including four conserved cysteines that form two intramolecular disulfide loops (Cys^{204}-Cys^{218} and Cys^{135}-Cys^{226}), and 18 highly conserved amino acid residues common to the C-type lectines (40). The three-dimensional structure of SP-A is not known, but the X-ray crystallographic structures of rat and human MBP fragments (22) and human SP-D fragments (21), as well as those from four other C-type lectins, are useful models for SP-A [see Ref. (22,41,42) for reviews].

Table 3.1 Requirement of a Structurally Intact Collagen-Like Domain for Structural and Functional Properties of SP-A

Structure	*In vitro* activities of SP-A		Potential *in vivo* functions of SP-A
Structural stability at physiological temperatures (human, dog, pig SP-A) (23–26)	• Tubular myelin formation (mouse SP-A) (27,28) • Prevention of surfactant inactivation by serum (mouse SP-A) (27,28)	→	Surfactant biophysical activity and integrity (protection against alveolar colapse)
↓			
Oligomerization (human and rat SP-A) (23,24,29)	• High affinity for lipid and carbohydrate binding (rat SP-A) (29,30) • Aggregation of bacterial lipopolysaccharide (human SP-A) (23,24) • Binding to the C1qRp (CD93) receptor in phagocytic cells (human SP-A) (31)	→	Host-defense binding to pathogen surface membranes and endotoxins
↓			
Ca^{2+}-dependent self-aggregation (pig, human SP-A) (23–25)	• Phagocytosis of Mycobacterium through SPR210 (human SP-A) (32) • Upregulation of Mannose receptor in alveolar macrophages (human and rat SP-A) (33) • Stimulation of chemotaxis in alveolar macrophages and neutrophils (human SP-A) (34,35) • Inhibition of lymphocyte proliferation and IL-2 secretion through SPAR210 (human, bovine and rat SP-A) (36)	→	Host-defense binding to receptors on cell membranes
	• Type II cell receptor binding (rat SP-A) (29,30) • Specific inhibition of lipid secretion by type II cells (rat SP-A) (29,30) • Lipid up-take by type II cells (rat SP-A) (29,30)	→	Regulation of type II cell function

The basic structure of the globular domain consists of a structural core made up of α-helical and β-strands. MBP and SP-D modeling predicts that one important structural domain is a hydrophobic cluster containing the conserved residues Phe-178, Tyr-188, Trp-191, Pro-196, Trp-213, and Val-205 (SP-A numbering). These aminoacids hold together the carbohydrate/calcium binding region (CRD). MBP modeling predicts that one of the two Ca^{2+} binding sites (named site 2) is located in the center of the sugar binding site. The binding of sugar involves hydrogen bonding and Van der Waal's interactions, and it is stabilized by coordination bonds to the calcium ion. MBP modeling also predicts that the SP-A residues Glu195, Glu202, Asn214, and Asp215 are responsible for those interactions. The two tryptophans (Trp-191 and Trp-213) are located near the calcium binding site 2 and are sensitive markers of conformational changes in this region. Using the fluorescent apolar probe *bis*-ANS, we recently found that hydrophobic sites in SP-A increase upon addition of calcium, indicating that the binding of calcium to the protein leads to a conformational change in the protein, which makes it more hydrophobic (Casals and García-Verdugo, unpublished data). This conclusion is confirmed by intrinsic fluorescence studies of human SP-A, in which the tryptophan fluorescence emission maximum of SP-A is blue-shifted upon addition of calcium (23,24). This conformational shift enhances lipid binding and allows carbohydrate binding, protein self-association, and SP-A-mediated lipid aggregation.

Carbohydrate Binding and Specificity

The collectins show preference either for D-hexoses with an equatorial orientation of the 3- and 4-hydroxyl groups (such as mannose, glucose, *N*-acetylglucosamine, or mannosamide) or for L-fucose with a similar arrangement of hydroxyl groups at positions 2 and 3 (40,43). Sequence analysis of C-type CRDs in comparison with monosaccharide specificity indicates that C-type lectins can be divided into two groups according to a three-residue motif in the CRD (carbohydrate/calcium binding region): (1) mannose/glucose-binding C-type lectins that contain a highly conserved sequence (Glu-Pro-Asn) in their CRDs that bind mannose/glucose. All collectins, except for SP-A, contain the Glu-Pro-Asn motif. In SP-A this sequence is Glu^{195}-Pro^{196}-Ala/Arg^{197}, where Ala^{197} is present in humans and Arg^{197} in other mammalians. (2) Galactose-binding C-type lectins contain the sequence Gln-Pro-Asp in their CRDs (40).

