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Seamus A. Rooney

Lung Surfactant: Cellular and Molecular Processing

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Lung Surfactant: Cellular and Molecular Processing

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PREFACE

Research on lung surfactant is constantly expanding. Ever since its discovery some 40 years ago, there has been a continuing interest and research activity concerning surfactant function, metabolism and regulation. Soon after its initial discovery, it was found that there was insufficient surfactant in premature infants who died from respiratory distress syndrome (RDS), at the time a major cause of death in premature newborns. The deficiency was established as being a consequence of lung immaturity with insufficient surfactant biosynthesis. Hormones—especially glucocorticoids—were found to be effective in accelerating fetal lung maturation and in stimulating surfactant synthesis in animals. Early clinical trials indicated that administration of steroids to pregnant women was effective in preventing newborn RDS. Today, steroids are widely used to prevent RDS in premature infants, and clinical trials with a combination of steroids and thyrotropin-releasing hormone are currently underway. A later development was the successful intratracheal administration of exogenous surfactant to prevent and treat RDS. Surfactant replacement is now a routine clinical therapy for prevention and/or treatment of RDS and several proprietary preparations are currently available. Although hormone and replacement surfactant therapies have dramatically reduced the incidence of RDS, they have not eliminated it completely. Hence research on the developmental regulation of surfactant production continues so as to devise additional therapeutic strategies.

A major function of surfactant is to lower surface tension at the air-liquid interface on the alveolar surface and thus facilitate lung expansion. Poor lung expansion can explain the symptoms of RDS. Surfactant consists of phospholipids and a number of specific proteins. It is well established that the major lipid component (phosphatidylcholine) and at least one of the proteins (surfactant protein B) are instrumental in the surface tension lowering function of surfactant. Recently, it has been realized that other surfactant components, surfactant proteins A and D, have a role in lung immunity and host defense mechanisms. Hence, it is possible that a deficiency in surfactant is a contributory factor to the susceptibility of premature newborn infants to infection. There is also surfactant deficiency or abnormality in adult lung disease. There are decreased amounts of surfactant in a number of adult lung diseases, although it is not clear if this is a cause of the disease or a consequence of it. Nevertheless, given its roles in lung expansion and lung defense, it is likely that impairment of the surfactant system has a deleterious effect on the course of lung disease.

Because of the clinical importance of RDS, the major foci of early research on surfactant were the regulation of its biosynthesis in developing fetal lung and the mechanism by which its production is accelerated by glucocorticoids and other hormones of potential therapeutic benefit. Although great strides have been made, there are still many unanswered questions relating to the developmental and hormonal control of the expression of the surfactant protein genes and of genes involved in phospholipid biosynthesis. Because of the

potential involvement of surfactant in a variety of lung diseases, a complete understanding of its overall metabolism, including biosynthesis, intracellular processing, secretion, removal and reutilization, is essential.

This book provides a current update on surfactant biology research. Its focus is on the function and overall metabolism of surfactant and on the genes that regulate its production. Two chapters review surfactant functions. The first chapter deals with the biophysical aspects of surfactant in relation to lowering surface tension and the final chapter explores its role in the host defense response of the lung. One chapter explores the regulation of surfactant phospholipid biosynthesis and four deal with the structure, biosynthesis and gene regulation of the four surfactant-associated proteins. Additional chapters review mechanisms and regulation of surfactant secretion and of its removal and reutilization.

Chapter 1

Surfactant Composition and Extracellular Transformations

Kevin M.W. Keough

Properly functioning pulmonary surfactant is essential for facile operation of the lung. It is a complex material of unusual composition that is secreted from the type II pneumocytes into the aqueous lining layer of the distal airspaces. It reduces the surface tension at the air-water interface in the alveoli and small airways and thereby contributes to critical physiological functions. The presence of the material leads to increased compliance, reduced work of breathing, reduced tendency for atelectasis at low lung volumes, and a decreased driving force for edema in the terminal airspaces. The absence of surfactant or a reduction in its functional capacity leads to respiratory distress syndromes in extreme circumstances, or a reduction in efficiency of breathing and gas exchange when its function is less severely compromised. Surfactant also plays a role in the host defense mechanisms of the lung. This chapter will deal with the role of surfactant in facilitating breathing, and how its special composition results in physicochemical properties that produce the effective physiology.

This chapter deals with selected examples that highlight critical factors related to the physicochemical functioning of surfactant. The reader is referred to some of the many recent reviews on surfactant composition and physical chemistry[1-8] for additional detail and insight. Surfactant and surfactant-like material appear in the lungs and related organs of many lower vertebrates,[9] but this discussion is almost exclusively confined to the surfactant of mammals.

Surfactant Composition

Pulmonary surfactant has a unique composition, consisting of some specifically associated proteins and an unusual lipid complement. It should be kept in mind that pulmonary surfactant is defined and characterized operationally by its ability to attain a certain biophysical performance and to take up some special physical forms. Surfactant is usually prepared in the laboratory from material lavaged from the lung by differential and density gradient centrifugations that are intended to remove extraneous material such as plasma proteins, which are considered unlikely constituents of normal surfactant, since they inhibit its actions. Depending upon the extent of "purification" of the material, however, it is possible, if unlikely, that some materials which may in effect be part of surfactant "as the cell sees it" might be lost in the process. The material prepared from different mammalian species, however, shows a high degree of consistency in composition.

There are four specific proteins associated with surfactant; these are termed surfactant protein A (SP-A) through surfactant protein D (SP-D) in the order of their discovery. The proteins constitute about 10% of the weight of surfactant. SP-A and SP-D are large multimeric

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water-soluble proteins which can bind to lipids and are members of the collectin protein family. SP-A constitutes the greatest amount by weight of the surfactant proteins (5-8% of the total weight of surfactant), with SP-D likely contributing the least to the protein mass (although its specific contribution to the total surfactant mass is not yet evaluated). SP-B and SP-C are quite hydrophobic and are of much smaller molecular weight than the other surfactant proteins. They contribute 1-2% each to the surfactant mass. SP-A is an octadecamer of subunits of an approximate monomeric molecular weight of 35 kDa, and SP-D is a dodecamer of 43 kDa monomers. SP-B is a dimer of 8.7 kDa monomers, and SP-C is the smallest of the four, being a monomer of about 4.2 kDa. SP-C contains two palmitates esterified to the side chains of cysteines 5 and 6 of its sequence. SP-C contributes the largest amount to surfactant protein on a molar basis, with SP-B, SP-A and SP-D contributing on a molar basis in that order.[3]

Lipids, of which over 80% is phospholipid, comprise about 90% of the mass of surfactant. Of the neutral lipids, cholesterol is the dominant constituent with acylglycerols, especially triacylglycerol, being next in order of importance. Cholesterol is present in the lipids in amounts of up to 8% by weight, or about 14 mol % of the lipids. Free fatty acids, particularly palmitic acid, are usually present only in small amounts in natural surfactant. However, they have been added in substantial amounts to some mixtures that are employed or have been tested as potential exogenous surfactants for use in treating respiratory distress syndromes. The amounts of glycolipids are small and their functions are not yet explored.

The major phospholipid is phosphatidylcholine (PC), it being about 80% of the phospholipid. Of the phosphatidylcholines, a very substantial portion contains two saturated fatty acids with the predominant molecular species being dipalmitoylphosphatidylcholine (DPPC). Some measures of the disaturated PC and DPPC contents have been as high as 70% of the total PC. However, these may be overestimates due in part to the fact that a commonly used technique which employs treatment of lipids with osmium tetroxide followed by chromatography to measure saturated phospholipids[10] has a propensity to overestimate saturated lipid content in the presence of lipids containing only one double bond.[11] Other estimates, not using this technique, suggest that the amount of saturated PC and DPPC is lower than may generally have been thought, it being about 40-50% of the total phospholipid.[1,11-13]

The surfactant of most mammals contains acidic phospholipids in the range of 10-15% of the phospholipid pool. The predominant negatively charged phospholipid is usually phosphatidylglycerol (PG) with phosphatidylinositol (PI) and phosphatidylserine making minor contributions to the total acidic lipid. The PG pool usually consists of both saturated and unsaturated molecular species.[1] The presence of PG is remarkable since it is not a substantial constituent of the phospholipids obtained from any other mammalian source. This finding has led to examination of its potential functional role in surfactant. When PG is low the level of PI is usually increased, and this is consistent with the possibility that there is a functional requirement for an acidic, and possibly a hydroxylated, phospholipid.

Surfactant Transformations in the Hypophase

Once surfactant is secreted into the hypophase, the aqueous lining layer of the airspaces, it undergoes a series of transformations, shown diagrammatically in Figure 1.1, that are associated with its biophysical function. Pulmonary surfactant is secreted from the lamellar bodies in which it is stored in the type II cell in the form of tightly packed bilayers. In this form the degree of hydration is likely quite low, the hydration state being barely enough to hydrate the lipid head groups fully.[14] The material then undergoes a transformation into a unique structure called tubular myelin.[15-17] Tubular myelin consists of an array of tubules of nearly rectangular cross section with bilayer walls. In the corners of the tubules the walls

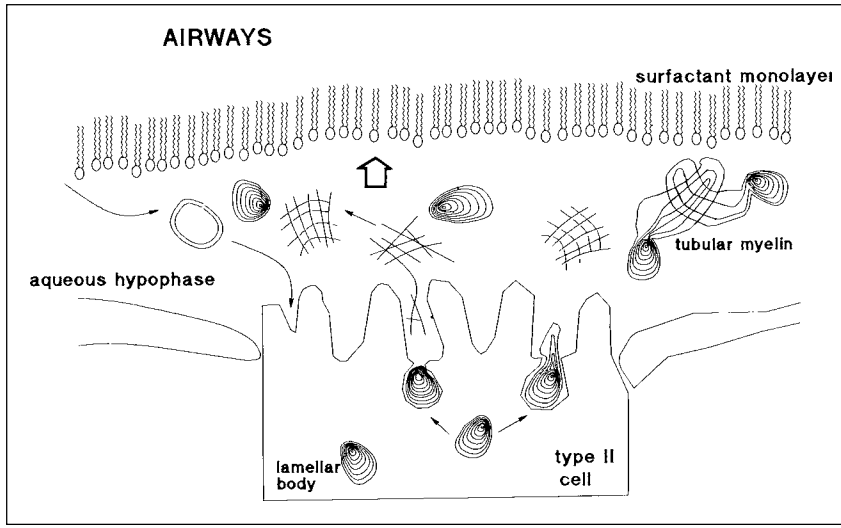

Fig. 1.1. Diagram of the extracellular transformation of pulmonary surfactant.

intersect or, in some instances, abut one another while they each undergo a 90° change in direction over a very small radius. At the regions of high curvature the bilayers must be highly strained, and at the points of intersection there must be some lipid which is not in bilayer phase. The transformation from bilayers in the lamellar bodies to tubular myelin occurs under the influence of an increase in calcium concentration in the hypophase. The presence of tubular myelin is associated with an ability of the surfactant to adsorb rapidly into the air-water interface, the next step in the extracellular processing.[18] For this reason, it is generally considered that in natural surfactant tubular myelin is the immediate precursor of the surface film. Calculations indicate that the transfer of material from the hypophase to the air-water interface must occur via the concerted movement of aggregates of surfactant molecules.[19,20] Evidence for a continuum between surfactant subphase structures and the surface film suggests a route by which this concerted transfer might occur.[21] The surface film is generally considered to be a monolayer, or a multilayer consisting of a monolayer and an associated bilayer(s).[5] The monolayer is thought to undergo refinement to enrich it in DPPC, either through processes of selective exclusion of non-DPPC materials or via selective insertion of DPPC. Material also leaves the surface either temporarily, being respread into the films, or permanently, for recycling back into the cell. The latter "spent" surfactant may be created by physical exclusion of materials from the surface, but there is evidence that an extracellular serine carboxylesterase contributes to this process.[22,23] Reuptake and reprocessing of material adds to that being synthesized de novo in the cell, and completes the "life cycle" of the surfactant.

