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ROLE OF SURFACTANT PROTEIN A (SP-A)/LIPID INTERACTIONS FOR SP-A FUNCTIONS IN THE LUNG

Cristina Casals, PhD □ *Department of Biochemistry and Molecular Biology, School of Biology, Complutense University, Madrid, Spain*

□ *Surfactant protein A (SP-A), an oligomeric glycoprotein, is a member of a group of proteins named collectins that contain collagen-like and Ca^{2+}-dependent carbohydrate recognition domains. SP-A interacts with a broad range of amphipathic lipids (glycerophospholipids, sphingophospholipids, glycosphingolipids, lipid A, and lipoglycans) that are present in surfactant or microbial membranes. This review summarizes SP-A/lipid interaction studies regarding the lipid system used (i.e., phospholipid vesicles, phospholipid monolayers, and lipids immobilized on silica or adsorbed on a solid support). The effect of calcium, ionic strength, and pH on the binding of SP-A to lipids and the subsequent lipid aggregation process is discussed. Current evidence suggests that hydrophobic-binding forces are involved in the peripherical association of SP-A to membranes. It is also proposed that fluid and liquid-ordered phase coexistence in surfactant membranes might favor partition of SP-A into those membranes. The binding of SP-A to surfactant membranes containing hydrophobic surfactant peptides makes possible the formation of a membrane reservoir in the alveolar fluid that is protected by SP-A against inactivation and improves the rate of surfactant film formation. In addition, the interaction of SP-A with membranes might enhance the affinity of SP-A for terminal carbohydrates of glycolipids or glycoproteins on the surface of invading microorganisms.*

Keywords membranes, phospholipids, pulmonary surfactant, SP-A, surfactant monolayer, vesicle aggregation

Surfactant protein A (SP-A) belongs to the collectin class of C-type lectins along with surfactant protein D (SP-D), mannose-binding protein (MBP), collectin-43 (CL-43), and conglutinin [1]. Collectins play important roles in innate immunity. They bind oligosaccharides on the surface of a wide range of microorganisms, including fungi, bacteria, and virus. Their roles initially appeared to be mainly as opsonins and agglutinins, but they now are considered to have wider actions in host defense.

Collectins are all large, extended oligomeric proteins composed of C-type lectin domains attached to collagen regions via α-helical coiled-coil neck

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Address correspondence to Cristina Casals, PhD, Dept. of Biochemistry and Molecular Biology, School of Biology, Complutense University of Madrid, 28040 Madrid, Spain. E-mail: casals@solea.quim.ucm.es

regions. MBP, CL-43, and conglutinin are serum proteins produced by liver whereas SP-A and SP-D are primarily found in the lung [2, 3]. SP-A and SP-D are synthesized mainly by alveolar type II cells and secreted to the alveolar surface. Thus, these lectins should be active in a surfactant lipid-rich environment. Unlike SP-D, SP-A is intimately associated with surfactant phospholipids in the alveolar space. Thus, the distribution of SP-A and SP-D in bronchoalveolar lavage (BAL) fluid is quite different. Fully 90% of the total SP-A recovered by BAL is distributed in the sedimentable form of surfactant, associated with large surfactant aggregates enriched in tubular myelin, a membrane lattice found in the alveolar spaces [4, 5]. In contrast, 70% of the total SP-D from BAL is not sedimented by prolonged high-speed centrifugation [5]. Mutagenesis studies are consistent with the location of the major lipid binding site(s) of SP-A to the globular lectin C-terminal domain [6, 7]. Recent studies using transmission electron microscopy confirm that the SP-A head region is responsible for interaction with lipid vesicles [8]. The regions critical for carbohydrate binding and lipid binding do not appear identical but do show overlap [6]. Whether these carbohydrate-binding regions in SP-A interact directly with the lipid is still not known. SP-D, in contrast, interacts with phosphatidylinositol (PI) and glycosphingolipids through a lectin-mediated binding [9, 10]. Likewise, MBP binds to phosphatidylserine (PS), PI, and phosphatidylcholine (PC) through its Ca^{2+}-carbohydrate-binding site. EDTA and monosaccharides inhibit MBP-lipid binding [11, 12]. The significance of lipid binding of collectins can only be conjectured, but collectins might recognize either phospholipid headgroups or carbohydrates on microbial surfaces. C-reactive protein (CRP), another Ca^{2+}-dependent lectin with a role in innate immunology, has high affinity for the headgroup of PC as does SP-A. However, while CRP inhibits surfactant biophysical activity [13, 14], SP-A enhances it and protects surfactant against inactivation by CRP or other serum proteins.

My review summarizes our knowledge about the nature and characteristics of lipid–SP-A interactions that seem to be different from other collectins. For a detailed discussion of the structure of SP-A and protein determinants for lipid binding, the reader is referred to the article by Francis McCormack also in this issue.