SP-A binds preferentially to mannose and fucose (44). These sugars are commonly found on fungal and micrococcal surfaces. Discrepancy has been reported on the affinity of SP-A for the galactose residue. It has been demonstrated that SP-A binds to galactose by affinity chromatography (44), but not by inhibition of SP-A binding to solid-phase mannan by specific sugars (45). On the other hand, SP-A binds galactosylceramide coated on a solid support (46). Galactosylceramide is a common glycolipid asymmetrically located in the extracellular face of mammalian cell membranes. Site-directed mutagenesis

of the CRD of rat SP-A indicated that substitution of *Glu*[195]-Pro[196]- *Arg*[197] by *Gln*[195]-Pro[196]-*Asp*[197] changed the specificity of SP-A from mannose to galactose. Curiously, the latter mutations inhibited the capability of SP-A to aggregate phospholipid vesicles in the presence of calcium but not the ability of SP-A to bind dipalmitoylphosphatidylcholine (DPPC) (47). Alanine mutations of residues within the calcium/carbohydrate coordination set blocked SP-A binding to phospholipids (48). Monoclonal antibodies against the CRD domain containing these residues also abrogated the binding of SP-A to phospholipids (49). These studies are consistent with the location of the major lipid binding site(s) of SP-A to the globular lectin C-terminal domain and indicate that the critical region for carbohydrate binding and the lipid binding domain might overlap. Recent studies using transmission electron microscopy confirm that the globular region of SP-A is responsible for interaction with lipid vesicles (50). Whether the carbohydrate binding region in SP-A interacts directly with the phosphocholine moiety of DPPC is still not known. However, the binding of SP-A to DPPC is Ca^{2+}-independent (51–53), and is not reversed or prevented by adding sugars (54) or galacotosylcermide (unpublished data) in the presence of calcium. In contrast, MBP and SP-D interact with phosphatidylinositol (PI) and glycosphingolipids through a lectin-mediated binding (55,56).

Lipid Ligands for SP-A and the Nature of SP-A/Lipid Interaction

SP-A interacts with a broad range of insoluble amphipathic lipids present in surfactant and cellular membranes or bacterial envelopes (6). Several studies indicated that SP-A preferentially binds to phospholipids whose headgroups are phosphocholine [phosphatidylcholine (PC) or sphingomyelin (SM)] and whose lipid moiety consists of long and saturated hydrocarbon chains. Both DPPC and SM fulfill these requirements (52,53,57). Several studies indicated that the binding of SP-A to DPPC vesicles is independent of Ca^{2+} but dependent on the physical state of the vesicle (52,53). SP-A interacts in a Ca^{2+}-independent manner with the interfacial region of saturated PC bilayers in the gel or ripple phase, which is characterized by a specific conformation of the phosphocholine moiety.

It remains questionable whether hydrophobic interactions occur between the aminoacid side chains of the protein and the phospholipid acyl chains in the bilayer. Several lines of evidence indicate the involvement of hydrophobic binding forces in the interaction of SP-A with DPPC vesicles or DPPC monolayers (52,58–61). It is reasonable to think that hydrophobic interactions of SP-A with DPPC-rich bilayers can only be explained if SP-A partly penetrates into the membrane interface due to the existence of lipid packing defects. We recently found partial solubilization of surfactant membranes in Triton X-100, suggesting that liquid ordered (Lo) and liquid disordered (fluid) (Lα) domains coexist in these membranes. Lipid analysis of detergent resistant membranes (DRMs) or triton-insoluble floating fractions (TIFFs) indicated that they were enriched in cholesterol and DPPC (C. Casals, unpublished data).

DRMs (or TIFFs) seem to function as platforms for the attachment of SP-A to surfactant membranes because SP-A was absolutely segregated in DRMs or TIFFS (C. Casals, unpublished data). Fluid and liquid ordered phase coexistence in surfactant membranes could favor partition of SP-A into those membranes. Interestingly, SP-A also interacts with the gel-like regions in monolayers of pulmonary surfactant lipid extracts (62) and causes a reorganization or rearrangement of solid domains in the surfactant monolayer. It is noteworthy that SP-A in the subphase only associates with the DPPC monolayer when gel-like domains begin to appear upon compression and liquid expanded (fluid) and liquid condensed (gel) domains coexist (59). Under these conditions, SP-A interacts with the monolayer in packing defects at fluid–gel boundaries (59). These results are consistent with the concept that SP-A recognizes the lipid in the gel phase but can only penetrate into the membrane interface in lipid packing defects at liquid disordered-liquid ordered boundaries. At a surface pressure of 10 mN/m (plateau region, in which there is phase coexistence), SP-A in the subphase is able to perturb the lipid packing of DPPC monolayers at neutral pH in the absence of Ca^{2+} (59,60). Globular domains of SP-A (comprising lipid binding domains) must interact with acyl chains of phospholipid monolayers sufficiently to perturb the usual lipid packing. It was recently demonstrated that SP-A induces a decrease in the average acyl chain tilt angle of DPPC monolayers (at a surface pressure of 10 mN/m) from 35° to 28° (61). This indicates that SP-A increases lipid packing efficiency and that hydrophobic interactions must be involved.