The Role of the Lipids

As most of the mass of pulmonary surfactant is contributed by lipids, especially phospholipids, it is hardly surprising that some of its major biophysical properties are dependent on these molecules and their interactions. The surface of the lung is covered with a thin continuous aqueous layer.[24] A most important function of surfactant is the reduction of surface tension in the air-water interface in a fashion where the surface tension is directly

related to the lung volume and surface area and where the value of surface tension is very low at low lung volume.[25-27] The reduction of surface tension is associated with a film of surfactant lipid at the air-water interface. Another important characteristic is the rapid formation of the film at a clean interface or the fast refilling of an expanding existing film.

It is useful to remark on the organizations that can be taken up by lipids in aqueous dispersions or in monolayers as a basis for the remainder of the discussion. Figure 1.2 shows the phases that exist when phospholipids are dispersed in aqueous media and that have or may have relevance to surfactant. These forms are dependent upon composition and temperature. The predominant forms are lamellar and consist of bilayers usually closed on themselves. At lower temperatures phospholipids in bilayers are highly ordered, with low inter- or intrachain motion, and their chains may be perpendicular (L_β) or tilted ($L_{\beta'}$) with respect to the surface of the bilayer. I will refer to these as rigid or gel phases. Above a temperature characteristic of the lipid the acyl chains "melt" and the bilayer lipid gains a high degree of inter- and intra-molecular motion. The L_α phase is a fluid or liquid crystalline phase. The transition temperature is dependent on the type of lipid and on its hydration. When maximally hydrated, dispersions of surfactant or of its lipids (plus hydrophobic proteins) are in a fluid phase or in a mixture of a fluid phase and the L_β phase at 37°C.[28-30] Depending on composition and temperature, it is possible that regions or domains of rigid or fluid lipid may be segregated laterally within a given bilayer.

Another lamellar phase that has been recently described in the lipid extract of pulmonary surfactant, which also contains the hydrophobic proteins SP-B and SP-C, is called the L_γ phase.[30] This phase is infrequently found in biological systems and its appearance in lung surfactant invokes thoughts of lipid segregation in both the bulk phase and in any multilayer at the surface.[5] A distribution of fluid and rigid lipid selectively into each leaflet of a bilayer as found in L_γ (Fig. 1.2) suggests ways in which regionalization of lipids may occur that could aid selective insertion of ordered lipids into air-water interface or selective absorption of fluid lipids.

Other phases that may be important in surfactant functioning are the nonlamellar hexagonal phases, particularly the H_{II} phase, and micellar arrangements. Some regions of H_{II} phase or inverted micelles may exist at intersections in tubular myelin. Other similar arrangements might exist transiently during the transformations of surfactant structures.

Monolayers at the air-water interface form the basis for productive modeling of the behavior of surfactant. Monolayers of lipids take up different phases that are also dependent on composition, temperature and surface pressure (π). Surface pressure is defined as the difference in the surface tension (γ) of a clean interface and that containing the monolayer. This means that for the relatively low surface tensions associated with the surface of the lung the monolayer needs to be sustaining a high surface pressure. Surface pressure can be increased by the two-dimensional compression of the monolayer. It is considered that compression and expansion of the lipid surface film in the lung is achieved through the breathing cycle. Figure 1.2 shows a compression isotherm of a film of DPPC at room temperature and the surface phases associated with it. The liquid-expanded phase containing fluid lipid, liquid condensed phases and liquid solid phase, containing rigid lipid, are relevant to surfactant. There is some correspondence between the rigid and fluid phases in monolayers and bilayers. Transitions in the monolayer forms can be achieved by temperature variation, but are usually obtained in monolayers by the application of external compressive force. The high surface pressure region of the isotherm may be associated with head group reorientation and dehydration.[31] The same types of phases exist for a monolayer of DPPC at 37°C, but the transition from liquid expanded to liquid condensed phase occurs at a higher surface pressure, in the range of 40 mN/m.[32]

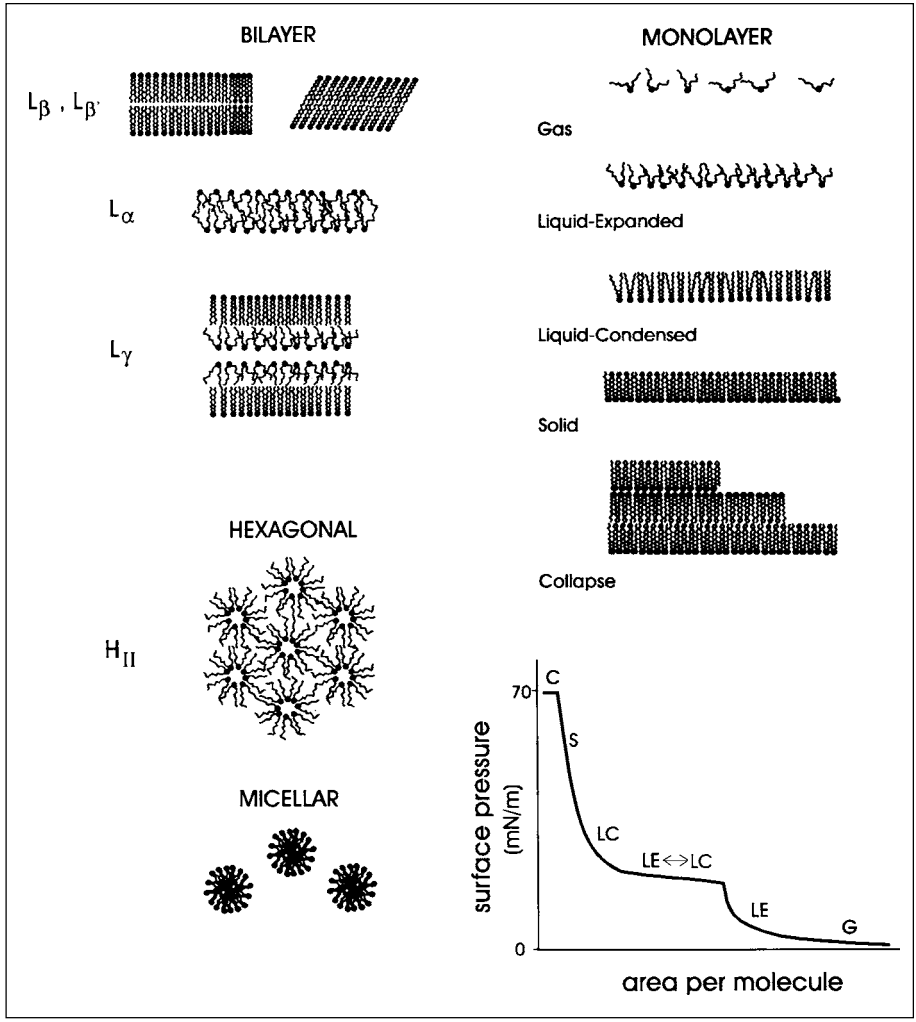

Fig. 1.2. Diagram of phases formed by phospholipids in aqueous dispersions (left) and in monolayers (right). A stylized isotherm of a DPPC monolayer at room temperature is shown in the lower right.

Attaining Low Surface Tension

For surfactant to lower the surface tension to the extremely low values seen in the lung, the material in the monolayer must be capable of sustaining very high surface pressures over a moderately long time. Of all of the components of surfactant, DPPC is the only one, in a monolayer at the air-water interface, that meets these requirements. To achieve the surface tensions usually associated with the functioning lung, values below 20 mN/m, the phospholipid film must be capable of attaining surface pressures of more than 50 mN/m and in most cases near 70 mN/m at low lung volume. These pressures are above the values of the equilibrium pressure for phospholipid monolayers, i.e., the surface pressure achieved

by a film of material that has been adsorbed from material dispersed in the bulk aqueous phase and which is in equilibrium with it. To achieve the high-pressure metastable state at the desired temperature, a PC which is rigid at the operational temperature and pressure is required.[33-38]

Unsaturated lipids are all above their transition temperatures at the body temperatures of mammals, and surface films composed of such lipids collapse at surface pressures at or very near their equilibrium surface pressures, about 45-50 mN/m, values much too low to stabilize the lung at low lung volumes. The characteristic temperature of transition for DPPC in bilayers at atmospheric pressure is near 42°C, and DPPC monolayers can achieve the high pressure state required for lung stability.[33-38] Mixing DPPC with other lipids that have lower transition temperatures alters the regularity of packing and lowers the temperature of the transition. Other saturated PCs with transition temperatures above 37°C are also capable of sustaining the necessary high surface pressure. Lipids such as palmitoyl-stearoyl PC, for example, meet the requirement, but it and other saturated PCs are found in only small amounts, if at all, in surfactant.[1,13] Dipalmitoyl PG (DPPG) also meets the requirements but it too is found in only small amounts in surfactant. It is likely not beneficial to have large amounts of lipids with transitions that are well above the operational temperature, as the system might remain too rigid and there might be difficulties with achieving sufficiently rapid entry of the material into the interface (see below). Monolayers of cholesterol collapse at much too low a surface pressure to sustain lung stability as do monolayers of proteins. The protein that sustains the highest surface pressures in monolayers is the surfactant protein SP-C, but by itself it produces monolayers that collapse at surface pressures in the range of 40-45 mN/m.[39,40]

Stability at Low Surface Tension

Since not all regions of the lung move in unison during the breathing cycle, there are at any time regions which are in different states of expansion or contraction. Maintenance of patency in the smaller airspaces requires that the surface tension remain relatively low for some period of time, a stability that has been observed experimentally at low lung volume.[24-26] Using the captive bubble technique, surfactant films at very low surface tension have been found to be stable over periods of minutes.[41,42] DPPC is the major component of surfactant which forms such stable monolayers. Films of lipids that are at a temperature above the transition temperature can sometimes be transiently compressed to pressures a few mN/m beyond the equilibrium surface pressure, but they collapse almost instantly back to the equilibrium pressure. On the other hand, films of DPPC at a temperature of 37°C or less can be compressed to very high surface pressures, up to 70 mN/m, and they collapse back to the equilibrium surface pressure very slowly.[33,34,36,43] The rate of collapse of the metastable DPPC film mimics that found in the surfactant films in the captive bubble. These concepts and observations by others [44] lead to the view that the surface film of surfactant in situ is enriched in DPPC.

Formation of the Surface Film

Despite the desirable qualities of films of DPPC given above, such films are difficult to form spontaneously. The formation of surface films from crystalline DPPC or from liposomes of pure DPPC is quite slow,[45-49] much too slow for pure DPPC to be an effective surfactant in situ. When monolayers are compressed beyond the point of collapse, material must leave the surface in three-dimensional layered aggregates, usually called collapsed phases. When the interface is reexpanded, material from the collapsed phases can potentially respread back into the interfacial monolayer. Such surface refilling may be an important part of replenishing surfactant films during the breathing cycle. Collapsed phases of pure DPPC are

very poor in undergoing such respreading, and that is the reason that large hysteresis in surface tension is seen in monolayers of DPPC that are compressed beyond their collapse points and then reexpanded.

The rate at which surfactant films can be formed by adsorption from the hypophase or by respreading from collapsed phases is increased by the addition of unsaturated phospholipids to DPPC.[50-57] The fluid state of the unsaturated lipids allows them to spread into the interface more rapidly than rigid lipids. The presence of fluid lipids or cholesterol disrupts the regular packing of DPPC and contributes to the increased ability of such mixtures to move rapidly into the interface.