POTENTIAL FUNCTIONS OF SP-A RELATED WITH ITS LIPID-BINDING PROPERTIES

Table 1 [15–36] summarizes in vitro activities of SP-A related with its ability to bind lipids and potential functions of SP-A in the lung as a lipid-binding protein. The recent availability of SP-A knockout mice allows re-examination of those SP-A potential functions.

TABLE 1. Activities and Potential Functions of SP-A in the Lung Related to its Lipid-Binding Properties (with References)

In Vitro Activities SP-A	Potential Functions of SP-A
Induces pH-dependent aggregation of acidic but not neutral phospholipid vesicles with or without SP-B or SP-C [15, 16].	Tight Bilayer Packing in Secretory/Endocytic Vesicles
Induces Ca^{2+}-dependent aggregation of lipid vesicles with or without SP-B or SP-C [16–21].	
Mediates transfer and exchange of phospholipids between the outer monolayers of membranes [22].	
Enhances adsorption of phospholipids along the air/liquid interface in a concerted action with SP-B [23, 24].	
Stabilizes the surfactant monolayer at low surface pressures and enhances elimination of non-DPPC lipids under compression [24].	Promotion of Surfactant Biophysical activity
Associates and perturbs pure DPPC monolayers at neutral pH but not at acidic pH [25, 26].	
Increases the phospholipid mixing activity of SP-B in the presence of Ca^{2+} or H^{+} [27].	
Mediates the formation of large ordered tubular myelin, when added to DPPC, PG and SP-B mixtures in the presence of Ca^{2+} [28, 29].	
Inhibits conversion of large (active) to small (inactive) surfactant aggregates [30].	Prevention of surfactant inactivation
Reduces inhibition of surfactant activity by foreign lipid binding proteins or serum lipoproteins [31].	
Enhances surfactant uptake into isolated type II cells [32].	Surfactant homeostasis
Mediates binding and aggregation of rough LPS [33, 34].	
Induces binding and aggregation of Gram-negative bacteria via interaction with lipid A [33, 35].	Host defense
Binds to mycobacteria via recognition of both carbohydrate epitopes and acyl residues of lipoglycans from mycobacterial envelope [36].	

Studies in SP-A–deficient mice have shown that SP-A is not absolutely required for breathing indicating that unlike SP-B, SP-A is not directly responsible for surface tension lowering properties of pulmonary surfactant [37]. However, the possibility that SP-A may participate in redundant regulatory mechanisms to increase the efficiency of surfactant biophysical activity cannot be ruled out. In fact, SP-A is necessary for the formation of tubular myelin [28, 29], a unique structure of surfactant in the alveolar spaces, whose presence can be correlated with high surface activity. SP-A-deficient surfactant isolated from SP-A knockout mice has impaired properties compared with normal SP-A-containing surfactant when assayed under limited concentration conditions [37]. This fact suggests that SP-A could improve surface activity of surfactant under certain circumstances such as pathologically limited availability of surfactant or the presence of inhibitory compounds in the airways.

Lungs from SP-A–deficient mice appear to have normal levels of SP-B, SP-C, and SP-D mRNA and protein [37]; normal surfactant phospholipid composition [37]; and normal mechanisms for secretion and clearance of PC [38]. This finding indicates that SP-A is not an important regulator of surfactant phospholipid metabolism in vivo under steady state conditions where compensatory mechanisms may function in the absence of SP-A.

The susceptibility of SP-A–deficient surfactant isolated from SP-A knockout mice to protein inhibition assayed in vitro [37] confirms previous conclusions that SP-A is involved protecting surfactant from protein inhibition. Recent in vivo studies from transgenic mice that overexpress SP-A [39] corroborates that SP-A enhances the resistance of surfactant to protein inhibition but does not affect resting surfactant phospholipid levels. Interestingly, lungs of SP-A–deficient mice have markedly decreased tubular myelin figures [37]. Tubular myelin structures seem unessential for the surface activity of pulmonary surfactant. However, it may fulfill a role in vivo as an extracellular surfactant reservoir protected by SP-A against inactivation.

The concept that SP-A plays an important role in innate immunity, protecting the lung from microbial infection and injury, comes from the finding that SP-A–deficient mice show an increased susceptibility to group B streptococcal and also to Pneumocystis and Pseudomonas infections [40]. SP-A binds to pathogens either through a lectin-mediated binding [41] or through interaction with the lipid moiety of bacterial lipopolysaccharide [33]. Alternatively, SP-A can recognize both the carbohydrate epitopes of lipoglycans from mycobacterial envelopes and acyl residues esterifying the glycerol moiety of lipoglycans [36]. On the other hand, the interaction of SP-A with surfactant membranes might induce some conformational changes in SP-A, enhancing the affinity of SP-A for the terminal mannose residues of carbohydrates on the surface of invading microorganisms.