In contrast, SP-A binds poorly to neutral or acidic phospholipid vesicles in the fluid phase, and detection of binding requires the presence of Ca^{2+} (53,63). The Ca^{2+}-dependent binding of immobilized SP-A to negatively charged phospholipid vesicles shows a preference for PI over phosphatidylglycerol (PG) (63). Similarly to SP-D or MBP, it is possible that the Ca^{2+}-dependent binding of SP-A to PI vesicles involves the CRD site. However, the inhibition of SP-A binding to PI by sugars has not been studied.

Lipomannan and mannosylated lipoarabidomannan, two major mycobacterial cell-wall lipoglycans, are also ligands for SP-A (64,65). The binding of SP-A to lipoglycans from the mycobacterial envelope seems to be dependent on Ca^{2+} (64). Both the terminal mannose residues and the fatty acids of lipoglycans are critical for binding. However, SP-A-lipoglycan interaction involves the CRD of SP-A. The carbohydrate binding site of SP-A seems to recognize the terminal mannosyl epitopes of lipoglycans from supramolecular assemblies of lipoglycan in solution. The lipid moiety of the lipoglycan seems to be necessary for the formation of those supramolecular assemblies. This supramolecular organization of these amphipathic molecules in solution might allow a repetitive and ordered presentation of terminal mannosyl epitopes, increasing recognition by the multiple CRDs of SP-A (65).

On the other hand, there are contradictory results about the Ca^{2+}-dependence of the binding of SP-A to rough lipopolysacchride (rough LPS) via lipid A (66–68) and to glycosphingolipids (46,69). It is also not clear whether

SP-A interacts with these lipids through a lectin-mediated binding. Our recent data indicated that SP-A is able to bind rough LPS in solution or rough LPS monolayers in a Ca^{2+}-independent manner (unpublished data).

III. SP-A Functions

A. Surfactant-Related Functions

Table 3.2 (51,53,54,58,70–82) summarizes *in vitro* lipid-related activities of SP-A and potential functions of SP-A in the integrity and biophysical activity of surfactant. The recent availability of SP-A knockout mice allows assessment of these functions. Evidence derived from SP-A knockout mice supports the concept that (1) SP-A does not directly contribute to surface properties of pulmonary surfactant, but the interaction of SP-A with surfactant membranes aids to maintain optimal surface activity in response to alterations in the alveolar microenviroment (83). *In vitro* experiments with surfactant isolated from transgenic mice that overexpress SP-A (78) or from SP-A knockout mice (27,83) corroborate that SP-A enhances the resistance of surfactant to protein inhibition. (2) SP-A is necessary for the formation of tubular myelin, a unique structure of surfactant in the alveolar spaces, whose presence has been correlated with high surface activity but is not absolutely required for breathing (27,83). (3) *In vivo* experiments from SP-A-deficient mice do not support a critical role of SP-A in surfactant homeostasis by controlling the secretion and uptake by alveolar cells (27,83). It is possible that some compensatory mechanism may function in the absence of SP-A.

Table 3.2 Surfactant-Related Functions of SP-A

"*In vitro*" surfactant-related activities of SP-A	Surfactant-related functions of SP-A
Induces Ca^{2+}-dependent aggregation of lipid vesicles with or without SP-B or SP-C (51,53,54,58,70,71)	Promotion of surfactant biophysical activity
Enhances adsorption of phospholipids along the air/liquid interface in a concerted action with SP-B (72,73)	
Mediates the formation of large ordered tubular myelin, when added to DPPC, PG, and SP-B mixtures in the presence of Ca^{2+} (74–76)	
Reduces inhibition of surfactant activity by foreign lipid binding proteins or serum lipoproteins (27,77,78)	Prevention of surfactant inactivation
Inhibits conversion of large (active) to small (inactive) surfactant aggregates (79)	
Enhances surfactant uptake into type II cells (80) and alveolar macrophages (81)	Surfactant homeostasis
Inhibits surfactant secretion by type II cells (82)	

The mechanism of stabilization and protection of surfactant mediated by SP-A is not known. One of the most interesting effects of SP-A on surfactant-like phospholipid vesicles is its ability to induce rapid aggregation of these vesicles with or without surfactant hydrophobic proteins SP-B and SP-C (51,53,54,58,70,71). This process is dependent on calcium, and predicts the surface active properties of the protein in concerted action with SP-B (72,73).