The unusual presence of PG has been associated with increased adsorption rates of surfactant. The interaction of calcium in the hypophase with PG is thought possibly to lead to separation of domains that are PG-rich and that might serve as focal points of bilayer disruption to speed interfacial film formation. Calcium can cause a redistribution of condensed and fluid domains of PG monolayers and it also induces an overall condensation of PG monolayers.[58]

The concerted transfer of material into the air-water interface requires some sort of local disruption in the usual lipid packing in bilayers. Regions of curvature-induced high strain or of nonbilayer phase may exist. The regions at the "corners" of tubular myelin could be associated with nonbilayer phase. It has been suggested that H_{II} phase formation may have a special role in the dynamics of the surfactant in the hypophase.[59] Although the usual lipids of surfactant do not form H_{II} phase in and of themselves, the special mixture in surfactant in the presence of calcium and surfactant proteins may partially and transiently segregate into such a phase. Recently mixtures of DPPC and substantial amounts of unsaturated phosphatidylethanolamine, lipids which readily form H_{II} phase, have been found to possess a number of desirable characteristics for an artificial exogenous surfactant.[60]

Refining of the Surface Film

If the operational surface film in situ were to have the same composition as that of surfactant in the hypophase it is highly likely that it would behave like a fluid rather than a rigid monolayer. In situ, however, the film has characteristics that are consistent with it being a rigid one enriched in DPPC.[24-26,44] It is therefore considered that the surface film undergoes some compositional refining through either a process that selectively inserts DPPC into the film or that selectively excludes non-DPPC components during compression or both. Monolayers of surfactant under compression in surface balances show plateaux that are consistent with selective exclusion. However, films adsorbed in captive bubbles show compression isotherms that could be consistent with a continual exclusion of lipids during compression or with a process of selective insertion of rigid lipid. For a detailed discussion of the refining process see reference 3. The amount of those non-DPPC component(s) that is selectively squeezed out is dependent on their amounts and physical characteristics and on the compression rate.[61-63]

Evidence for selective insertion is more tenuous than that for selective exclusion. Any selective insertion of components, of DPPC in particular, would most likely require the participation of one or more of the surfactant proteins (see below). It is interesting that monolayers made from lipid extracts of surfactant (which contain SP-B and SP-C) show some unanticipated properties upon investigation by epifluorescence microscopy and Brewster angle microscopy. As the monolayers are compressed, they undergo internal separation into domains which are apparently DPPC-rich and condensed. These condensed domains are maximal in amount near 35 mN/m and then, as surface pressure is further increased, they seem to disappear again, with the monolayer taking on a homogenously fluid appearance.[64] The apparent fluid characteristics of the monolayer at higher pressures

are unexpected, but they are in keeping with properties of a simple mixture of an unsaturated PC and DPPC.[65] Some recent observations suggest that in the higher pressure regions there may be very small probe-excluded (rigid) domains embedded in a lattice-work of probe-containing domains that had not been discerned in previous studies.[66] The amount of rigid phase in the lattice is difficult to quantitate but may be sufficiently high to provide the low surface tension and metastability associated with surfactant monolayers under high compression. The finely divided meshwork or intermingled array of very small rigid domains and encompassing fine lattice of what might be fluid-like domains is reminiscent of a metal alloy. A "two-dimensional alloy" in the surfactant film at high pressures, or low surface tensions, might impart strength to the film under compression while allowing it to remain somewhat flexible, potentially desirable characteristics in the dynamic conditions occurring in the lung.

The Role of the Proteins

The hydrophobic proteins SP-B and SP-C are strongly associated with the lipids of surfactant. Water-soluble SP-A is also lipid-associated in natural surfactant prepared from lung lavage, but it can be readily removed from the lipid by a variety of techniques, the simplest of which is addition of calcium chelating agents.[67] SP-B and SP-C, however, are soluble only in detergents or organic solvents and so are present in extracts of surfactant lipids made with organic solvents. SP-D is found in soluble form in bronchoalveolar lavage.

SP-A and SP-D are members of the family of collectins, lectins with collagenous domains. They are C-type, or calcium-dependent, lectins. Both proteins are oligomeric, with each monomer consisting of a short N-terminal sequence that may be involved in disulfide formation followed by a collagenous portion, a "neck" region which connects the collagenous portion to a carboxy terminal region with the associated lectin activity. The collagenous regions form triple helices and six triple helical units are assembled into octadecamers in the case of native SP-A, whereas four such units form dodecamers in SP-D. Their shapes have been determined by rotary shadowing on electron microscopy[68,69] and are shown diagrammatically in Figure 1.3. Some larger aggregates have been observed for SP-D[69] from normal lungs and for SP-A in bronchoalveolar lavages from patients with alveolar proteinosis.[70]

SP-B and SP-C are in a class of proteins termed proteolipids because of their high hydrophobicity and solubility in organic solvents. They are much smaller than the two water-soluble proteins (Fig. 1.3). They have been implicated in a number of processes in surfactant biophysics and are essential components of the working surfactant system in the hypophase and may be associated with the surfactant film at the air-water interface.

SP-A augments certain activities such as surfactant adsorption and is required for tubular myelin formation. When the SP-A gene was targeted to produce SP-A deficient mice, surfactant function was partially deficient when tested in vitro but the mutation did not detectably alter pulmonary function or survival after birth.[71] On the other hand, the congenital absence of SP-B causes lethal respiratory failure in full term infants[72,73] and its partial deficiency results in milder lung disease and longer survival.[74] The clinical deficiency of SP-B has been reproduced in a mouse model where targeted disruption of the SP-B gene caused respiratory failure at birth.[75] The disruption of the SP-B gene also leads to incomplete processing of SP-C, so that an aberrant 8.5 kDa SP-C accumulates and the amount of normal 4.2 kDa SP-C is markedly reduced.[75] These observations are consistent with SP-B, and possibly SP-C, having an essential role in surfactant physiology and for SP-A having a modulating and optimizing role in surfactant dynamics in the airspaces.

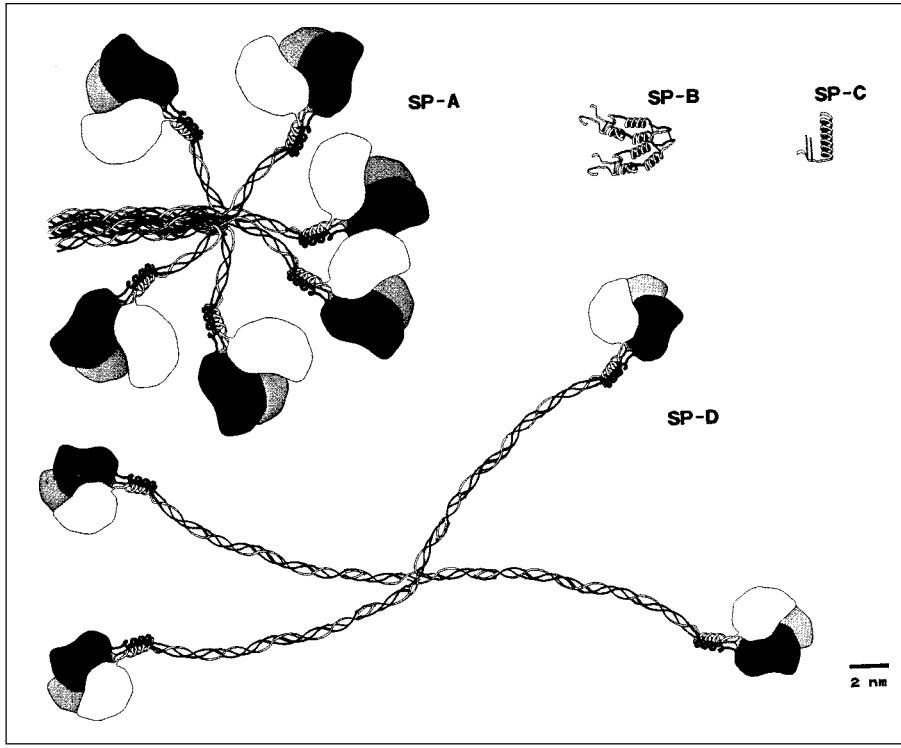

Fig. 1.3. Diagrams of the four surfactant proteins drawn to scale.

The Water Soluble Proteins

SP-A

The recognition that proteins play an important role in surfactant dynamics came about 25 years ago when King and Clements[76] reported that a water-soluble protein associated with surfactant increased the ability of surfactant lipids to enter the air-water interface.[76] A large body of work has subsequently been carried out on that protein, now named SP-A.

SP-A binds to dispersions of the extracted lipid-soluble materials of surfactant and various simpler mixtures of lipids. Of the phospholipids present in surfactant, SP-A shows a preferential interaction with PC and especially with DPPC.[77-80] SP-A also binds to neutral glycolipids in surfactant,[81,82] although the role of glycolipids in surfactant dynamics is undetermined.

The function of DPPC is, as noted above, fairly well understood. Not only does SP-A have a selective interaction with DPPC, but it preferentially interacts with the lipid in the gel phase in bilayers. In liposomes the greatest interaction between SP-A and lipid occurs not only in the presence of gel phase lipids but when packing dislocations or domain separations are present.[79] The binding of SP-A to lipid is primarily driven by hydrophobic forces whether the lipid is in bilayers,[79] monolayers,[82] on thin layer chromatograms[80] or on plastic surfaces.[81] Monolayer studies indicate that SP-A interacts with DPPC but not with DPPG, from which it appears to be completely excluded.[83] The association of SP-A with DPPC

monolayers induces the production of more, smaller, domains of rigid lipid than are seen in equivalent monolayers without protein (Fig. 1.4),[84] a seemingly common phenomenon when proteins are inserted into phospholipid monolayers. A greater number of nucleation sites for formation of condensed phases occurs, but the condensed regions cannot grow as large as in the absence of SP-A as the protein interferes with packing into regular arrays, a phenomenon also seen with SP-B and SP-C. Using fluorescently-labeled SP-A it has been observed that the protein adsorbed from the subphase into a DPPC monolayer only when there is a mixed phase region produced by compression of the film so that rigid and fluid DPPC domains coexist. SP-A accumulates at the solid domain edges but it also spreads to a limited extent into the fluid phase (Fig.1.5).[84] On the other hand, when the fluorescent SP-A adsorbed to a monolayer of DPPC/DPPG the protein was distributed in large aggregates in the packing dislocations at the domain boundaries, consistent with a lack of interaction between SP-A and the acidic PG at neutral pH. Exclusion of SP-A into aggregates is probably due to natural repulsion of the two negatively charged molecules PG and SP-A.[83] Those studies were done in the presence of solutions containing 2 mM $CaCl_2$ in which self-aggregation of SP-A might be expected.[85,86] The extent of aggregation is much greater with SP-A adsorbed to DPPC/DPPG monolayers than to DPPC monolayers, a finding also consistent with repulsion of SP-A by DPPG.

At concentrations of calcium found in the lung, hypophase SP-A induces the aggregation of phospholipid vesicles, a process mediated by its C-terminal carbohydrate recognition domain[87] but requiring an intact protein for maximal activity.[88] The lipid aggregating activity of SP-A can be triggered by much lower concentrations of calcium (in the μM range) than those required for self-aggregation of lipid-free SP-A (mM concentrations).[89]

A recent study has shown by epifluorescence microscopy on monolayers of the lipid extract of surfactant that SP-A can induce aggregation of the domains of condensed lipid (DPPC-rich domains),[66] a finding consistent with SP-A associating with edges of solid DPPC and with SP-A self-associating in the presence of calcium.

The binding of SP-A to phospholipid vesicles is facilitated by a "neck" region between the collagenous and globular carbohydrate recognition domains.[90,91] Studies on recombinant proteins and synthetic peptides replicating partial sequences of SP-A also implicate not only the neck region but part of the carbohydrate recognition domain in lipid binding.[92-94]

The lipid binding characteristics of SP-A are undoubtedly related to its functional roles in the hypophase. SP-A is associated with tubular myelin[95,96] and is located at the corners of the tubular myelin matrix.[97] The presence of tubular myelin in surfactant preparations is associated with rapid adsorption and surface refining. SP-A, SP-B, DPPC, PG and calcium are minimal essentials for the formation of tubular myelin structures in vitro.[98-100] SP-A is essential for the maintenance of surfactant in the large aggregate forms that contain tubular myelin and which are associated with rapid surface film formation. During the process of surface cycling, known to cause conversion of the large aggregates to small vesicles, the presence of SP-A resists such conversion.[22,23,101,102] The large aggregates are considered to be the surface forming materials and the small aggregates a "spent" form of surfactant that is produced upon the cycling of material in the surface.

