The Structure and Function of Surfactant Protein-A*

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The interface between the lung and the atmosphere poses major challenges for all air-breathing organisms. The pulmonary alveolus, the gas-exchanging unit of the lung, is a delicate structure that is prone to collapse under the influence of natural intermolecular forces at the air-tissue boundary. The patency of the airspaces is maintained by an oily substance called pulmonary surfactant, which reduces surface tension within the aqueous lining layer that blankets the pulmonary epithelium. Surfactant is composed of both phospholipids and proteins, but most of the surface activity is attributable to the major phospholipid component, dipalmitoylphosphatidylcholine (DPPC). Surfactant phospholipids assemble at the air-liquid interface into a tightly packed, noncompressible monomolecular membrane with a very low surface pressure. The three surfactant proteins that are unique to the lung and intimately associated with surfactant lipids are surfactant proteins A, B, and C (SP-A, SP-B, SP-C, respectively). As a group, the surfactant proteins function in the delivery of surfactant phospholipids to the monolayer, the spreading of phospholipids at the interface, and the structure of surfactant aggregates (for review see Weaver and Whitsett). A more recently described polypeptide in the alveolar space, SP-D, has no clear role in the surface active functions of surfactant and is probably a host defense protein.

The focus of this review is the most abundant protein component of surfactant, SP-A. To more fully appreciate the role of SP-A in surfactant function, it is useful to briefly review the life cycle of surfactant in the airspace. Surfactant components are synthesized by alveolar type II cells, packaged into intracellular organelles called lamellar bodies (LB), and secreted into the alveolar lining layer. The LB unfold to form the lattice-like array, tubular myelin (TM), which requires surfactant proteins A and B for formation and/or stabilization. SP-B and SP-C, and to a lesser extent SP-A, each contribute to the rapid delivery of DPPC from TM and other surfactant aggregates to the monomolecular phospholipid membrane at the alveolar air-liquid interface. The composition of the monolayer is refined with each respiratory cycle, squeezing some surfactant components below the surface and enriching the film in DPPC. Vesicles of surfactant proteins and lipids in the subphase are internalized into type II cells and routed through the endocytic pathway to LB for rescervation.

Metabolism of surfactant lipids and proteins by type II cells and macrophages constitutes the major routes of clearance, but the mechanisms involved and the role of surfactant proteins in the process are not fully understood.

SP-A is a 26- to 38-kd (reduced molecular weight) oligomeric glycoprotein that is expressed by the Clara cells and alveolar type II cells of the distal respiratory epithelium. The deduced primary structure and amino terminal sequencing of rat SP-A reveal five discrete structural domains including the following: (1) amino terminal domain (Asn1-Ala7); (2) collagen-like sequence of Gly-X-Y repeats (Gly3-Pro60); (3) hydrophobic neck region (Gly78-Val114); (4) carbohydrate recognition domain (CRD) (Gly115-Phe228), which shares 14% invariant and 18 conserved amino acids with the C-type lectins, especially mannose binding protein (MBP); and (5) two differentially occupied consensus sites for asparagine-linked glycosylation at Asn1 and Asn197. Subunits of SP-A form trimers through the folding of their collagen-like domains into triple helices and further associate through thiol-dependent and nonthiol-dependent interactions into an octadecamer (molecular weight=approximately 700 kd), similar in organization to C1q (Fig 2).

Posttranslational modifications of SP-A include cleavage of the signal peptide, interchain disulfide linkage at the amino-terminus, proline hydroxylation in the collagen-like region, and asparagine-linked glycosylation (for review see Weaver and Whitsett).

An expanding body of data from several laboratories supports roles for SP-A in receptor-mediated regulation of surfactant secretion and uptake by alveolar type II cells, surfactant biophysical function and integrity, TM formation, and host defense. This discussion briefly reviews the role of individual structural domains of SP-A in the functions of the protein that are measurable in the laboratory. The data are derived from the work of several groups and are based on analyses of the functional consequences of biochemical modifications of native SP-A and site-specific amino acid mutations of recombinant SP-As.