CHARACTERISTICS OF LIPID–SP-A INTERACTIONS

Lipid-Binding Studies

Numerous biochemical and biophysical methods have been used to elucidate the various aspects of the interaction of SP-A with membrane lipids (Table 2) [4, 8, 15–22, 25, 26, 28, 29, 42-53]. Studies reported in the literature differ not only in the choice of the lipid system (bilayers, monolayers, lipids immobilized on silica or adsorbed on a solid support), but also in the state of SP-A in the stock solutions and other experimental conditions such as temperature, amounts of calcium, pH, and ionic strength that influence both protein structure [54] and properties of bilayers and monolayers [55].

The physical state of phospholipids or glycolipids in a silica matrix or a microtiter well is not understood. However, it is conceivable that lipids form aggregates, such as monolayers or sheets of monolayers. Phospholipids such as dipalmitoyl phosphatidyl choline (DPPC) or sphingomyelin (SM), that have saturated acyl chains and a uniform shape, may form higher order aggregates on a silica matrix than unsaturated PC, phosphatidylethanolamine (PE), or phosphatidylglycerol (PG)-Ca^{2+}. The application of these solid phase binding

TABLE 2. SP-A–Lipid Binding Studies

Lipid System	Method	References
Bilayers (phospholipids)	Centrifugation	[42, 43]
	Density gradient centrifugation	[16, 17]
	Intrinsic and extrinsic fluorescence	[19, 22]
Multilamellar vesicles	Circular dichroism	[16]
Unilamellar vesicles	Photometric aggregation	[15–21, 44–45]
	Resonant mirror spectroscopy	[44–45]
	Differential scanning calorimetry	[42]
	Infrared spectroscopy	[46]
	Transmission electron microscopy	[8, 47, 48]
	Electron microscopy	[4, 28, 29]
Monolayers (phospholipids)	Dynamic surface balance	[49]
	Epifluorescence microscopy	[25, 26]
	Transmission electron microscopy	[50]
Immobilized on silica by thin layer chromatography (TLC) (phospholipids and glycolipids)	Direct binding of ^{125}I-SP-A to lipid spot on TLC	[51–53]
Coated on a solid support	Solid phase binding assays (phospholipids, glycolipids, lipopolisaccharide, or lipoglycans were added to microtiter wells in ethanol)	[34, 36, 52, 53]

assays to study the binding of SP-A to lipids allows us to state the interaction of SP-A with a specific lipid structure in defined experimental conditions but does not give information about the nature and character of the interaction.

The physical state of phospholipids in model membranes, such as multilamellar vesicles (MLV), small (SUV), or large (LUV) unilamellar vesicles, depends not only on the chemical structure of the lipid (type of acyl chains, and size, hydration and/or charges in the headgroup), but also on dynamics of rotational and translational motions as well as motions of the acyl chains [55]. A characteristic property of lipid vesicles is their ability to undergo phase transitions that can be induced by a number of factors, such as temperature, ions, or pH. Lipid vesicles cannot be regarded as rigid structures but rather as highly dynamic entities whose molecular organization is susceptible to change [55]. For instance, they are osmotically active: they shrink or swell with changes in osmolarity. Therefore, ionic strength of the milieu in which lipid vesicles reside is a factor that should be controlled. In lipid vesicles, osmotic shrinkage and swelling leads to increase and decrease, respectively, of the lipid lateral packing pressure. Lipid vesicles are adequate lipid systems for studying membrane/SP-A interactions. The binding of SP-A to lipid vesicles in the presence of Ca^{2+} leads to a rapid aggregation of those vesicles [16–21]. Vesicle binding and aggregation are closely coupled processes, although they appear to be different phenomena [16, 56].

Experiments with lipid monolayers residing on an air/water interface allow us to study exclusively the process of SP-A/phospholipid binding but not the process of vesicle aggregation. Lipid monolayers show liquid expanded (LE) to liquid condensed (LC) transitions on compression. These phases are often considered equivalent to liquid-crystalline ($L\alpha$) and crystal (L_β) phase in bilayers, although they are not completely so. Experiments with lipid monolayers allow determination of the lipid lateral packing pressure (π) that normally influences the binding of peripherical membrane proteins to films [57]. Compression isotherms also provide information on the effect of SP-A on lateral phase transitions indicating whether protein/phospholipid monolayer interactions are sufficient to perturb the usual lipid packing [25, 49]. Furthermore, the use of fluorescently labeled proteins allows analysis of the preferential interaction of a given protein with LE or LC domains of the monolayer [25, 26].