Using a captive bubble surfactometer, it has been observed that SP-A could enhance the adsorption into the interface of a lipid extract of surfactant from which most of the neutral lipids had been removed. The observations also suggest that SP-A could be aiding the elimination of non-DPPC lipids from the interface during cycling.[103] Recent studies also suggest a potential role for SP-A in selective insertion of DPPC into the surface from the same lipid extract of surfactant to which neutral lipids had been added.[104] This surface enhancement may be augmented by the fact that SP-A could limit the adsorption of cholesterol to the interface.[105] Since cholesterol could fluidize the surface lipids, its selective

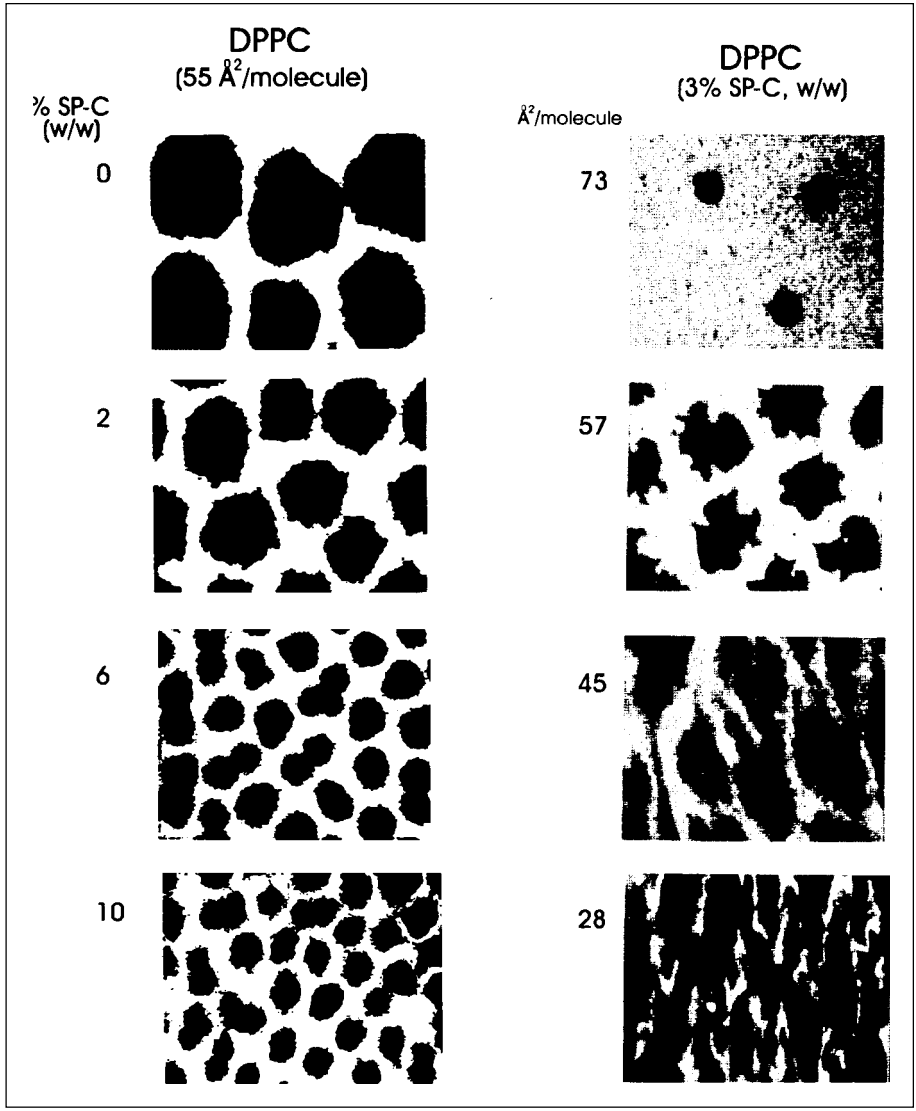

Fig. 1.4. The influence of incorporated proteins on the distribution of domains in the liquid-expanded (light) and liquid-condensed (dark) coexistence phases of DPPC monolayers. (Left) Effect of increasing concentrations of protein at one surface pressure. (Right) Effect of increasing surface pressure at one concentration of protein.

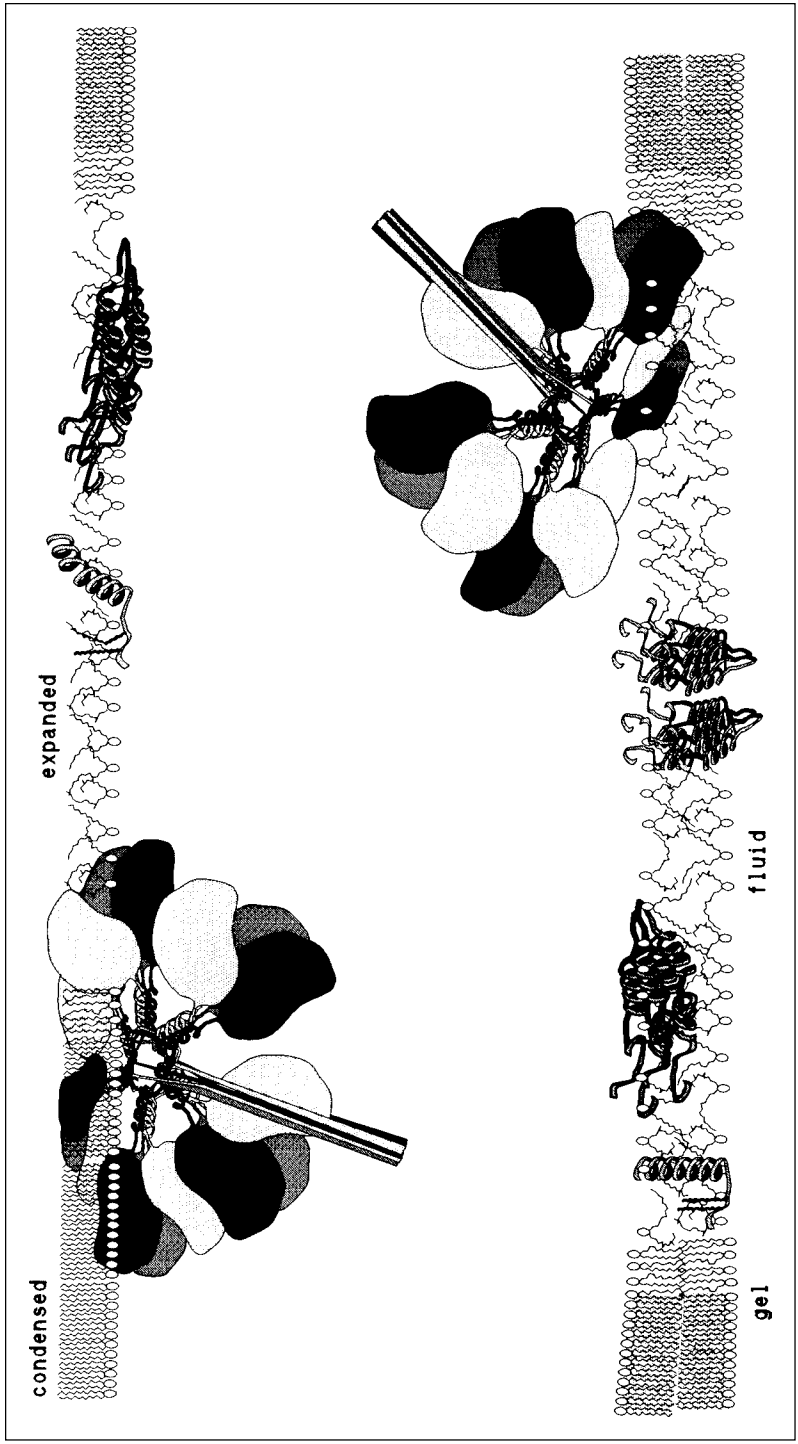

Fig. 1.5. Selections of possible orientations of SP-A, SP-B and SP-C in bilayers and monolayers. The proteins are depicted as in Figure 1.3.

elimination could add to surface stabilization by DPPC. DPPC aggregates were observed in the surface during the process of SP-A promoted DPPC insertion.[104] This phenomenon is consistent with the association of SP-A and the boundaries of solid DPPC domains in monolayers.[84,89]

The ability of SP-A to promote film formation and enrichment with DPPC is likely the major reason for its ability to aid in overcoming the inhibition of surfactant surface activity by plasma proteins that compete with surfactant for the surface space.[106-108]

SP-D

To date no specific role for SP-D in the extracellular biophysics of surfactant has been determined. The recent observations that SP-D and PI may substitute for SP-A and PG in forming tubular myelin-like figures in vitro suggest that in some circumstances SP-D may have a role in trafficking in the hypophase.[109] SP-D binds specifically to PI and glucosylceramide[110-112] on thin layer or microtiter plates and to liposomes containing PI. In the presence of calcium, SP-D can induce the aggregation of liposomes containing PI.[110] A recent study also showed that SP-D binds not only to PI but also to DPPC, although not to unsaturated PC, on microtiter plates.[113] When recombinant rat SP-D was allowed to adsorb into monolayers of unsaturated PC, PG or PI and mixtures of DPPC and either PI or PG, no headgroup specific differences were detected.[114] That suggests that under those conditions the interaction between SP-D and the lipids was primarily driven by hydrophobic forces. Various studies have shown that the binding of SP-D to lipids requires the carbohydrate recognition domain, especially the "neck" region, but it appears to be modulated by the presence of the collagen-like region.[92,113,115] More study will be required to determine if the SP-D lipid interactions noted have a major role in extracellular surfactant biophysics in relation to the lowering of surface tension or if they are related to other factors such as the interaction of SP-D with surfaces of foreign particles. The fact that PI is usually present in surfactants in amounts near that of the more usual PG when PG is missing makes the SP-D interaction with lipids continue to intrigue many investigators.

The Hydrophobic Proteins

The presence of the hydrophobic proteins SP-B and SP-C in surfactant has been known for some time.[116,117] These proteins are extracted from surfactant by organic solvents along with the lipids. They can be separated from the lipids by various forms of chromatography, the most common being hydrophobic gel exclusion chromatography. In some studies, especially earlier ones, SP-B and SP-C were obtained in the same fraction, whereas in later work the two proteins are obtained separately.

SP-B is a disulfide-linked homodimer, the monomers of which contain 79 amino acids. Each monomer has seven cysteines, six of which are involved in intramonomer disulfides, and the seventh forms the link to the other monomer. The primary sequence and the positioning of the disulfides imparts to SP-B some "kringle-like" structure.[118] The sequence and placing of the cysteines also provides SP-B with a fairly high degree of homology with the family of lipid binding proteins called the saposins[119] and the lytic polypeptide NK-lysin.[120] The structure of SP-B is a little less than 50% α-helical in solvents and when it is in bilayers or monolayers.[39,121-124] The protein contains an excess of basic residues which are located primarily in two regions that have been calculated to be potential amphipathic helices. These regions have been variously calculated to be approximately 10 amino acid stretches starting about 10 residues from either terminal with a third smaller amphipathic helix postulated to be about in the middle of the monomer sequence.[125,126]

SP-C is extremely hydrophobic. It is 35 residues long with 23 hydrophobic amino acids leading to the C-terminal, a region particularly rich in valine. The 12-residue N-terminal

region contains a variety of side chains including two positively charged ones at positions 11 and 12. SP-C from all but one species, the dog, has two vicinal cysteines at positions 5 and 6, each of which are acylated with palmitoyl chains. Canine SP-C has one palmitoylated cysteine and a phenylalanine in those positions. The protein has a high content (70% or more) of α-helix in solvents, bilayers and monolayers.[39,123,126-129] The determination of the high resolution structure of SP-C in organic solvents and in micelles has confirmed that the hydrophobic C-terminal region and the preceding 2-4 amino acids constitute a regular α-helix.[130,131]

There is a dimeric form of SP-C that is composed of SP-C monomers linked through disulfides. The dimers are not palmitoylated and they possess a variable amount of α-helix and β-structure depending upon their source.[131-133]

It has been recognized for some time that the hydrophobic proteins have the important property of being able, either alone or in combination, to substantially enhance the ability of surfactant lipids and various combinations of synthetic lipids to adsorb into the air-water interface, a function considered to be essential for optimal surfactant dynamics in the lung.[134-137] When added to lipids, SP-B and SP-C also aid resistance to inhibition of surfactant activity by various plasma proteins. The overall consensus of a large body of work is that both proteins enhance these activities and that SP-B is more effective than SP-C. In promoting these effects the two proteins are additive but not synergistic.[138] Both SP-B and SP-C can be spread in monolayers alone or with lipids. The two proteins also appear to act independently in these monolayers with one exception found to date. In DPPG-containing monolayers, SP-B and SP-C behave cooperatively in their exclusion under increasing pressure and in removing lipids out of the DPPG monolayer.[139] The cooperativity is abolished, however, in the presence of calcium.[40] The hydrophobic protein-promoted adsorption of lipids from the subphase into the interface is nonspecifically dependent on the presence of monovalent or divalent cations.[140] When either SP-B or SP-C is present in monolayers of phospholipids they can induce the adsorption of additional phospholipids from small or large unilamellar vesicles that are injected beneath the monolayer. Contacts between the monolayers and the vesicles are necessary for the process, which is independent of the lipid type, to occur. In keeping with other findings related to adsorption, SP-B is more potent than SP-C in promoting the lipid transfer.[39,140] A number of studies have been carried out in monolayers and bilayers to elucidate the ways in which the two hydrophobic proteins modify the distribution and packing properties of lipids so that they can catalyze the dynamic properties described.