The mechanism involved in the vesicle aggregation phenomenon is poorly understood. It was suggested that the process of lipid aggregation mediated by SP-A could be correlated with that of self-association of the protein (74). Recent evidence indicates that vesicle aggregation and SP-A self-association might be related phenomena:

1. The calcium activation constant ($K_a^{Ca^{2+}}$) for both processes is similar. It is in the micromolar range in the presence of physiological saline (0.74 ± 0.2 and 2.4 ± 0.5 μM, for SP-A-induced lipid aggregation and protein self-association, respectively) (25,54).
2. The extent of SP-A-mediated lipid aggregation depends on proline hydroxylation in the collagen domain and the degree of SP-A oligomerization (23,24). Likewise, the ability of SP-A to self-associate depends on the stability of the collagen-like domain, which is correlated to proline hydroxylation and the degree of oligomerization (23,24). In addition, self-association activity of human or porcine SP-A is completely inhibited by unfolding of the collagen-like domain (24,25). SP-A self-association depends on calcium, and Ca^{2+} induces a conformational change on the globular domain of the protein identified by intrinsic fluorescence (23,24). Thus, it is possible that SP-A–SP-A association occurs among globular heads. A structurally intact collagen domain would ensure the grouping and orientation of globular heads in the oligomer.
3. Tubular myelin or multilamellar vesicles from native surfactant contain arrays of SP-A (76,85). Those structures seem to remain intact when the lipid is partially removed with acetone (76,85), and their spacing is comparable to the size of SP-A. These results suggest that interconnected SP-A molecules form the skeleton of these multilamellar structures or tubular myelin.

Figure 3.5 illustrates self-associated SP-A molecules connecting surfactant membranes by interaction of their globular heads with membrane surfaces of contiguous bilayers. The SP-A protein network interacting with DPPC monolayers is visible by transmission dectron microscopy (86) and fluorescence microscopy (59,60). This type of supraquaternary organization of SP-A and cooperative interaction with surfactant membranes could stabilize large surfactant aggregates, decrease surfactant inactivation in the presence of serum protein inhibitors, and, more importantly, prevent adherence of endotoxin or bacteria to the alveolar epithelium. Interestingly, Palaniyar et al. (87) showed that

F5

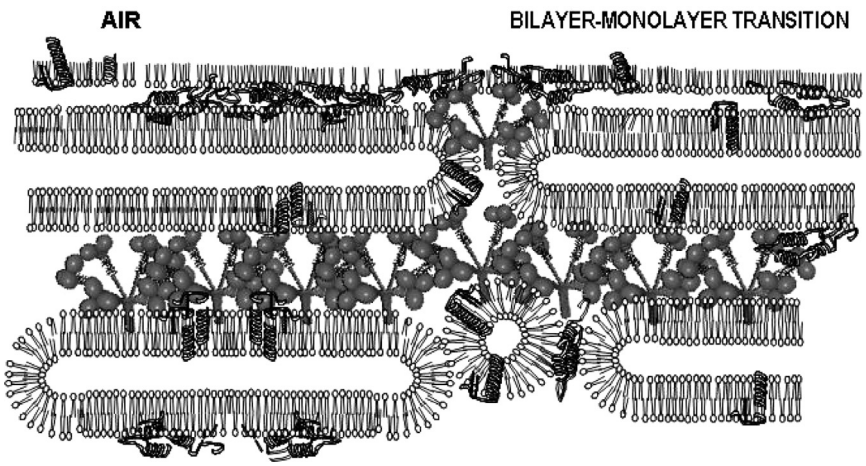

Figure 3.5 Model of the interaction of self-associated SP-A with surfactant membranes containing the hydrophobic surfactant proteins SP-B and SP-C.

recombinant rat SP-A with a deletion of the collagen-like domain failed to form protein networks and interacted with lipid monolayers in an unorganized manner. The collagen-like region and/or full oligomeric assembly of SP-A actually play an important role in the accommodation of SP-A in the alveolar fluid, because *in vivo* experiments demonstrate that the collagen-deficient mutant converts SP-A into an inhibitor of surfactant function (27). Deletion of the collagen-like domain also disrupts tubular myelin formation (27).