**RECEPTOR BINDING AND INHIBITION OF SURFACTANT SECRETION**

The intra-alveolar levels of surfactant lipids are maintained within narrowly defined limits, and several lines of evidence indicate that SP-A acts as an autocrine regulator of surfactant homeostasis. SP-A enhances the uptake of surfactant liposomes into type II cells in vitro and in vivo, and inhibits the secretion of surfactant from isolated type II cells. These activities of SP-A are mediated through calcium-dependent binding to one or more receptors.

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Figure 1. Primary structure of rat SP-A. Solid line indicates signal peptide; broken solid line, collagen like region; dotted line, neck region; and double line, carbohydrate recognition domain. Subscripts refer to amino acid number relative to the reported amino terminus (Asn1).
The contributions of amino terminal domains of SP-A to receptor binding and regulation of surfactant secretion have also been explored. Wright et al.37 demonstrated that the collagenase-resistant fragment (CRF), composed of the neck and CRD of SP-A, binds to the type II cell receptor with lower affinity than the native molecule and is only a weak inhibitor of surfactant secretion.27 It was not possible to determine whether the loss of the collagen-like domain or perturbations in oligomeric assembly were primarily responsible for the loss of SP-A function. To attempt to differentiate between these mechanisms, a mutant recombinant form of SP-A with the collagen-like domain deleted but with an intact amino terminal domain was produced. The collagen-deletion mutant form of SP-A competed poorly with native rat SP-A for occupancy of the receptor on alveolar type II cells, but retained the ability to inhibit surfactant secretion under the same assay conditions that were employed for the CRF.35,36 The different behaviors of the collagenase-derived and recombinant collagen region-deficient SP-As may have been related to the preservation of the amino terminal interchain bonds in the latter protein. To assess the role of the interchain cross-link in SP-A/type II cell interactions, the activities of the protein were determined after substitution of Ser for Cys in the full-length molecule. The disulfide bond-deficient mutant recombinant SP-A only weakly competed for receptor occupancy and inhibited the secretion of surfactant poorly.35 Thus, it is likely that the intermolecular disulfide bond and collagen-like domains of SP-A both contribute to receptor binding and surfactant regulation by stabilizing the oligomeric form of the protein and increasing the valency for receptor/ligand interactions.

**SP-A/Lipid Interactions**

The properties of SP-A to bind and aggregate lipids contribute to the roles of SP-A in surfactant biophysical function and the structure of surfactant aggregates. SP-A exhibits specific and Ca\(^{2+}\)-dependent binding to DPPC on solid supports37 or in liposomes38 and several domains of SP-A been implicated in the interaction. Hydrophobicity plots initially suggested a potential lipid-binding domain in the neck region35 and an early study using proteolytic fragments of SP-A provided support for this notion.38 More recently, attention has focused on the CRD as the major lipid