SP-B

Result of studies on SP-B in PC and PG have led to equivocal interpretations of its specific location with respect to the chains and the polar groups of the lipids. Investigation of the influence of natural SP-B on the properties of fluorescent lipid probes in model bilayer systems suggests that the protein interacts near the surface of the bilayer and causes little perturbation of the acyl chains deep within the bilayer.[141] Studies with a synthetic monomeric version and a natural dimeric form of SP-B that employed techniques of deuteron magnetic resonance spectroscopy and differential scanning calorimetry showed that SP-B perturbs the chain packing very little in systems containing DPPC, DPPG or a mixture of the two and that the perturbations that do occur are essentially the same for all parts of the hydrocarbon chains.[122,142] The presence of SP-B modifies the slow motions of lipids in bilayers. The data from these latter experiments could be interpreted as being consistent with two models for SP-B lipid interactions. In one model SP-B interacts through its amphipathic helices at the lipid head groups with minimum perturbation of the chain packing, and in the second SP-B aligns at the edges of bilayer disks by virtue of its amphipathic

character (Fig. 1.5). The disks would subsequently aggregate into bilayer sheets. The second model, which is based on earlier electron microscopic studies,[99] is preferred.[142] The latter interpretation is also supported by the observations that SP-B promotes mixing of lipids between, and fusion of, lipid vesicles.[143-145]

The positively charged SP-B molecule, when interacting with bilayers containing negatively charged phospholipids such as PG, produces changes such as elevation of the temperature for chain melting and a broadening of the transition from the gel to the liquid crystal state.[142,146,147] In bilayers containing mixtures of DPPC and PG of various acyl chain composition it appears that SP-B shows some preference for interaction with, and possible partial segregation of, the PG component.[141,142,144,146,147]

When SP-B is introduced into monolayers of DPPC or DPPG it expands the films at lower surface pressures[148] and modifies the packing so that more, smaller condensed domains of lipid are produced during the compression of the monolayers through their liquid-expanded to liquid-condensed states into solid states at high surface pressures (Fig. 1.4).[149] SP-B is excluded from monolayers of either DPPC or DPPG at a surface pressure a little above that where monolayers of the pure protein collapse, and in neither case does it carry a significant amount of lipid molecules out of the monolayer with it.[123,148] In DPPG but not DPPC films in the presence of calcium, SP-B is excluded at a lower pressure than in the absence of calcium, likely due to the direct effect of calcium on the lipid.[40] Direct observation of fluorescently labeled SP-B in monolayers shows that at lower pressures the protein is associated with the liquid-expanded state, but some of it remains associated with the monolayer even when it is compressed to very high pressures and into the collapse phase.[149] The association of the protein with the collapse phase helps respreading of the lipid into the monolayer when it is reexpanded.[150,151] The presence of cholesterol in the protein-lipid film lowers stability at high pressure and detracts from the respreading ability of the collapse phase.[151] When SP-B is distributed in monolayers of DPPC, where it is usually found in the liquid-expanded or fluid phase at intermediate surface pressures, the addition of SP-A to the subphase appears to cause a redistribution of SP-B to match that of SP-A (Nag K, Keough KMW, unpublished observations).

A series of studies with synthetic peptide portions of SP-B and other peptides that mimic some portions of the molecule in various lipid environments have been carried out to correlate peptide structure with biophysical properties. A general view of the findings indicates that peptides that include the regions near the N and C terminals which are predicted to contain amphipathic helices tend to assume helical character in the presence of lipids, and that they perturb packing and promote adsorption of lipids to the air-water interface. Some combinations of peptides with lipids also serve to improve compliance of surfactant-deficient lungs.[124,152-155]

Addition to lipid mixtures of a regular repeating positively-charged peptide, $(KLLL)_4K$, the sequence of which was suggested by the C-terminal portion of SP-B, promotes improved biophysical properties and compliance.[156] The peptide, which was originally thought to be surface associating and which shows some preferential interaction with DPPG in DPPC/DPPG mixtures,[157] has recently been shown to be a bilayer-spanning helix.[158]

The peptide containing the first 25 amino acids of SP-B, and most likely the entire SP-B molecule, has been suggested to play a special role in stabilizing palmitic acid in monolayers of artificial surfactants in which it is present in relatively high amounts.[159,160]

SP-C

As noted above, SP-C can promote the transfer of lipids from bilayer forms into monolayers at the air-water interface and enhance the respreading of lipids from excluded or collapsed forms of monolayers. In most cases where direct comparisons have been made,

SP-C has been found to be less effective than SP-B in promoting lipid adsorption, but its role in these functions is not to be discounted by the currently available evidence. The role of the palmitoylated vicinal cysteines in adsorption and respreading has been studied, as has that of mixtures of lipids plus SP-C with and without palmitates on pressure vs. volume properties of surfactant deficient lungs. Deacylated SP-C has been produced by both recombinant technology and by chemical modifications. Both approaches have also been used to remove the positive charges on the side chains of residues 11 and 12 (and position 2 in porcine SP-C) of the SP-C sequence. Models[161] and measurements[162] place these charged residues in the region of the lipid head groups.

Depalmitoylation of SP-C causes little change in its α-helical content[129,163] and nonpalmitoylated recombinant SP-C also has a high content of α-helix.[164] A synthetic nonpalmitoylated form has a reduced amount of α-helix.[163] There are mixed indications about the effect of removing the positive charges on the α-helical region, with both decreases and increases in biophysical activity being reported.[163,165] Differences in the methodologies for removing the charges to obtain the modified SP-C, and whether it is located in either monolayers and bilayers, may account for the differences in the calculated amounts of helix in the two studies.[163,165]

Like SP-B, SP-C in lipid monolayers can promote the insertion of additional lipid from vesicles below the monolayer and this activity involves the adherence of the vesicles to the monolayer.[39,140] The absence of the palmitates does not substantially influence this process.[164] Similarly, the absence of palmitates does not substantially affect adsorption rates of synthetic lipid mixtures to which nonpalmitoylated SP-C is added[163,166,167] or the ability of lipid-protein mixtures to improve the compliance of surfactant-deficient lungs in comparison to mixtures containing intact SP-C.[167,168] Nonpalmitoylated recombinant SP-C retains an ability to offset the inhibition of surfactant biophysical activity usually induced by fibrinogen[169] and to inhibit the plasmic cleavage of fibrinogen.[170] On the other hand, the presence of the palmitates substantially improves the stability of monolayers and lipid respreading upon surface expansion after monolayer overcompression and collapse.[171]

While depalmitoylation does not reduce the ability of SP-C in a lipid-protein monolayer to cause adsorption of additional lipid from vesicles in the subphase, SP-C in which the positive charges are blocked by chemical modification can no longer carry out this activity.[165] This suggests that the positive charges on SP-C are necessary for the adherence of the phospholipid vesicles to the monolayer, considered to be an important part of the mechanism mediating this process.[39] The SP-C with a reduced number of positive charges is able to promote lipid mixing between negatively charged vesicles,[165] a process that is not influenced by the native protein.[143] The deletion of the positive charges in a synthetic SP-C somewhat reduces its ability to promote lipid adsorption, as does the absence of palmitates in some synthetic analogues.[163] The ability of these modified peptides to promote adsorption correlates with the amount of α-helical structure, suggesting that α-helical structure is an important determinant of the adsorption-promoting ability of SP-C.[163] This view is borne out by the observations that synthetic peptides lacking the first few N-terminal amino acids, but containing the positive charges near the lipid headgroups and the complete helix, produce good biophysical and physiological properties in lipid mixtures.[167,168]

The dimeric form of SP-C that is obtained in extracts of natural surfactant is not acylated at the cysteine positions and the monomers are linked by a disulfide bond at those residues.[132,133] The bovine dimer is slightly less effective at promoting adsorption of lipids from the subphase into an interface than is monomeric SP-C.[132] When included in a monolayer of lipids plus proteins formed over a subphase containing lipid vesicles, the canine dimeric form and the monomeric form are about equally efficient in promoting the adsortion of additional lipid from the vesicles into the monolayer.[133] The bovine SP-C dimer in bilayers

has a substantial proportion of β-structure, whereas the canine form shows a high amount of α-helix when it is spread in a monolayer at the air-water interface.[132,133] Those inconsistencies may represent species differences or may just have resulted from the various differences in preparation and assay.

The SP-C molecule is a transbilayer peptide. The hydrophobic α-helical portion extends across the bilayer, with the helix axis slightly tilted with respect to the acyl chains at an average angle of orientation of 24° with respect to the bilayer normal.[127-129,162] Removal of the palmitoyl groups does not substantially affect that orientation.[129] The possibilities that the palmitate chains are inserted in a bilayer adjacent to the one containing the helix or that those two portions orient with one hydrophobic part in a monolayer and the other in a bilayer are intriguing, but moot. SP-C perturbs the packing of the lipids in bilayers and leads to a reduction of acyl chain order in the usually ordered gel state and to a small increase in order of the chains in the fluid liquid-crystalline state.[127,128,146,147,172-174] In pure lipids the effects of SP-C on chain motions of DPPC and DPPG are similar, although the positively charged protein does cause an increase in the transition temperature of pure DPPG bilayers.[146,174] SP-C also modifies slow motions in both the fluid and gel states of the lipids and the addition of calcium can substantially reverse this effect.[127,174] SP-C shows little self-association in lipid bilayers in the fluid state, whereas it self-aggregates in the gel state[175] with some increase in its amount of β-structure.[128] In mixed lipid systems containing saturated PC and PG, SP-C shows no strong preference for either type of lipid, but in the gel state there is a greater effect of SP-C on the order of the PG component than on that of PC, suggesting that there is some preferential interaction or that SP-C can induce a nonrandom distribution of those lipid types, at least in the gel state.[172,173]

As in the case of SP-B, SP-C can be spread in monolayers with and without lipids at the air-water interface or adsorbed into the interface with lipids from lipid-protein vesicles below the surface. Epifluorescence microscopy used to observe fluorescently labeled SP-C in DPPC monolayers spread from solvents or adsorbed from appropriate lipid-protein vesicles indicates that the two types of monolayers are virtually equivalent.[176] This observation validates the use of spread monolayers for certain studies of surfactant properties since the films formed in situ are more likely to resemble adsorbed ones. This observation of near equivalence of the types of films has recently been extended to the lipid extract of surfactant which also contains the two hydrophobic proteins.[66] The addition of SP-C to monolayers of DPPC, DPPG, or a mixture of the two, expands the monolayers at lower pressures, consistent with the protein perturbing the lipid packing. At surface pressures above the collapse pressure of the protein alone, and above that of SP-B with or without lipids, there is a partial exclusion of the SP-C protein. In contrast to SP-B, where little or no lipid is removed with the protein, the excluded SP-C molecules are accompanied by about 7-10 lipid molecules, with the amounts being somewhat higher from DPPG than from DPPC monolayers.[177] As in the case of SP-B, the presence of calcium lowers the exclusion pressure and removes the ability of SP-C to carry DPPG out of the monolayer on its exclusion.[40] The difference between the exclusion pressures and the amounts of lipids accompanying SP-B and SP-C is consistent with SP-C having greater hydrophobic interactions with the lipids than does SP-B. The interactions of SP-C with either lipid in the monolayer are dominated by hydrophobic forces. Some SP-C remains in the monolayer up to very high surface pressures, where the monolayers collapse. The protein that is present in both the excluded and the collapsed phases augments the respreading of the lipid into the air-water interface on monolayer expansion.[150]

The presence of cholesterol at physiological concentrations in films of DPPC and SP-C increases the amount of SP-C that is retained in films up to the collapse point, although it makes the films more unstable at that stage. As in the case of SP-B, the presence of cholesterol reduces the ability of SP-C to promote the respreading of lipids from the collapse phase.[151]

SP-C has been found to modify the rheological properties of lipid films in such a way as to be consistent with its ability to promote adsorption and respreading of lipids.[128,178]

The properties seen in studies on films containing SP-C by conventional techniques have been confirmed by epifluorescence microscopy of monolayers containing fluorescently labeled lipids and proteins and by scanning force microscopy on Langmuir-Blodgett films prepared from monolayers of SP-C and lipids.[149,177-180] SP-C occupies the fluid regions of monolayers at low pressures and is excluded from the condensed phases. It causes the appearance of more, but smaller, condensed phase regions, as its concentration is increased in the monolayer (Fig. 1.4). At intermediate pressures (~30 mN/m) such films become separated into regions where there are large lipid-rich domains occurring in an extended protein rich lattice-work. At still higher pressures there is segregation and exclusion of the protein rich domains. Some of the protein, however, remains at or in the interface at pressures above the exclusion pressure. The appearance of the films on reexpansion to lower pressures very closely resembles that of films during their compression stages. This implies that the protein is reversibly excluded in the cycling process.[149,179,180]

These observations are consistent with SP-C being more firmly embedded in bilayers and monolayers than either of the other proteins and its being removed only under conditions where lipid can accompany it (Fig. 1.5). This suggests that SP-C could have a significant role in refining and respreading lipids.