Lipid Ligands for SP-A

SP-A interacts with a broad range of insoluble amphipathic lipids present in surfactant membranes or bacterial envelopes (Table 3). Some lipid binding studies suggest that SP-A binds to both the polar group and acyl chains of amphipathic lipids (phospholipids, glycolipids, or lipoglycans). Direct binding of ^{125}I–SP-A to phospholipids immobilized on silica [51] indicated that SP-A

TABLE 3. SP-A–Lipid Binding at Neutral pH

Lipid Ligands for SP-A	Ca^{2+} Requirement with References
Diacyl-glycerophospholipids:	
DPPC (bilayers or monolayers)	No [17, 19, 25, 42, 45]
(immobilized on silica)	Yes [51]
DPPC/other unsaturated PLs (bilayers)	Yes [43, 44]
PC, PI, OG (bilayers) (low binding)	Yes [44, 45]
Sphingophospholipids	
Sphingomyelin (immobilized on silica)	Yes [51]
(bilayers)	Yes [45]
Glycosphingolipids	
Galactosylceramide and Asialo-GM_2	No [52]
(immobilized on silica or coated on a solid support)	Yes [53]
Rough lipopolisaccharide or lipid A from gram-negative bacteria (coated on a solid support and forming aggregates in solution)	Yes [33, 34] No [59]
Lipoglycans from mycobacteria envelope (coated on a solid support)	Yes [36]

specifically binds phospholipids whose headgroups are phosphocholine (PC or SM) and whose lipid moiety consists in long and saturated hydrocarbon chains. Both DPPC and SM fulfill these requirements. It is curious, however, that when binding experiments are carried out with unilamellar vesicles, kinetic measurements indicate that the protein does not exhibit high specificity but still a preference for either the polar group or the hydrophobic moiety of phospholipids [19, 45]. The interaction of SP-A with DPPC is more pronounced than with other phospholipids such as dipalmitoyl (DP)PG (with the same acyl chains and temperature phase transition (Tm) but different headgroup) or egg-PC (with the same polar headgroup but different acyl chains and Tm). SP-A seems to show preference for interacting with the specific headgroup/backbone conformation of high-order DPPC vesicles at 37°C [19] but also interacts, with reduced strength, with PC, PI, PG, or PE vesicles in the presence of Ca^{2+} [45]. The preferential interaction of SP-A with DPPC over DPPG is supported by epifluorescence microscopy studies of fluorescent Texas Red-labeled SP-A adsorbed to monolayers of either DPPC or DPPC/DPPG. In these experiments, SP-A is first injected in the subphase, at neutral pH and physiological ionic strength but in the absence of Ca^{2+} ions, and then the monolayer is formed. Fluorescent SP-A associates with LE regions of DPPC monolayers and accumulates in packing defects at

boundaries between LE and LC domains under conditions of phase coexistence [25]. In DPPC/DPPG monolayers, SP-A is effectively excluded from LE regions, accumulating in discrete patches at the LE–LC boundaries [25]. This immiscibility has been explained as resulting from electrostatic repulsion between negatively charged phospholipids and the surface charge on the protein contributed by carboxyl groups [19, 49].

On the other hand, solid phase binding assays suggest that the interaction of the hydrophobic lipid moiety of both glycosphingolipids and mycobacterial lipoglycans with the lipid-binding domain of SP-A appears to ensure the anchoring of the saccharide moieties to the lectin domains of SP-A [36, 52, 53]. Lectin domains of SP-A have a relatively broad specificity with measurable binding to mannose, fucose, glucose, and galactose, which is Ca^{2+}-dependent. Interestingly, there is no evidence that SP-A can bind carbohydrates in the absence of a carrier molecule such as a lipid or a protein [53]. It is possible that the interaction of acyl chains with a potential hydrophobic cavity or groove in the globular heads of the protein may induce some conformational changes in the carbohydrate recognition domain (CRD) that enhances the affinity of SP-A for saccharide moieties. The interaction of DPPC or DPPC/DPPG vesicles with SP-A causes conformational changes in the globular head of the protein that increases its intrinsic fluorescence [19] and decreases its accessibility to trypsin degradation [21].

Influence of Ca^{2+} on Lipid Binding

While there is no question about the Ca^{2+} dependence of the phospholipid vesicle aggregation activity of SP-A, there are contradictory reports about the Ca^{2+} dependence of lipid binding (Table 3). Kuroki and Akino [51] reported that the specific binding of rat ^{125}I-SP-A to DPPC adsorbed to silica gel is Ca^{2+}-dependent. However, a recent study with immobilized SP-A indicated that SP-A binds to DPPC vesicles at 15°C in the presence of EDTA, unless 9% cholesterol and 1% α-tocopherol are included in the composition of the DPPC vesicle [45]. Both cholesterol and α-tocopherol increase fluidity and decrease order and lipid packing defects in gel state membranes, and α-tocopherol has a larger influence on DPPC bilayers than does cholesterol [58]. Several studies indicate that the binding of SP-A to DPPC vesicles is independent of Ca^{2+} but dependent on the physical state of the vesicle [19, 42]. King et al. [42] demonstrated by sedimentation methods that the binding of SP-A to dimyristoyl (DM)PC, DPPC, or distearoyl (DS)PC occurs in the presence of EDTA when vesicles are in the gel phase. Furthermore, the increase in intrinsic fluorescence of SP-A on addition of DPPC vesicles is much stronger when the vesicles are in the gel state than when they are in the liquid-crystalline state and this effect is independent of Ca^{2+} [19]. The Ca^{2+}-independent binding of SP-A to DPPC bilayers is also demonstrated for DPPC monolayers