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**Figure 2.** Domain and oligomeric structure of rat SP-A. SP-A is composed of a series of discrete structural domains. Monomers of SP-A form trimers by triple-helix formation in the collagen-like domains. The fully assembled molecule is an octadecamer, stabilized by intertrimeric and intratrameric disulfide bonds at the amino terminus. More high-affinity receptors on the surface of the type II cell.26-28 The structural domains of SP-A that interact with the receptor have been investigated recently. The CRD of SP-A was identified as an important receptor-binding domain in studies that demonstrated that epitope-specific monoclonal antibodies to the CRD of SP-A blocked receptor binding.29,30 More precise mapping of the receptor-binding site was provided by point mutations of amino acids within the putative carbohydrate-binding pocket of the CRD.31 The selection of specific amino acids as targets for mutagenesis of SP-A was based on comparisons with the calcium and carbohydrate coordinating residues of the homologous lectin, MBP, complexed with an oligosaccharide.32 The corresponding amino acids within the CRD of rat SP-A included Glu\(^{105}\), Arg\(^{197}\), Glu\(^{202}\), Asn\(^{214}\), and Asp\(^{215}\). Using a strategy adapted from studies of MBP,33 the motif within the CRD of SP-A which is known to confer mannose-binding specificity to C-type lectins (Glu-Pro-Asn or Glu-Pro-Arg), was replaced with a motif that confers galactose-binding specificity (Gln-Pro-Asp). Substitutions of residues Glu\(^{105}\) → Glu and Arg\(^{197}\) → Asp within the CRD of rat SP-A resulted in enhanced affinity for galactose and blocked type II cell receptor binding and inhibition of surfactant secretion by SP-A.31 Most recently, these studies have been extended by alanine mutagenesis of each of the charged (Glu\(^{105}\), Glu\(^{202}\), Asp\(^{215}\)) and polar (Asn\(^{214}\)) amino acids of the putative carbohydrate binding site of SP-A.34 These more subtle mutations of the CRD also markedly reduced the binding of SP-A to carbohydrate and lipid ligands. Since the targeted amino acids also coordinate binding to calcium, it is possible that alanine substitutions adversely affected divalent cation-induced conformational shifts that expose remote ligand binding sites in the CRD. Nevertheless, collectively these data provide evidence that receptor binding and regulation of surfactant secretion and uptake are coupled and are mediated by the CRD of SP-A.
interaction site. Ogasawara et al. demonstrated that a chimeric molecule composed of the CRD and the neck region of SP-A grafted onto the collagen-like region of the non-DPPC binding protein, SP-D, acquired DPPC binding properties. In a companion article, Kuroki et al. showed that an epitope-specific monoclonal antibody (ID6) that recognized a region within the CRD of SP-A (Cys304-Cys218) blocked binding to phospholipid, but a second antibody (6E3) that recognized the neck region of the molecule did not affect binding. Structural mutant forms of SP-A were used to more finely map the lipid-binding region. The tandem mutations of Glu105→Gln and Arg197→Asp, which altered carbohydrate-binding specificity of SP-A, did not significantly affect lipid binding, but alanine substitutions of selected residues within the CRD of SP-A reduced binding affinity in the rank order Asn214→Ala<Glu202→Ala<Asp215→Ala<Glu195→Ala. These results indicate that the CRD of SP-A contains a lipid-binding site, although precise localization of that site is difficult to achieve with mutagenesis studies alone. The affinity of SP-A for lipid ligands is also dependent on amino terminal domains that contribute to complete oligomeric assembly, since deletion of the amino terminal disulfide bond (Cys3→Ser) or the collagen-like region reduced binding to lipid. Collectively, these data suggest that lipid binding requires the acidic amino acids near and within the Cys3-Cys218 intramolecular disulfide loop of the CRD, and that the authentic oligomeric assembly is important for lipid binding.

SP-A aggregates phospholipid liposomes in the presence of calcium. Given the finding that the CRD contains an important lipid-binding site, it is not surprising that this region also participates in lipid aggregation. There is general concordance between amino acid substitutions of CRD, which most effectively block lipid binding (Glu105→Ala, Glu202→Ala and Asp215→Ala), and the loss of lipid aggregation properties. However, other mutations (Glu105→Gln and Arg197Asp), which leave lipid binding intact, block lipid aggregation. The explanation for the dissociation between lipid binding and aggregation activities is not clear, but it is likely that amino acid substitutions of the CRD can have conflicting effects on the calcium-dependent conformation of the domain that contribute to aggregation and the integrity of ligand-binding sites. Since SP-A has a tendency to self-aggregate, one of the possible mechanisms for lipid vesicle aggregation is a protein-protein interaction that cross-links SP-A molecules within proximate SP-A/liposome complexes. It has been proposed that asparagine-linked oligosaccharides of SP-A may function as ligands to produce aggregation, through binding to lectin domains of neighboring SP-A molecules. This hypothesis is based on the loss of aggregation properties of the human protein after glycosidase treatment. However, nonglycosylated rat SP-A molecules produced by single amino acid substitutions with the consensus sequences for glycosylation aggregated lipids and the wild-type recombinant protein. The discrepant results in these two studies may have been related to species differences or to variables introduced by the use of glycosidases in the former study. For the rat protein, our preferred interpretation is that SP-A mediates lipid vesicle aggregation through multivalent interactions of the CRD with phospholipids within adjacent liposomes.