Summary

The unique composition of pulmonary surfactant has an important role in its achieving the physical transformations that impart its essential function in the airspaces. Such transformations are achieved external to the cell without the direct input of metabolic energy and are driven by the free energy gains imparted by the rearrangements of the constituents in response to the different milieus inside and outside the cell. Additional energy can be obtained from mechanical forces of the breathing lung.

The most prominent component, DPPC, is essential in the surface film in order for it to achieve the metastable, densely packed state which permits the high surface pressure and low surface tension seen in the lung at low volume. Other lipid components detract from this property because of their phase characteristics, but some of them, including unsaturated PC and PG and cholesterol, are helpful in attaining the rapid transfer of surfactant lipid from the bilayer phase into the film at the interface.

At least three of the proteins, SP-A, SP-B and SP-C, have important roles in enhancing the processes of adsorption and respreading of lipids in the surface. All three interact with phospholipids to different extents and likely in a different manner. SP-A and SP-B are essential for formation of tubular myelin in vitro and are thus implicated in the lipid transfer process. There is some evidence that SP-A and SP-B may interact with one another. SP-B by itself is capable of promoting the adsorption of lipids into the air-water interface or their respreading from collapsed phases, but it can be augmented by SP-A and SP-C in carrying out those processes. Either SP-A or SP-C alone can also promote the transfer of lipids from the hypophase or from collapsed phases into the monolayer but they are not as efficient as SP-B. SP-A may have a role in sorting surface lipids, in that there is some evidence that it promotes the selective transfer of DPPC into the monolayer. SP-A has a high degree of preferential interaction with solid DPPC, likely at the edges of solid domains, especially when they are mixed with fluid ones. This could help surface sorting. Neither SP-B nor SP-C show strong selectivity for lipid types, but both proteins have some preference for PG when it is present with PC. The preference can be abolished by calcium, likely because it preferentially associates with the PG headgroup. For the most part the effects of the proteins appear to be additive, especially when calcium is present.

SP-A interacts at the surface of bilayers and monolayers with an unknown amount of incorporation of the very large molecule into the lipid. SP-B might interact at the surface of lipid arrays through its partial amphipathic character, but data obtained to date are also consistent with it being arranged with its hydrophobic parts abutting the hydrophobic chains of lipids. This orientation would produce small discs which aggregate into larger sheets. This arrangement may be especially important in the formation of tubular myelin. SP-B promotes fusion of lipid vesicles, in keeping with this role. SP-C is a transbilayer peptide in the bulk phase arrays of surfactant and its orientation in monolayers may be nearly parallel to the surface. When excluded from a monolayer under compression, it carries with it up to 10-11 molecules of lipid with a slight preference for PG. This may give it a significant role in surface refining after monolayer formation.

This remarkable, complex system continues to intrigue and surprise investigators. Surfactant is elegantly designed for multiple purposes, and we have begun to achieve understanding of their molecular foundations. Subsequent chapters will expose much more of these fascinating properties.

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Chapter 2

Regulation of Surfactant Phospholipid Biosynthesis

Seamus A. Rooney

The goal of this chapter is to summarize information on the regulation of surfactant phospholipid biosynthesis. As discussed in chapter 1, lipids comprise about 90% of the total mass of surfactant and phospholipids account for the bulk of them. Phosphatidylcholine (PC) is by far the most abundant phospholipid in surfactant and its disaturated species (DSPC), principally dipalmitoyl PC, has a critical role in lowering surface tension. PC accounts for as much as 80% of the total phospholipid in surfactant preparations and at least half of that is disaturated.[1] Phosphatidylglycerol is the second most abundant phospholipid in surfactant and accounts for up to 10-12% of the total.[1]

The major focus of this review is on the biochemical pathways of PC and DSPC biosynthesis and their regulation. Because of the early realization that lung immaturity with a consequent deficiency in surfactant synthesis is a major factor in the respiratory distress syndrome (RDS) in premature infants, much of the information on the regulation of surfactant phospholipid synthesis has been obtained in developing fetal lungs.[2-5] Hormones, particularly glucocorticoids, have long been known to accelerate fetal lung maturation and to stimulate surfactant phospholipid biosynthesis, and studies on the influence of glucocorticoids on fetal lung have been instrumental in elucidating regulatory steps in surfactant phospholipid biosynthesis.[2-8]

The type II alveolar epithelial cell is the cellular source of surfactant phospholipids.[2,9] The lipids are synthesized in the endoplasmic reticulum, processed though the Golgi apparatus and stored in lamellar bodies from which they are ultimately secreted to the alveolar lumen.[9,10] A general problem in studying surfactant phospholipid biosynthesis is that, although surfactant does have a unique phospholipid composition, it contains no unique lipids.[1] Surfactant is enriched in PC, DSPC and phosphatidylglycerol[1] but none of those lipids is unique to surfactant, as they are all found in nonsurfactant fractions of the lung[11] and type II cell.[12] Indeed, DSPC[13] and phosphatidylglycerol[14,15] are found in organs other than the lung, whereas PC is ubiquitous in mammalian cells. Hence it is difficult to specifically study the synthesis of surfactant-associated phospholipids. Measurement of substrate incorporation into phospholipids isolated from lamellar bodies or other surfactant-enriched fractions can give an indication of rates of surfactant synthesis. However, lamellar bodies themselves do not have the biosynthetic machinery necessary to synthesize phospholipids,[2] so that studies on enzyme activities, enzyme content or gene expression cannot be carried out on those organelles. Even studies on isolated type II cells do not specifically address surfactant phospholipid synthesis, as such cells synthesize lipids for incorporation into membranes and for cell signaling functions as well as for surfactant. Much information on

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the mechanism and regulation of PC and DSPC biosynthesis was obtained in developing fetal lung and few of those studies were carried out on isolated type II cells.[2-8] Type II cells do not appear in fetal lung until relatively late in gestation[16] when many of the developmental and hormonal-induced changes in surfactant synthesis have already occurred.[17] In reality, it is not possible to distinguish between synthesis of membrane phospholipids and those associated with surfactant in most studies and the data must be interpreted accordingly.

Biochemical Pathways

The biochemical pathways of PC and phosphatidylglycerol biosynthesis are shown in Figure 2.1. PC is a glycerophosphatide consisting of a glycerol backbone, two fatty acids and a choline phosphate molecule. Phosphatidylglycerol has the same structure except that choline is replaced by a second glycerol moiety. The glycerol backbone arises from dihydroxyacetone phosphate, an intermediate in glycolysis. Dihydroxyacetone phosphate is first converted to 1-acylglycerol-3-phosphate by either of two pathways: initial reduction to glycerol-3-phosphate followed by acylation or initial acylation followed by reduction. Both pathways exist in the lung[18] and take part in PC and phosphatidylglycerol biosynthesis in the type II cell.[19] Addition of a second acyl group to 1-acylglycerol-3-phosphate results in the formation of 1,2-diacylglycerol-3-phosphate, phosphatidic acid, the first lipid intermediate in the pathway.

Phosphatidic acid occupies a central position in glycerolipid biosynthesis. Several nonlipid precursors can be incorporated into phospholipids during the synthesis of phosphatidic acid.[1] Glycerol is first metabolized to glycerol-3-phosphate in a reaction catalyzed by glycerol kinase. Glucose is similarly phosphorylated by hexokinase to the glycolytic intermediate glucose-6-phosphate. Degradation of glycogen by the action of glycogen phosphorylase leads to the formation of glucose-1-phosphate, which is converted to glucose-6-phosphate by the action of phosphoglucomutase. Fatty acids, which may be synthesized de novo in the lung (see subsequent section) or supplied from extrapulmonary sources, can be incorporated into phospholipids via the above listed acylation reactions as well as in subsequent reacylation steps.

PC and phosphatidylglycerol are synthesized from phosphatidic acid by different mechanisms (Fig. 2.1). In PC biosynthesis, phosphatidic acid is first dephosphorylated to a diacylglycerol by the action of phosphatidate phosphatase. The diacylglycerol is combined with cytidine diphosphocholine (CDPcholine) in a reaction catalyzed by cholinephosphotransferase to form PC. CDPcholine arises from choline after initial phosphorylation and subsequent transfer to cytidine triphosphate (CTP) in reactions catalyzed by choline kinase and choline-phosphate cytidylyltransferase (CYT), respectively. Choline is essentially a vitamin that is obtained from dietary sources, often in the form of PC.[20]

In the synthesis of phosphatidylglycerol, phosphatidic acid reacts with CTP to form CDPdiacylglycerol. This intermediate then reacts with glycerol-3-phosphate to form phosphatidylglycerophosphate, which is immediately dephosphorylated to phosphatidylglycerol.

DSPC may be synthesized by the above de novo mechanism as well as by remodeling of the 1-saturated-2-unsaturated molecular species. Although a substantial portion of DSPC in lung tissue[21-23] and type II cells[24] may be synthesized de novo, there is evidence that remodeling mechanisms exist in the lung and type II cells[25,26] and account for synthesis of at least 50% of DSPC in type II cells.[27] As shown in Figure 2.1, there are at least two remodeling mechanisms. Both involve initial deacylation of de novo synthesized 1-saturated-2-unsaturated PC by the action of phospholipase A_2. The resulting 1-acyl-2-lyso-PC is then either reacylated with a saturated acyl-CoA or transacylated in a reaction in which two molecules of lyso-PC react to form one molecule each of PC and glycerophosphocholine.