[25, 49]. Calcium ions in the subphase did not alter the properties of SP-A/DPPC films, whereas they improve the ability of SP-A to mix with DPPG or DPPC/DPPG monolayers [49]. Dehydration on the binding of Ca^{2+} to DPPG could affect its effective molecular geometry.

Collectively, data indicate that SP-A interacts in a Ca^{2+}-independent manner with the interfacial region of saturated PC bilayers in the gel phase that is characterized by a specific conformation of phosphocholine moiety, glycerol backbone, ester carbonyls, and the first methylene segments. Lipid packing defects in gel phase PC bilayers would likely decrease the energy barrier for SP-A association and possible partial penetration into the bilayer. The discrepancy between results from experiments performed with DPPC vesicles [17, 19, 42, 45] and those performed with DPPC immobilized on silica gel by thin layer chromatography [51] could be due to the different experimental systems used. The detection of the binding of ^{125}I-SP-A to immobilized DPPC [51] might be enhanced in the presence of 2 mM Ca^{2+} by self-aggregation of SP-A on the lipid-binding site.

In contrast, the binding of SP-A to unsaturated PC vesicles increases in the presence of Ca^{2+} [19, 45]. These vesicles are in fluid phase. The binding of immobilized SP-A, which can bind but not aggregate phospholipid vesicles, to negatively charged phospholipid vesicles is also Ca^{2+}-dependent [45], and there is a preference for PI over PG. Similarly to SP-D or MBP, it is possible that the Ca^{2+}-dependent binding of SP-A to PI vesicles involves the carbohydrate recognition binding site. However, the inhibition of SP-A binding to PI by sugars has not been studied. On the other hand, there are contradictory results about the Ca^{2+} dependence of the binding of SP-A to both lipopolisaccharide [33, 34, 59] and glycosphingolipids [52, 53]. The binding of SP-A to lipoglycans from mycobacterial envelope seems to be dependent on Ca^{2+} [36].

Involvement of Hydrophobic-Binding Forces

Several lines of evidence indicate that the interaction of SP-A with DPPC vesicles is hydrophobic in nature [16, 25, 42]. The extent of DPPC aggregation mediated by SP-A does not decrease as the concentration of salts increases indicating a contribution of hydrophobic-binding forces [16]. Aggregation of DPPC/DPPG vesicles at micromolar concentrations of Ca^{2+} does not occur at low ionic strength because there is charge repulsion between DPPG and SP-A [16, 21]. When charge repulsion is reduced in the present of physiologic saline, then hydrophobic binding likely occurs because the extent of DPPC/DPPG aggregation mediated by SP-A does not decrease as the concentration of salts highly increases [16].

SP-A is able to perturb the lipid packing of DPPC monolayers at neutral pH in the absence of Ca^{2+}. SP-A is able to decrease the total amount of

condensed area on compression and to produce more, smaller condensed domains in monolayers of DPPC. Globular domains of SP-A (comprising lipid-binding domains) must interact with acyl chains of phospholipid monolayers sufficiently to perturb the usual lipid packing [25]. Similar perturbations in the condensation of the monolayer under compression have been described for the surfactant hydrophobic peptide SP-C [60]. However, the magnitude of the perturbation caused by SP-A appears relatively small on the basis of mass of protein but could be significant on the basis of the molar amount of the protein associated with the monolayer in comparison with a smaller protein such as SP-C. Filaments of protein aggregates, formed in the presence of Ca^{2+}, can interact with the DPPC monolayer as has been visualized by transmission electron microscopy [50].

The thermotropic properties of multilamellar vesicles of DMPC in the presence of SP-A (1:1, lipid to protein weight ratio) and in the absence of Ca^{2+} indicates that the interaction of SP-A induces some perturbation of the lipid molecular packing so that the cooperativity of the transition is reduced [42]. However, the effect of SP-A on the thermotropic behavior of DPPC/DPPG vesicles (1:1, lipid to protein weight ratio) in the absence of Ca^{2+} is small and larger proportions of protein are required to see any effect [46].