Finally, as with lipid-binding activity, the amino terminal domains also play a role in lipid aggregation. The comparison of the aggregation activity of biochemically and genetically modified SP-A is useful for determining the contribution of the collagen-like region to liposome aggregation. Phospholipid vesicle cross-linking is inducible with a mutant recombinant form of SP-A that contains a deletion of the collagen-like region but not by the CRF. The major difference between these two proteins is the presence of the seven amino acid NH2-terminal segment and disulfide bond at Cys6 in the former construct. Intermolecular bonds have been shown previously to be important for lipid aggregation by SP-A, since partial reduction of the protein to dissociate the amino terminal disulfide linkage at Cys6 blocks aggregation. These results are consistent with observations that the substitution of Cys6→Ser in recombinant rat SP-A blocks lipid aggregation. Thus, covalent interchain linkage but not the collagen-like domain appears to be required for lipid aggregation. Collectively, the available literature indicates that (1) the CRD of SP-A mediates lipid aggregation, (2) the lipid binding and lipid aggregation domains of SP-A may overlap, but they are not identical, and (3) disulfide-dependent oligomeric assembly but not the collagen-like domain of SP-A is required for lipid aggregation.

**SP-A, Macrophages, and Host Defense**

Accumulating evidence suggests that SP-A mediates interactions between microorganisms and macrophages in the alveolar space. This role for SP-A was initially suggested by sequence homology with MBP, which has been shown to act as an opsonin by binding to mannose-rich oligosaccharides which decorate microorganisms. The group of collagen-lectin hybrid molecules with putative roles in innate immunity has expanded to include bovine conglutinin, CL-43, and SP-D, recently dubbed the “collectin” family. SP-A has been shown to bind to the lipid A moiety of endotoxin and to a variety of pulmonary pathogens, including Haemophilus influenzae type A, Streptococcus pneumoniae, Mycobacterium tuberculosis, Pneumocystis carinii, and influenza A virus. The mechanism of binding in some cases is mediated by the ligation of microbial oligosaccharides by the CRD and in others by the interaction of an unknown microbial ligand with the asparagine-linked oligosaccharides of SP-A. SP-A also binds to alveolar macrophages and stimulates chemotaxis and release of toxic oxygen species. The binding of SP-A to at least one receptor population on the phagocytes is mediated by the collagen-like domain. Following opsonization of the microbe or direct interaction with the macrophage, SP-A has been variably reported to enhance or to have no effect on the adherence and/or phagocytosis of respiratory pathogens. The property of SP-A to enhance the adherence of Mycobacterium to macrophages is mediated by the asparagine-linked oligosaccharides of SP-A. Thus, SP-A may function as an alveolar opsonin to defend hosts without specific antibody or for immediate protection against remotely
encountered pathogens that require the clonal expansion of lymphocytes for adequate response. However, SP-A may also act as a virulence factor for some intracellular organisms, such as *M tuberculosis*, by permitting access to a compartment or niche where proliferation occurs.

Despite decades of research, the understanding of the principal physiologic role of SP-A in the alveolar space remains elusive. Over time, the list of putative functions based on *in vitro* observations has grown quite large and implausibly diverse (Fig 3). Indeed, only a few of the reported activities of SP-A were confirmed by the initial phenotype of the recently developed SP-A<sup>-/-</sup> (knockout) mouse model, which had decreased levels of TM and surfactant surface-active properties that were easily inhibited by dilution. As with all knockout strategies, it will be important to rigorously exclude the possibility that adaptive or compensatory mechanisms, or undetectable levels of SP-A expression, may have obscured the functional consequences of ablation of the SP-A gene. Nevertheless, the surprising absence of abnormalities in resting respiration, alveolar phospholipid pool sizes, or survival in the SP-A knockout mice raised serious questions about the physiologic relevance of the laboratory-assigned roles for SP-A in pulmonary homeostasis. The extensive sequence conservation of SP-A across species and the abundance of the protein in the airspace of all air-breathing mammals attest to the evolutionary importance of SP-A in pulmonary function. One hypothesis that is consistent with the preliminary SP-A knockout phenotype and the reported roles for SP-A is that the protein is essential for maintaining alveolar integrity in the presence of environmental or pathophysiologic stressors, such as infection, fever, pulmonary edema, etc. Alternatively, it is possible that SP-A is primarily a host defense protein. These questions are currently being addressed in several laboratories using reengineered animal models.