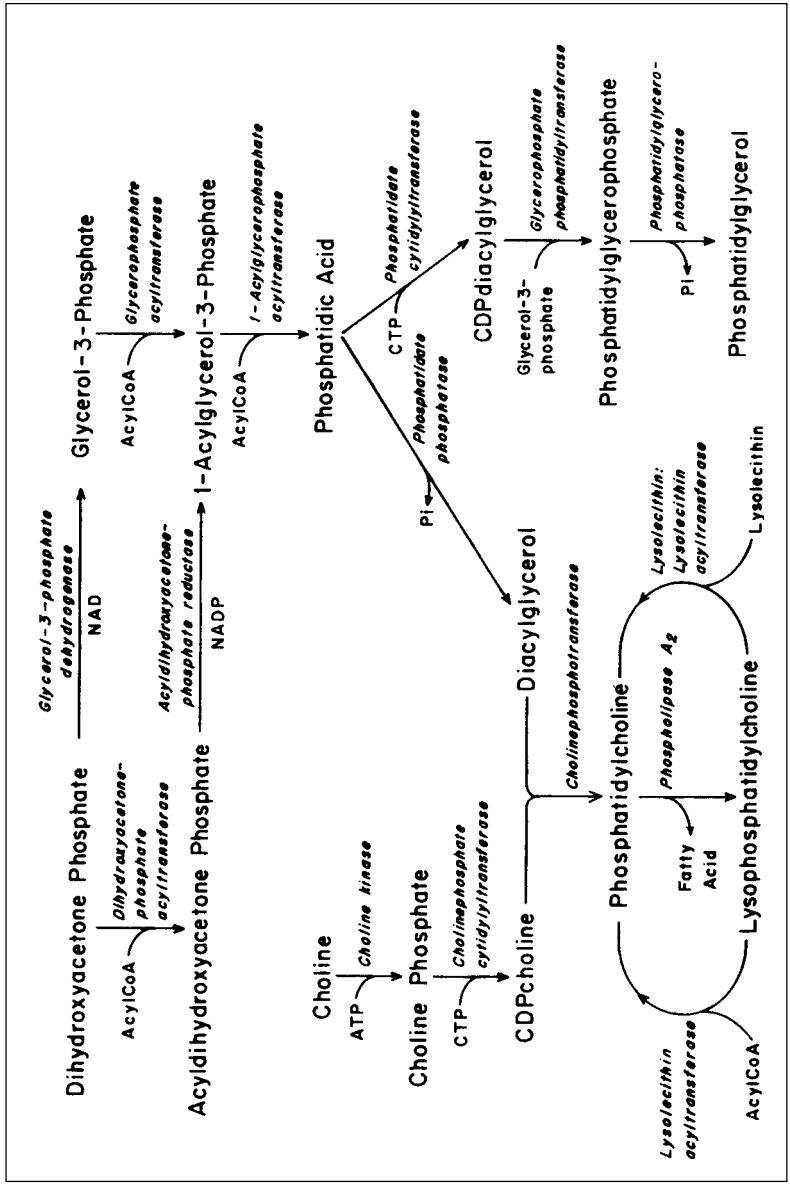

Fig. 2.1. Pathways of phosphatidylcholine and phosphatidylglycerol biosynthesis. Reproduced with permission from Rooney SA. Biosynthesis of lung surfactant during fetal and early postnatal development. Trends Biochem Sci 1979; 4:189-191.

All available evidence suggests that, of the two remodeling mechanisms, only the reacylation pathway is quantitatively important in the synthesis of lung DSPC.[2,5] The phospholipase A_2 involved in the remodeling pathway is a calcium-independent enzyme with an acidic pH optimum that has recently been cloned.[28]

Developmental and Hormone-Induced Changes in Lung Phospholipid Biosynthesis

There is a surge in surfactant production toward the end of gestation as the fetus prepares for extrauterine life. There is a 10-fold increase in the amount of surfactant phospholipid in lung lavage from fetal rabbits during the final 13% of gestation and an increase of similar magnitude in the first few days after birth.[2,5] The postnatal increase is due to increased secretion in response to labor and ventilation (see chapter 7) but the prenatal increase is largely due to increased synthesis. There is a developmental increase in rates of substrate incorporation into PC and DSPC in the late gestation fetal lung and there are also developmental increases in the activities of enzymes involved in the biosynthesis of those and other surfactant phospholipids.[2,5]

Developmental increases in the activities of many of the enzymes listed in Figure 2.1 were reported in the fetal lungs of several species late in gestation.[5] However, with the exception of two enzymes, there are considerable inconsistencies between different studies, even among those conducted in the same species.[5] The two enzyme activities that are consistently increased in late gestation fetal lung are CYT and fatty-acid synthase (FAS). CYT is involved in the de novo synthesis of PC (Fig. 2.1), whereas FAS is involved in the biosynthesis of long chain fatty acids. As discussed subsequently, CYT and FAS have pivotal roles in the regulation of surfactant PC biosynthesis.

CYT activity increases in fetal rat, rabbit, and mouse lung either at the end of gestation or immediately after birth.[2] Consistent with its involvement in the biosynthesis of surfactant PC, there is also a developmental increase in CYT activity in type II cells isolated from fetal rats[29] and that increase in activity is accompanied by increased enzyme mass and mRNA content.[30] The increase in CYT mRNA content in fetal type II cells is due to an increase in mRNA stability rather than increased transcription.[31]

De novo fatty acid biosynthesis, as determined by the rate of ^3H_2O incorporation into fatty acids, increases in the lung during late fetal life.[17,32] Starting from citrate, an intermediate in the citric acid cycle, long chain fatty acid biosynthesis is accomplished by just three enzymes: ATP-citrate lyase, acetyl-CoA carboxylase and FAS.[6,33] ATP-citrate lyase converts citrate to acetyl-CoA, acetyl-CoA carboxylase converts acetyl-CoA to malonyl-CoA and FAS completes the process in a series of seven distinct biochemical reactions, the first six of which are repeated for the addition of each two-carbon unit. The increase in fatty acid biosynthesis in fetal lung appears to be largely due to increased expression of the FAS gene. FAS activity,[34-36] mass[34] and mRNA content[37,38] all increase developmentally in late gestation fetal lung and the time courses of the increase in de novo fatty acid biosynthesis and FAS activity are almost identical.[35] Developmental increases in the activities and/or mRNA levels of fetal lung ATP-citrate lyase[37,38] and acetyl-CoA carboxylase[32,36] have also been reported, but the increases in ATP-citrate lyase were much less pronounced than those in FAS,[37,38] and the increases in acetyl-CoA carboxylase were not confirmed.[35,38]

There is a developmental increase followed by a subsequent decrease in the amount of glycogen in fetal lung,[5] and there are corresponding developmental changes in enzymes of glycogen synthesis and degradation.[35,39,40] The temporal relationship between glycogen depletion and increased PC synthesis, which has also been reported in isolated fetal type II cells,[41] led to speculation that glycogen provides substrate or energy for surfactant phospholipid biosynthesis in the fetal lung.[42] A direct relationship between glycogen depletion and in-

creased phospholipid synthesis has not been proved, however. Indeed the reciprocal relationship that exists between fetal lung glycogen content and PC biosynthesis also exists between glycogen and fatty acid biosynthesis,[17] so that glycogen is just as likely to provide substrate or energy for the biosynthesis of fatty acids as for that of phospholipids.

Lung maturation and surfactant production in the fetus can be accelerated by a number of hormones and growth factors.[2,4,5,7] Glucocorticoids were the first hormones reported to stimulate fetal lung surfactant production[5] and they are used clinically to prevent RDS in premature human infants.[4] Glucocorticoids accelerate all parameters of surfactant phospholipid biosynthesis. Such parameters include increased surfactant phospholipid content, increased synthesis of PC and DSPC, increased activities of CYT and FAS and acceleration of the normal developmental profile of glycogen content.[5] In addition to CYT and FAS, glucocorticoids have also been reported to increase the activities of other phospholipid biosynthetic enzymes, but, as in the case of normal development, many such reports were often not confirmed.[2,5] The stimulatory effects of glucocorticoids are directly on the fetal lung. Glucocorticoid receptors exist in the lung and the type II cell and stimulatory effects of glucocorticoids have been demonstrated in explants of fetal lung in organ culture as well as in intact animals in vivo.[5] However, the stimulatory effects of glucocorticoids are very modest in fetal[43,44] and adult[45] type II cells, so it appears that the effect of glucocorticoids on surfactant phospholipid biosynthesis is an indirect effect on the type II cells mediated by mesenchymal factors, possibly from fibroblasts.[7,9,44,46,47]

Other hormones, growth factors and cytokines that have been reported to accelerate parameters of surfactant production in fetal lung include thyroid hormone,[2,4,5,7] thyrotropin releasing hormone (TRH),[4,5] estrogen,[2,4,5,7] prolactin,[2,4,5,7] parathyroid hormone,[48] epidermal growth factor (EGF)[5,49-51] and keratinocyte growth factor.[52] Effects of estrogen are largely confined to the rabbit, whereas the effects of prolactin have not generally been confirmed,[4,5,7] The stimulatory effect of thyroid hormone is a consistent finding in several species, and maternal administration of TRH, a tripeptide that readily crosses the placenta and increases fetal thyroid hormone production,[4] has been used clinically in combination with glucocorticoids to prevent RDS.[53-56] Thyroid hormone[2] and estrogen[2] stimulate CYT activity in fetal lung, and EGF has a similar effect in the newborn.[57] However, the mechanism by which other agents stimulate surfactant phospholipid production has not been established.

Regulation of Phospholipid Biosynthesis

Two enzymes have major roles in the regulation of PC biosynthesis in the lung, particularly during late fetal development. The enzymes are CYT (EC 2.7.7.15) and FAS (EC 2.3.1.85).

Choline-Phosphate Cytidylyltransferase (CYT)

CYT, the enzyme that catalyzes formation of CDPcholine from choline phosphate and CTP (Fig. 2.1), is a rate-regulatory enzyme in PC biosynthesis in all systems examined,[58-60] including the lung.[2,5] Consistent with a rate-regulatory role for CYT, the size of the choline phosphate pool is larger than that of choline or CDPcholine in both whole lung and type II cells.[61-63] Pulse-chase studies with radiolabeled choline have also established that the choline phosphate pool is larger than that of the other choline compounds in the pathway.[63-65]

Altered CYT activity invariably accompanies altered PC biosynthesis[58,60] and the developmental increase in PC biosynthesis in developing fetal lung is accompanied by increased CYT activity.[2,5] Glucocorticoids, thyroid hormone and estrogen, hormones that increase fetal lung PC biosynthesis,[2,5] also increase CYT activity in fetal rat, rabbit, mouse and human lung.[66-71] Such data are consistent with a major rate-regulatory role for CYT in lung PC biosynthesis. As noted above, developmental and hormone-induced increases in the

activities of other enzymes of PC biosynthesis have also been reported but such effects are not consistent. However, the possibility that enzymes other than CYT may also have regulatory roles, at least in some circumstances, cannot be excluded.[59]

The mechanism by which CYT activity is increased in response to glucocorticoids in fetal lung has been extensively investigated. The glucocorticoid induced increase in CYT activity is a direct effect on the lung as it is apparent in fetal lung explants treated with the hormone in culture[67,69,70] as well as in lung tissue[66,67,71] and isolated type II cells[72] from fetuses whose mothers were injected with the hormone in vivo. Glucocorticoids generally act by binding to a cytosolic receptor that is translocated to the nucleus where it binds to regulatory regions of genes thereby promoting expression of the gene and synthesis of new mRNA and protein. The increase in CYT activity in fetal lung in response to dexamethasone is mediated by the glucocorticoid receptor, as the dissociation constant (K_d) for dexamethasone binding to the nuclear receptor is identical to the concentration eliciting 50% of its maximum effect (EC_{50}) on CYT activation.[73] In addition, the potency order of steroids in binding to the receptor and in stimulating CYT are the same.[73] The time course of glucocorticoid stimulation of CYT activity is consistent with that of new protein synthesis, as the increase does not become detectable until about 20 hours after addition of dexamethasone to explants of fetal lung[74] or to mixed fetal lung cells in culture.[75] The stimulatory effects of glucocorticoid on both PC synthesis and CYT activity are abolished by actinomycin D,[73] an inhibitor of transcription, and the effect on CYT activity is strongly antagonized by cycloheximide, an inhibitor of protein synthesis.[75] The latter data are again consistent with the effect of CYT being due to increased mRNA and protein synthesis.