Models for Peripheral Association of SP-A with Membranes

SP-A preferentially interacts with DPPC bilayers in a gel or ripple phase. Hydrophobic interactions of SP-A with those bilayers can only be explained if SP-A partly penetrates into the membrane interface due to the existence of lipid-packing defects. It is noteworthy that SP-A in the subphase only associates with the DPPC monolayer when LC domains begin to appear on compression and LE and LC domains coexist [25]. Under these conditions, SP-A first interacts with the monolayer in packing defects at LC–LE boundaries followed by diffusion of the protein to LE regions [25]. These results are consistent with the concept that SP-A recognizes the lipid in the gel phase but can only penetrate into the membrane interfase in lipid-packing defects or in the fluid phase.

It is interesting to note that surfactant membranes cannot exist in an entirely fluid phase (L_α). Instead, they likely present DPPC-rich microdomains that must be in gel phase (L_β) or in a liquid-ordered phase (β) if DPPC is mixed with cholesterol and high-Tm lipids with saturated acyl chains such as sphingolipids [61–63]. Fluid and liquid-ordered phase coexistence in surfactant membranes could favor partition of SP-A into those membranes.

An alternative model for SP-A association with membranes would involve the accommodation of one of the acyl chains of unsaturated phospholipids in fluid phase into a proper hydrophobic cavity in the protein. The other chain

would remain within the lipid bilayer. This hydrophobic interaction does not involve penetration of the protein into the bilayer. Additional contribution from electrostatic interactions between the phospholipid headgroup and the protein surface may be further involved. This model has been suggested by Kinnunen et al. [64] for the attachment of several peripheral membrane proteins with verified hydrophobic cavities such as lipocalins [65] or lipid-transfer proteins [66]. The existence of a hydrophobic cavity in SP-A has not been explored.

Ca^{2+}-Dependent Lipid Vesicle Aggregation Mediated by SP-A and Ca^{2+}-Dependent Protein Self-Aggregation

The ability of SP-A to aggregate phospholipid vesicles has been widely studied [15–21]. However, the mechanism involved in the vesicle aggregation phenomenon is poorly understood. Phospholipid vesicle aggregation mediated by SP-A requires phospholipid binding, but the binding of phospholipids to SP-A does not always lead to vesicle aggregation [16, 56]. A mutant of SP-A with tandem mutations in the CRD (Glu195Gln, Arg197Asp) binds but does not aggregate phospholipid vesicles [56] and porcine, canine, or human SP-As binds but does not aggregate DPPC or DPPC/PC vesicles at acidic pH (16).

It was suggested that the process of lipid aggregation mediated by SP-A could be correlated with that of self-association of the protein [67]. We recently reported that both processes require micromolar concentrations of calcium in the presence of physiologic saline [21, 54]. The calcium activation constant (K_a^{Ca2+}) for the process of Ca^{2+}/Na^{+}-dependent lipid vesicle aggregation mediated by porcine SP-A is 0.74 ± 0.2 μM [21] and that for the process of Ca^{2+}/Na^{+}-induced self-aggregation of porcine SP-A is 2.4 ± 0.5 μM [54], suggesting they might be related phenomena.

SP-A self-aggregates by the collagen-like domain in the presence of Ca^{2+} [54]. Temperature-dependent experiments indicate that a structurally intact collagen-like domain is required for Ca^{2+}/Na^{+}-induced self-aggregation of the protein [54] but not for its lipid vesicle aggregation activity (unpublished results). These data suggest that native SP-A with unfolded collagen-like regions aggregates lipid vesicles in the presence of Ca^{2+} but is unable to self-aggregate. Baculovirus-expressed collagen-deletion SP-A mutants are as capable of aggregating phospholipid vesicles as wild-type SP-A, although not as effective as rat SP-A [68]. Mutagenesis studies on SP-A indicate that the N-terminal segment, interchain bridging, and CRD, but not collagen-like region, seem to be critical for the lipid aggregation function of SP-A [7]. It is not known whether lipid/protein aggregates induced by SP-A with either unfolded or deleted collagen-like domains are similar or not to those aggregates induced by SP-A, containing an intact collagen region.

Carbohydrate and lipid-binding domains of SP-A are located in the globular head of the protein. Therefore, these domains are not buried by self-aggregation of the protein because SP-A self-associates by the collagen-like domain. We suggest that both processes, protein self-aggregation and lipid vesicle aggregation mediated by SP-A, can occur simultaneously. Self-aggregation of SP-A might amplify lipid vesicle aggregation mediated by SP-A at physiologic concentrations of NaCl and Ca^{2+}.