Figure 3.6 shows a schematic model of SP-A/SP-B-dependent tubular myelin structure. The mechanism involved in the formation of tubular myelin is poorly understood. However, it is likely that the formation of these complex structures requires (1) close contacts between opposing DPPC-rich membranes mediated by SP-A; (2) SP-A self-association mediated by calcium; and (3) fusion of membranes mediated by SP-B and facilitated by nonbilayer lipids such as unsaturated-PG-Ca^{2+} (these cone-shaped lipids are likely present in the corners of tubular myelin structures). F6

The functional significance of these complex structures is not known. Interestingly, tubular myelin-rich fraction is the most active fraction of all surfactant subfractions assayed *in vitro* (88), and morphological studies indicate that tubular myelin figures are in close proximity to the surface layer of the alveolar fluid. These structures seem to function as a membrane reservoir in the alveolar fluid in which SP-A is highly concentrated at the interface in a configuration that does not disrupt but optimizes the biophysical activity of surfactant lipids. Because of the high concentration of SP-A in these membrane traps close to the surface layer, McCormack and Whitsett (5) suggested that tubular myelin could have a primary antimicrobial function. Tubular myelin may function as

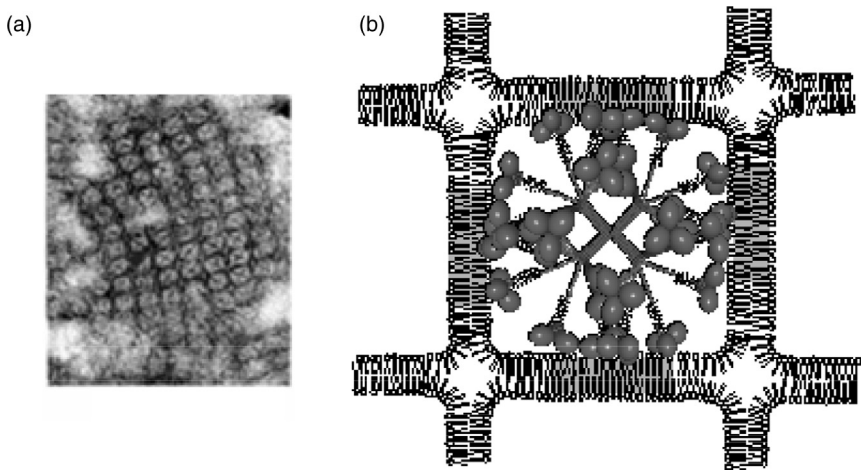

Figure 3.6 Tubular Myelin (TM). (a) Typical electron microscopy (EM) micrograph of tubular myelin. The EM image shows X-shaped structures (probably protein) in the square-lattice regions. [From Nag et al. (76) with permission]. (b) Scheme of TM: the globular heads of self-associated SP-A likely interact with DPPC-rich bilayers. Unsaturated PG-Ca^{2+} and SP-B (not shown) are likely present in the corners of TM. They probably make possible nonbilayer structures at the membrane intersection.

an extracellular surfactant reservoir that serves to collect inhaled microbes at the air–liquid interface due to the high concentration of SP-A in these structures. It seems possible that surfactant membranes and their apolipoproteins simultaneously function as the primary antimicrobial defense in the alveolar fluid and as a protective layer against alveolar collapse.

B. Host-Defense and Immunomodulation of the Inflammatory Response in the Alveolus

SP-A binds to a variety of nonself molecular structures including allergens, lipopolysaccharides, and other components of bacteria, viral, and fungi surfaces. This binding neutralizes, agglutinates, and/or enhances the uptake of pathogens by phagocytes of the innate immune system such as alveolar macrophages and neutrophils. Moreover, SP-A is capable of direct interaction with immune cells through binding to the cell membrane receptors resulting in modulation of immune cell functions such as phagocytosis, chemotaxis, proliferation, cytokine production, respiratory burst, and expression of surface receptors (Fig. 3.7). F7

Binding of SP-A to Pathogen Surfaces

SP-A recognizes complex arrays of polysaccharides and other glycoconjugates, including polysaccharide constituents of capsules, Gram-negative (GN)