**Figure 3.** Partial functional map of SP-A. Schematic representation of the domains of SP-A that have been implicated in the oligomeric assembly and biological functions of the protein. *M. tb* = *M tuberculosis.*

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Coordination of Genetic, Epigenetic, and Environmental Factors in Lung Development, Injury, and Repair*

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Lung development is regulated by a number of genetic and epigenetic factors including transcriptional factors, peptide growth factor receptor-mediated signaling, and environmental influences including lung fluid volume, mechanical strain, and hyperoxia. These inputs are integrated during the normal process of lung organogenesis to determine organized patterns of cellular proliferation and cell lineage differentiation and to correlate structure with physiologic function. Lung development extends from branching morphogenesis in early embryonic life, through the critical transition from fetal life to air breathing, up to the completion of alveolarization which occurs postnatally. Lung organogenesis can be positively or negatively impacted by environmental factors.

Transcription of master genes is necessary in the process of pulmonary organogenesis. The pattern-forming events that control the initial and subsequent steps of pulmonary organogenesis are only now beginning to emerge. We and others have recently demonstrated that the thyroid transcription factor-1 (TTF-1) family, also called the thyroid enhancer binding protein (T/ebp) transcriptional factor family, is essential for the induction of embryonic lung branching morphogenesis.1,2 TTF-1 co-
sensus recognition sites are found in the 5′ promoters of several peripheral lung cell lineage specific genes, including surfactant protein-A (SP-A), SP-B, SP-C, SP-D, CC-10, and TTF-1 itself.3,4 However, while TTF-1 expression appears to be necessary for lung morphogenesis, other families of transcription factors are also clearly involved.

The hepatocyte nuclear (HNF) family of transcription factors are related to the forkhead family of Drosophila and are known to play key roles in regional specification of epithelial cell fates in the gastrointestinal tract and liver. HNF-a is expressed in the gut epithelium anterior to the liver and consensus HNF binding sites are found in the 5′ promoter regions of peripheral lung-specific genes including SP-A, SP-B, SP-C, SP-D, and CC-10 in close proximity to TTF-1 sites.5,6 Thus, the HNF family appears to cooperate with the TTF-1 family to determine pulmonary epithelial cell lineage fates.7,8,9

Very recently, trachealless (trh) has been identified as necessary to direct tubulogenesis in the respiratory organs and salivary glands of Drosophila.10 In trh mutants, tube-forming cells of the trachea, salivary gland, and filzkörper fail to invaginate to form tubes and remain on the embryo surface. The trh expression is controlled by Sex comb reduced (scr) and forkhead (fkh), and is homologous to the human hypoxia-inducible factor-1α.11 It will be interesting to determine whether mammalian homologues of these additional Drosophila gene families also play a role in pulmonary organogenesis.

Peptide growth factor signaling is also necessary for lung morphogenesis. Branching morphogenesis and cell lineage differentiation also occur spontaneously in mouse early embryonic lung under serumless chemically defined conditions.12-14 Soluble factors released by peripheral lung mesenchyme can induce ectopic branching from the trachea of early mouse embryonic lung explants, as well as inducing expression of a complete repertoire of genes specific to peripheral lung epithelium including SP-A, SP-B, SP-C, and CC-10.15 These data strongly suggest that endogenous factors can activate both morphogenesis and lung-specific gene expression. Candidate inductive peptide growth factors include epidermal (EGF), insulin-like (IGF), basic fibroblast (bFGF), platelet-derived (PDGF), hepatocyte (HGF), keratinocyte (KGF), and transforming (TGF)-β3, all of which exert inductive or permissive influences on lung development as demonstrated by gain and loss of function experiments in early embryonic mouse lung organ culture, transgenic mice, and null mutant mice.16-20

In general, peptide growth factor cognate receptors with tyrosine kinase intracellular signaling domains such as EGF stimulate lung morphogenesis, while those cognate receptors with serine/threonine kinase intracellular signaling domains, such as the TGF-β family are inhibitory.21-24 We have recently found that branching morphogenesis is reduced by 50% both in vivo and in vitro in the lungs of epidermal growth factor receptor (EGFR) null mutant mice.25 While addition of exogenous EGF stimulates branching threefold and SP-C levels 50-fold in wild-type embryonic lungs in culture, whereas the EGFR null mutants do not respond at all to exogenous EGF. In contrast, exogenous TGF-β1 or TGF-β2 suppresses branch-

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