Recent experiments with native human SP-A and human recombinant SP-A expressed either from baculovirus-mediated insect cell system or from mammalian CHO-K1 cells indicate that the efficiency of both SP-A self-aggregation and lipid vesicle aggregation mediated by SP-A depends on the thermal stability of the protein (Floros & Casals, unpublished work). Recombinant hSP-A from insect cells exhibits a lower melting temperature of the collagen-like domain compared with native or recombinant SP-A expressed from mammalian cells, which is likely a consequence of the defect in posttranslational hydroxylation of proline residues [43]. Recombinant hSP-A from insect cell systems is only able to self-aggregate and mediate lipid vesicle aggregation at 25°C but not at 37°C, and at a protein concentration much higher than that required for native hSP-A or recombinant hSP-A from mammalian cells. The quaternary structure of SP-A, which depends on the collagen-like region, the N-terminal segment, and intermolecular disulfide bonds, seems to be important for SP-A self-aggregation and its lipid aggregation function.

The physiologic importance of lipid aggregation activity of SP-A is not fully understood. Multilamellar vesicles or common tubular myelin from native surfactant contain arrays of SP-A [47]. Those multilamellar structures seem to remain intact when the lipid is partially removed with acetone [47] and their spacing is comparable with the size of SP-A (8). These results suggest that interconnected SP-A molecules form the skeleton of these multilamellar structures.

Close contacts between opposing membranes mediated by SP-A allow the exchange of phospholipids between the outer monolayers of the membranes in contact. This would permit selective exchange of lipid species between membranes [22]. However, those close contacts are not sufficient to induce fusion unless bilayers are somehow perturbed by the insertion of the amphipathic surfactant peptide SP-B [27]. Extensive membrane structural rearrangements induced by SP-A, SP-B, and calcium result in the formation of tubular myelin-like lattices [27–29]. The marked structural rearrangement of SP-B/phospholipid vesicles in the presence of SP-A and calcium is also accompanied by a significant enhancement of the rate of surface film formation. Collectively, these data indicate that SP-A affects surfactant membrane structure and dynamics suggesting that SP-A might be involved in redundant mechanisms that improve the efficiency of surfactant biophysical activity.

pH Dependence to Interaction of SP-A with Phospholipids and H^+ -Dependent Protein Self-Aggregation

The structural properties of SP-A and its interaction with phospholipids have been studied mainly at neutral pH. However, SP-A encounters a progressive decrease in pH along its exocytic/endocytic pathway (see below). SP-A is a protein rich in negatively charged amino acids with isoelectric point varying between pH 4.5 and 5.2. We found that the aggregation state of the protein changed with the progressive acidification of the medium [16]. The protein undergoes a marked self-aggregation at mildly acidic pHs, which is associated with changes in the circular dichroism spectra of the protein [16, 54]. Self-aggregation induced by H^+ is not affected by unfolding of the collagen domain [54] suggesting that this domain is not required for this type of SP-A self-aggregation. The presence of salts decreases the extent of H^+-induced self-aggregation [54] indicating that SP-A self-aggregates at acidic pH by electrostatic interactions. Protonation of carboxyl groups of SP-A, at acidic pH, would reduce the negative surface charge on the neck and CRD regions of the protein. Interestingly, when both NaCl (or KCL) and $CaCl_2$ are present in the medium, the level of self-aggregation decreases even more as the pH is reduced in the range of 4.2–6.5 [54] and self-aggregation becomes partially dependent on the collagen-like domain (unpublished results). These results suggest that Ca^{2+} and H^+ ions induce different types of protein aggregates.

It seems likely that carbohydrate and lipid-binding domains are buried in the center of a protein aggregate induced by H^+. At acidic pH, the binding of SP-A to carbohydrates is abrogated [69] as well as aggregation of DPPC or DPPC/PC vesicles induced by SP-A [16]. A regulatory element in pH-dependent self-aggregation is the interaction of SP-A with phospholipid monolayers or vesicles containing acidic phospholipids. The binding of SP-A to DPPC/DPPG, but not to DPPC vesicles, leads to a decrease in protein aggregation induced by H^+ as deduced by the effect of those vesicles on the circular dichroism spectrum of the protein [16]. This finding is consistent with the view that the adsorption of fluorescent Texas-Red labeled SP-A, which is in a self-aggregated form in the subphase at pH 4.5, to DPPC/DPPG, but not to DPPC, monolayers reverses protein self-aggregation [26]. Lipid/protein interactions seem to be favored over protein/protein interactions at acidic pH provided that vesicles [16] or monolayers [26] contain acidic phospholipids. The interaction of acidic vesicles with SP-A leads to a conformational change in the protein that affects its secondary structure and its accessibility to endoproteinase C degradation at acidic pH [16].

SP-A induces aggregation of acidic vesicles either with or without SP-B or SP-C or both proteins. However, SP-A binds, but poorly aggregates, DPPC or DPPC/PC vesicles at pH 4.5 in the presence of Ca^{2+} [16]. It is likely that

SP-A–induced vesicle aggregation requires that the protein not form large protein aggregates. This hypothesis may explain why SP-A binds to neutral vesicles but poorly aggregates them as the protein is forming large aggregates at acidic pH.