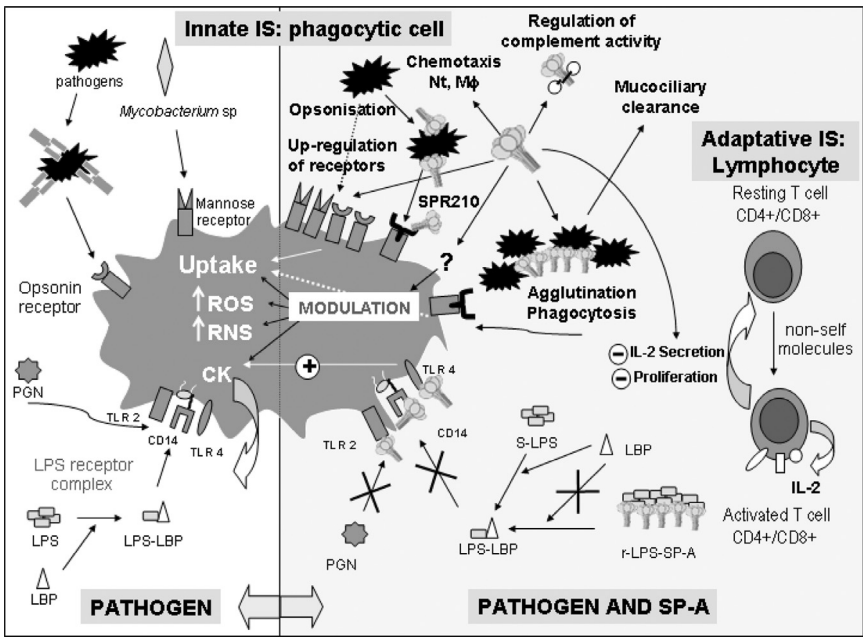

Figure 3.7 Role of SP-A in innate immunity. CK, cytokine; IS, immune system; LPS, smooth or rough lipopolysaccharide; LBP, LPS binding protein; Mϕ, macrophage; Nt, neutrophil; PGN, peptidoglycan; r-LPS, rough LPS; s-LPS, smooth LPS; ROS, reactive oxygen species; RNS, reactive nitrogen species; SP-AR, SP-A receptor; TLR, Toll-like receptor.

lipopolysaccharides, lipoglycans, and glycoproteins that are present in pathogen surfaces (4). The C-terminal globular domain of SP-A seems to be responsible for these interactions. As we discussed earlier, this domain is involved in the binding of SP-A to lipids, Ca^{2+}, and carbohydrates. The globular domain also contains a conserver Asn187 which is posttranslational glycosylated (Fig. 3.1). Glycosylation in Asn187 is important in the binding of SP-A to certain viruses.

Table 3.3 shows different mechanisms of SP-A interaction with pathogen surfaces (65,66,89–99). The binding of SP-A to influenza virus A involves the sialic acid residues on Asn^{187}-linked oligosaccharide moiety of SP-A (90,91). Deglycosylation of SP-A or enzymatic digestion to remove only sialic acid residues inhibits the binding of SP-A to influenza virus A, whereas mannan, which binds to the CRD of SP-A, has no effect (91). In contrast, SP-A binds to cytomegalovirus (CMV) proteins in a Ca^{2+}-dependent manner. In addition, the binding of SP-A to CMV proteins is inhibited by mannan, suggesting that interaction between SP-A and CMV proteins involves the carbohydrate recognition activity of SP-A (92). Participation of the CRD–lectin activity of SP-A has also been

Table 3.3 Interaction of the C-Terminal Domain of SP-A with Pathogens

Structural motif in SP-A	Structural motif in the pathogen surface	Pathogen type
Asn^{187}-linked carbohydrates	Lectin	Herpes Simplex virus 1 (89) Q2 Influenza A virus (90,91)
Lectin domain	Envelope glycoproteins	Cytomegalovirus (92)
	G,F-Glycoproteins	Respir. Syncitial Virus (93,94)
	Major surface glycoprotein	*Pneumocystis carinii* (95,96)
	Capsular polysaccharide	*Klebsiella pneumoniae* (97)
	Mannosylated lipoarabinomannan	*Mycobacterium sp.* (65) *Aspergillus fumigatus* (98)
	Lipomannan	
	Glycoproteins (gp45, 55)	
Lipid binding domain	Lipopolysaccharide (lipid A)	Gram-negative bacteria (66)
Undefined	Peptidoglycan (?)	Gram-positive bacteria (99)

reported in the interaction of SP-A with lipoglycans of mycobacteria (65) and with the major surface glycoprotein of *Pneumocystis carinii* (95,96) (4).

The interaction of SP-A with GN and Gram-positive (GP) bacteria is not fully understood. Some authors have suggested that SP-A may recognize peptidoglycan or lipoteichoic acid from the GP cell wall but convincing results have not been published yet (99,100). SP-A has been described as binding to rough but not to smooth LPS from GN strains (66,67). Bacteria with rough LPS phenotypes are most common among species that colonize the surfaces of the respiratory tract (101). Binding studies in the presence of mannan or deglycosylated SP-A indicated that neither the carbohydrate binding region nor the carbohydrate moiety of SP-A are involved in its binding to rough LPS (66,68). It is likely that SP-A binds to the lipid A moiety of rough LPS by the lipid binding domain of SP-A instead of through a lectin-mediated binding (66). Interaction of SP-A with rough LPS seems to interfere with the subsequent binding of rough LPS to LPS binding protein (LBP) (102). LBP binds to the lipid A domain of LPS, catalyzes the binding of LPS to CD14, and enhances CD14-mediated cell activation. The complex CD14/TLR4/MD2 leads to stimulation of cells via induction of NF-κB (103). The presence of SP-A results in significant inhibition of NF-κB activation in alveolar macrophages stimulated with rough LPS (102) (Fig. 3.7).

Once SP-A has recognized the nonself structure in the pathogen surface, different mechanisms are involved in the neutralization and clearance of pathogens. SP-A is able either to opsonize nonself structures for disposal by immune cells (104,97) or to agglutinate various microorganisms, including bacteria, fungi, and viruses (104–106). Agglutination facilitates the mechanical removal of bacteria from the lungs by mucociliary clearance and also increases the phagocytosis of bacteria by alveolar macrophages (107) (Fig. 3.7).

Interaction of SP-A with Immune Cell Membranes

SP-A may have three modes of binding to immune cells: (a) through a lectin-mediated event to glycoproteins present in the surface of monocytes and macrophages (108,109); (b) through the N-linked carbohydrate on the C-terminal domain of SP-A, which binds to a lectin present in the plasma membrane of alveolar macrophages (110); and (c) through the collagen-like domain of SP-A, which binds to a protein receptor on the surface of alveolar macrophages (111,112) (Fig. 3.7).

Several cell surface proteins that bind SP-A have been identified (Table 3.4) (31,32,36,67,100,113–115). However, the specific contributions of these molecules to the biological activities of SP-A remain unclear. T4

SPR210

Chroneos et al. (114) described a specific receptor of 210 kDa, named SPR210, that binds to SP-A with high affinity in a Ca^{2+}-dependent manner but independent of the carbohydrate binding activity of SP-A. The collagen-like domain of SP-A has been suggested as the putative domain that interacts with SPR210 (36,114). This receptor mediates SP-A-induced inhibition of phospholipid

Table 3.4 Potential SP-A Receptors in Cells Present in the Alveolar Fluid

SP-A domain	Receptor	Expression in alveolar cells	SP-A function
nd	Calreticulin/ CD91	Macrophages[a]	Clearance of apoptotic cells (113)
Collagen-like	C1qRp (CD93)	Monocyte/ macrophages Type II cells (murine)	Phagocytosis (31)
Collagen-like	SPA receptor (SPR210)	Lymphocytes Macrophages (Mϕ) Type II neumocytes	Inhibition of T cell proliferation and IL-2 production (36) Enhanced uptake of BCG by Mϕ (32) Inhibition of phospholipid secretion (114)
Neck	CD14 m	Macrophages[a]	Modulation of LPS response (67)
nd	Toll-like 4	Macrophages[a]	Activation of macrophages (115)
nd	Toll-like 2	Macrophages[a]	Inhibition of cytokine-PGN induced response (100)

[a]Expressed in more cell types; nd, not determinated; BCG, bacillus Calmette–Guerin; PGN, peptidoglycan

secretion by type II cells (114), SP-A-induced inhibition of T-cell proliferation (36), and SP-A-enhanced uptake of bacillus Calmette–Guérin by macrophages (32).

C1qRp (CD93)

C1q receptors (C1qR) trigger effects on a wide range of immune cells. C1qRp has recently been identified as the leukocyte antigen CD93 and is expressed in human endothelial cells, monocytes, and immature dendritic cells (116). It has been suggested that SP-A, MBL, and C1q directly bind to C1qRp through their collagen-like domains and enhance phagocytosis (31). Recent studies suggest that C1qRp is involved primarily in adhesion events rather than C1q-mediated phagocytosis (117). The role of C1qRp in phagocytosis requires further studies.

CD14

CD14 is a 53 kDa GPI-anchored protein that also exists as a soluble form. Nowadays, CD14 stands as a major receptor for various bacterial components, and is considered as a pattern-recognition receptor (103). SP-A directly interacts with CD14 via its neck domain (118). The binding of SP-A to CD14 might prevent the binding of smooth LPS to CD14 (67). This would explain the inhibition mediated by SP-A of TNF-alpha release from rat alveolar macrophages stimulated with smooth LPS (119), because SP-A poorly binds to smooth LPS. SP-A also reduced the cytokine release from human alveolar macrophages (120) and human buffy coat cells (121) stimulated with smooth LPS. Consistent with these data, *in vivo* experiments showed that SP-A-deficient mice intratracheally challenged with smooth *Escherichia coli* 026:B6 LPS produce significantly more tumor necrosis factor TNF-alpha than the wild-type mice (122). In contrast, Bufler et al. (123) recently reported that SP-A has no major effects on the response of a macrophage cell line to smooth and rough *Pseudomonas aeruginosa* strains.