DIFFERENT INTRACELLULAR AND EXTRACELLULAR ENVIRONMENTS AND THE STRUCTURE AND LIPID-BINDING PROPERTIES OF SP-A

SP-A is both a secretory and endocytic protein. Thus, mature SP-A is always present in vesicular compartments that sequester it from the cytosol. SP-A is present in significant amounts in secretory granules of type II cells (lamellar bodies) together with surfactant lipids and hydrophobic surfactant proteins B and C [70–72]. The lamellar body not only functions as a classic secretory granule, but it also intersects with the endocytic pathway. Like the storage granules in other secretory cells, lamellar bodies have an acidic internal environment (pH 5.5) and high calcium and K^+ content [73]. There are contradictory reports about how SP-A reaches the lamellar body.

Voorhout et al. [70] reported that SP-A travels together with the precursors of the hydrophobic surfactant proteins SP-B and SP-C through the same pathway from the Golgi complex to lamellar bodies. Alternatively, in vitro [72] and in vivo [74] studies suggest that newly synthesized SP-A is secreted by a constitutive pathway to the extracellular space before entering the lamellar body by endocytosis. The progressive decrease in pH along either the exocytic pathway or the endocytic pathway prior to its accumulation in lamellar bodies influences the structure of SP-A [16, 54]. We found that the aggregation state of the protein changes with the progressive acidification of the medium. Furthermore, SP-A becomes immunologically unreactive under these conditions [75] and its lipid-binding properties change [16]. The H^+-dependent self-aggregation property of SP-A in the presence of salts and calcium, together with its ability to bind to membranes at mildly acidic pH, might be important for the sorting of SP-A to the lamellar body by the sorting-by-retention hypothesis [76].

This hypothesis indicates that the critical step for the targeting of a protein to the regulated pathway would reside in the capacity of that particular protein to aggregate on acidification. This mechanism is less effective than that dependent on a receptor. A sophisticated mode of targeting of SP-A to the regulated pathway might not be necessary because any missorted, apically secreted SP-A would end up in the lamellar body by endocytosis of surfactant components from extracellular space [72, 74]. Interestingly, in SP-B–deficient patients, lamellar bodies are rare, and lipids, SP-A, and pro-SP-C are secreted via simple vesicles at the apical and basal membranes of type II cells [77]. Surfactant secretion seems to be nondirectional in these patients. Furthermore,

SP-A and pro-SP-C might be transported through a constitutive-like, non-regulated secretory pathway that is not utilized when SP-B is present and lamellar bodies are abundant.

The presence of acidic phospholipids in lamellar bodies or endocytic vesicles might be needed for tight contacts between membranes mediated by SP-A, because SP-A aggregates acidic phospholipid vesicles but hardly aggregates neutral vesicles of DPPC or mixtures DPPC/egg PC at acidic pH [16]. In vitro studies indicate that a nearly total segregation of neutral phospholipids and SP-A might occur at acidic pH in the absence of acidic phospholipids [16, 26]. On the other hand, the packaging of newly synthesized PC in lamellar bodies is also influenced by acidic pH [78]. The PC of the lamellar body compartment is enriched with respect to DPPC when compared with PC of other membranes in type II cells [79]. It is not known how the type II cell realizes the enrichment of DPPC and the depletion of unsaturated-PC in the lamellar body. SP-A does not associate preferentially with DPPC at acidic pH [16, 26] therefore it is unlikely that SP-A may prevent equilibration of DPPC with other PC pools via phospholipid exchange by phospholipid-transfer proteins [80].

SP-A structure and SP-A/lipid interactions markedly change with the shift of pH from acidic to neutral [16], indicating that the change from the acidic lamellar body to the neutral alveolar subphase could be a factor involved in the reorganization of surfactant material after secretion. Neutral alveolar subphase contains Na^+ (150 mM) and Ca^{2+} (1.5 mM) ions [81]. Under these conditions, SP-A octadecamers are thought to be in an opened-bouquet structure [8], which means the full exposure of its 6 trimeric globular domains permitting multiple attachments of a single molecule with its ligands. Furthermore, SP-A could self-aggregate by the collagen-like domain and interact with surfactant phospholipids, carbohydrates, or glycolipids on microbial surfaces and cell receptors in a self-aggregated form, resulting in a multivalent presentation of SP-A to its ligands. The relatively broad specificity of SP-A to interact with different amphipathic ligands present in either surfactant or microbial membranes and the nature of SP-A/lipid interactions allow SP-A to fulfill its threefold roles: in innate immunity, in prevention of surfactant inactivation, and in improvement of surfactant biophysical activity.

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