Toll-Like Receptors

SP-A also interacts with toll-like receptors (TLR). TLR are pattern recognition receptors that participate in signaling a variety of microbial infections (103). The interaction of SP-A with the extracellular domain of TLR2 inhibits peptidoglycan-mediated response (100). On the other hand, SP-A from alveolar proteinosis patients seems to interact with TLR4 complex, inducing activation of the NF-kB pathway and up-regulation of cytokine synthesis (115). However, the detailed mechanism by which SP-A interacts with TLR4 receptor requires further studies. Phelps and co-workers also demonstrated that human SP-A from alveolar proteinosis patients and recombinant human SP-A stimulate TNF-alpha secretion by THP-1 cells (124,125). The precise mechanism of the interaction of SP-A with this monocytic cell line has not been described yet.

Beside cytokine modulation, SP-A itself can modulate other functions in monocytes/macropahges such as reactive oxygen (ROS) and nitrogen species

(RNS) production. Both ROS and RNS are involved in antibacterial and antiviral defense. However, these reactive species, as well as cytokines, have the potential to exacerbate an inflammatory response if their levels are not tightly regulated. Alveolar macrophages incubated with SP-A have a decrease in superoxide production, indicating a dampening of the respiratory burst and suggesting a protective role against the oxidant injury caused by alveolar macrophages in the lung (126,127). Others, however, have found SP-A to stimulate the respiratory burst (128). The reasons for these different findings are not completely understood but may be related to different methods used to purify SP-A (128). On the other hand, SP-A seems to enhance the production of nitric oxide by alveolar macrophages (129) although removal of endotoxin from SP-A preparation reverses this effect (130). In contrast, SP-A with low endotoxin level has been shown to enhance the production of nitric oxide metabolites by alveolar macrophages activated with IFN-gamma and challenged *in vitro* with *Mycoplasma pneumoniae* (131). *In vivo* studies using SP-A-deficient mice indicate that these mice produce more nitric oxide upon intratracheal challenge of *P. aeruginosa* or LPS (122,132). The response to SP-A seems to vary with the pathogen challenge, the state of cell activation (133), cell source (134), and SP-A nature (135). SP-A domains involved in the regulation of the cited inflammatory mediators are unknown and requires further studies.

Finally, there are different studies that support an activating ligand role of SP-A. SP-A enhances phagocytosis of IgG or complement-coated sheep erythrocytes, presumably due to up-regulation of Fc and CR1 receptors by SP-A (136). The treatment of human monocytic cell line THP-1 with SP-A leads to a significant increase of the expression of CD14, ICAM1, and CD11b (137). Pretreatment of macrophages with SP-A stimulates phagocytosis of *M. tuberculosis* probably by up-regulation of mannose receptor (108). More recently, it was shown that SP-A increases the surface expression of functional mannose receptor on macrophages, as demonstrated by both flow cytometry and confocal microscopy (33). Using recombinant mutants of rat SP-A, these authors demonstrate a critical role for both the CRD and the collagen-like region of SP-A in mediating up-regulation of mannose receptor.

IV. Concluding Remarks

Lung SP-A is part of the naturally occurring innate immune system which provides an immediate defense against a wide range of lung pathogens (viruses, bacteria, and fungi). The high affinity of SP-A to surfactant membranes allows the concentration of this protein in the alveolar fluid. Levels of SP-A have been reported to fall during infections and lung inflammation. Therefore, the use of recombinant forms of human SP-A together with surfactant lipids may alleviate the need for administration of antibiotics and/or anti-inflammatory drugs, especially in the very young and in the immunocompromised adults. One of

the open questions in surfactant molecular biology is why there are two functional genes (SP-A1 and SP-A2) in humans, corresponding to two different SP-A cDNA sequences. A complete understanding of the structure and function of human SP-A will allow the production of recombinant SP-A to be used in human therapies.

Acknowledgment

We thank Dr. M. L. F. Ruano for drawing the three-dimensional model of SP-A monomer, trimer, and octadecamer. This work was supported by PB98-0769-C02-01 from CICYT and QLK2-CT-2000-00325 from the European Community.

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