The above data are consistent with the stimulatory effect of CYT being due to increased gene expression. However, all available evidence suggests that expression of the CYT gene is not increased by glucocorticoids in fetal lung and that the stimulatory effect of the hormone is due to activation of existing enzyme rather than synthesis of new CYT. The amount of CYT protein, as measured by immunotitration[69] and immunoblotting,[71] is not increased by glucocorticoids in fetal rat lung. CYT mRNA levels are not increased by glucocorticoids in lung tissue and type II cells from fetuses of pregnant rats injected with dexamethasone in vivo[31] or in fetal lung explants[76] and fetal type II cells[44] cultured with glucocorticoids. CYT activity can be increased several fold by inclusion of phosphatidylglycerol and other lipids in the assay mixture,[68,77] and when fetal lung CYT is assayed in the presence of sufficient lipid to achieve maximum activation in vitro the stimulatory effect of glucocorticoids is considerably reduced or completely abolished.[67,69,71,73,74] As the lipids would be expected to activate any newly synthesized CYT just as much as existing enzyme, those data strongly suggest that it is the catalytic activity rather than the amount of CYT that is increased by glucocorticoids. It is likely that other hormones that stimulate CYT activity also activate existing enzyme rather than increase expression of the CYT gene, as the stimulatory effects of thyroid hormone[67] and estrogen[61,68,78] on CYT activity in fetal lung are also abolished by inclusion of lipids in the in vitro assay and the increase in CYT activity in response to EGF in newborn rat lung is not accompanied by an increase in enzyme mass.[57]

CYT activity in fetal lung cytosol is markedly decreased by extraction of lipids with solvents that do not cause protein denaturation.[68,71] Lipid extraction under the same conditions completely abolishes the stimulatory effects of betamethasone in the rat[71] and of estrogen in the rabbit.[68] CYT activity and the stimulatory effects of the hormones are fully restored on readdition of the appropriate lipid extract.[68,71] Such data establish that the stimulatory effects of those hormones are mediated by a lipid factor(s). Free fatty acids[79] as well as a number of phospholipids[68,77,80] are known to activate CYT activity in vitro. Betamethasone elevates the levels of a number of phospholipids and fatty acids in fetal rat lung.[71] Estrogen also increases phospholipid levels in fetal rabbit lung but its effect on fatty acids has not

been examined.[68] As discussed in detail below, glucocorticoids enhance fatty acid biosynthesis and increase expression of the FAS gene in the late gestation fetal lung. Inhibition of fatty acid biosynthesis in fetal rat lung explants completely abolished the stimulatory effect of dexamethasone on CYT activity.[74] That suggests that the stimulatory effect of glucocorticoids on CYT activity in fetal lung is mediated by induction of the FAS gene. Whether the effect of the hormone is mediated by free fatty acids, by lipids into which they become incorporated or possibly by other fatty acid metabolites is not clear at present. The fact that linoleic acid is the fatty acid that was increased to the greatest extent by betamethasone in fetal rat lung and the same fatty acid was the most effective in activating CYT in vitro led to the suggestion that glucocorticoid stimulation of fetal lung CYT activity is mediated by linoleic acid.[81] However, linoleic acid is an essential fatty acid that is not synthesized by mammals and that must be provided from nutritional sources. It is consequently difficult to reconcile the notions that glucocorticoid activation of CYT is mediated by both induction of FAS and by linoleic acid. In summary, although there is compelling evidence that the stimulatory effect of glucocorticoids on CYT activity is mediated by endogenous lipid(s), the precise nature of the activator(s) remains to be established.

CYT is an ambiquitous enzyme in that it is found in more than one subcellular compartment. CYT exists in the cell cytosol and has also been reported in a number of membrane fractions including endoplasmic reticulum, Golgi apparatus and nucleus.[59] The precise subcellular distribution is controversial. Some distribution differences may be due to technical considerations, but there also may be tissue to tissue variation.[82,83] There is evidence that activation of CYT in many systems is due to translocation of the enzyme from a relatively inactive cytosolic form to a microsomal fraction where it is activated.[2,59] In the fetal lung,[2] however, and in a number of other systems,[59] activation of CYT is not mediated by subcellular translocation. The stimulatory effects of glucocorticoids on fetal lung CYT activity in the rat[73] and of estrogen in the rabbit[68] are not accompanied by translocation of the enzyme from cytosol to microsomes. In most studies, the stimulatory effects of glucocorticoids and estrogen are entirely on cytosolic CYT with little increase in microsomal activity.[68,70,71,73] In some studies, however, the stimulatory effects of glucocorticoids were observed in the microsomes alone[72] or in both cytosol and microsomes.[75] However, the increased microsomal CYT activity was not accompanied by a decrease in cytosolic activity[72,75] and cannot therefore be attributed to translocation. Cytosolic CYT in the lung has been reported to exist in an inactive low molecular weight form and in an active high molecular form.[84] In some studies, glucocorticoid stimulation of cytosolic CYT was accompanied by a change form low to high molecular size forms[71,81] and that process was also mimicked by fatty acids.[85] Recently, specific amino acid residues have been reported to be essential in mediating association of CYT with membranes.[86] Deletion or mutation of such regions of the CYT molecule led to altered enzyme activity and PC biosynthesis in A549 and L-2 lung epithelial cell lines.[86] However, it has not been established if such regions of the CYT molecule or association of CYT with membranes have any role in the developmental or hormonal regulation of CYT activity in developing fetal lung.

Despite the fact that there is no evidence of increased CYT mass[69,71] or mRNA content[31,76] in fetal lung in response to hormones, the possibility of increased expression of the CYT gene cannot be completely discounted. Although the stimulatory effects of glucocorticoids and other hormones were largely eliminated when the CYT assay was carried out under conditions of maximum in vitro activation,[66-69,71,73,74] small increases in activity remained in a number of glucocorticoid studies[66,69,71,73-75] and were sometimes statistically significant.[66,73,75] Indeed, the increase in CYT activity in response to dexamethasone in human fetal lung explants was more pronounced, and only significant when phosphatidylglycerol was included in the assay mixture.[70] An approximately 30% increase in CYT

mRNA content was reported in fetal rat type II cells exposed to both cortisol and fibroblast-conditioned medium.[44] However, CYT activity was not measured, so it is unclear if such a small increase in mRNA level is meaningful as, in a subsequent study from the same laboratory, dexamethasone did not increase CYT mRNA content in lung explants.[76] In contrast to hormonal stimulation, it is clear that the normal developmental increase in CYT activity in fetal type II cells is due to increased gene expression, as the amounts of CYT protein and mRNA are correspondingly increased.[30]

Fatty-Acid Synthase (FAS)

FAS catalyzes the synthesis of long chain fatty acids from acetyl-CoA and malonyl-CoA. The reaction involves a series of seven distinct biochemical steps, the first six of which are repeated for the addition of each two-carbon unit to the growing fatty acid chain. In animals, the 16-carbon fatty acid, palmitic acid, is the usual product of the FAS reaction. In prokaryotes and plants the seven reactions are carried out by separate, loosely associated proteins, but in animals and yeast they are carried out by single multifunctional proteins.[87,88] Animal FAS (EC 2.3.1.85) is a homodimer (M_r approximately 570,000) of two identical subunits, whereas yeast FAS (EC 2.3.1.86) is an $\alpha_6\beta_6$ multimer (M_r approximately 2.3×10^6) of two nonidentical polypeptides each of which have M_r of approximately 200,000.[87,88] Animal FAS is encoded by a single gene that is 18 kilobases (kb) in the rat,[88,89] ~19 kb in the human[90] and 50 kb in the goose.[91] The rat FAS gene contains 43 exons from which two equally abundant mRNAs, a consequence of two different polyadenylation signals, are transcribed.[88,89,92,93] Two mRNAs are also transcribed in goose[91,94] and chicken[95] but there is only one transcript in some species,[96,97] including humans.[90,98] The FAS gene has been localized to chromosome 17q in the human[98,99] and to syntenic distal chromosome 11 in the mouse.[98]

Fatty acids are integral components of glycerophosphatides and consequently key substrates in the synthesis of surfactant lipids. It is clear that fetal and adult lungs have the ability to synthesize fatty acids de novo.[6] However, fatty acids can also be supplied via the blood[6,8] and the relative importance of the two sources in the provision of fatty acids for surfactant synthesis is not known. As there is little blood supply to the lungs during fetal life, it is likely that de novo synthesis is of quantitative importance at that stage of development. That notion is supported by the findings, discussed in a previous section, of a developmental increase in the rate of de novo fatty acid biosynthesis in fetal lung at the time of increased surfactant synthesis.[17,32] However, the most compelling evidence for the importance of fatty acids in surfactant PC biosynthesis in fetal lung is the finding that the stimulatory effect of glucocorticoids on activation of CYT is completely abolished by inhibition of fatty acid biosynthesis.[74]

The developmental increase in the rate of fatty acid synthesis is accompanied by increased FAS activity and increased expression of the FAS gene,[34-38] with relatively little or inconsistent changes in the activities and expression of ATP-citrate lyase and acetyl-CoA carboxylase,[32,35-38] the other two enzymes involved in de novo fatty acid biosynthesis. Glucocorticoids stimulate the rate of ^3H_2O incorporation into fatty acids in developing fetal rat,[17] rabbit[100] and human[101] lung. As in the case of normal development, the increase in de novo fatty acid biosynthesis in response to glucocorticoids is also due to increased FAS activity. Glucocorticoids increase FAS activity in fetal rat[35,38,102] and human[101] lung but they do not increase the activities of ATP-citrate lyase[38] or acetyl-CoA carboxylase.[35] Taken together, the above data suggest that FAS has a pivotal role in the regulation of surfactant PC biosynthesis in fetal lung.

The glucocorticoid effect is directly on the lung, as increased fatty acid biosynthesis and FAS activity occur in cultured fetal lung explants[38,100,101] as well in lung slices from

fetuses whose mothers are injected with glucocorticoid in vivo.[17,35] It is clear that the effect is mediated by the glucocorticoid receptor as the EC_{50} (0.5 to 1.5 nM) for stimulation of fatty acid biosynthesis,[100] and FAS activity[101,102] is similar to or less than the K_d for nuclear receptor binding. In addition, the potency order of steroids in stimulating FAS activity and in binding to the receptor are the same.[101,102] Maximum stimulation of FAS activity is not achieved until 20-30 hours after exposure of fetal rat lung explants to dexamethasone,[74,102] and the effect of the hormone is abolished by actinomycin D.[102] Such data suggest that the glucocorticoid effect is due to increased mRNA and protein synthesis. In contrast to CYT, there is convincing evidence that the stimulatory effect of glucocorticoid on FAS activity is indeed due to increased expression of the FAS gene. In fact, FAS is the only gene for a lipogenic enzyme in fetal lung that has been shown to be induced by glucocorticoids.

Immunotitration[102] and immunoblotting[103] established that dexamethasone increases the amount of FAS in fetal rat lung. The increase in FAS activity and mass are both approximately 2-fold.[103] Glucocorticoids also increase FAS mRNA content in both fetal rat[38,76] and human[104] lung explants. As in the case of enzyme activities, glucocorticoids do not increase ATP-citrate lyase or acetyl-CoA carboxylase mRNA levels in fetal rat lung.[38,76] There is a biphasic increase in FAS mRNA content in response to dexamethasone in fetal rat lung, an early increase that is maximal (almost 3-fold) 5 hours after addition of the hormone followed by a later increase of similar magnitude that reaches a plateau after 28 hours.[38] The early increase is antagonized by actinomycin D, suggesting that it is due to increased transcription.[38] However, the later increase cannot be attributed to increased transcription, as it is not antagonized by actinomycin D.[38] Nuclear run-on assays confirmed that glucocorticoids do indeed increase transcription of the FAS gene in fetal rat lung.[103] The increase in the rate of transcription is maximal 1 hour after addition of the hormone and is still apparent for at least 48 hours. The rate of transcription of the FAS gene[103] and the amount of FAS mRNA[38,103,105] are both maximally increased 2- to 3-fold by the hormone. Glucocorticoids also increase FAS mRNA stability.[105] The apparent half-life of FAS mRNA in fetal rat lung is about 4 hours and it is increased 84% by dexamethasone.[105] These data establish that glucocorticoids increase expression of the FAS gene in developing fetal lung. The increased expression occurs by two mechanisms: an increase in the rate of gene transcription and an increase in the stability of the mRNA transcripts.

Increased FAS activity is also due to increased gene expression in all other systems examined. There are no indications that the enzyme is subject to short term regulation by covalent modification, metabolites or other allosteric factors. Expression of the FAS gene in liver and adipose tissue is regulated by developmental[106,107] and nutritional factors.[33,108-110] Gene expression is also increased by insulin[111-113] and thyroid hormone[107,111,114] and decreased by adenosine 3',5'-cyclic monophosphate (cAMP),[96,114,115] glucagon[114,115] and growth hormone[97,116] in liver and fat cells and increased by progesterone[117] and androgens[118] in breast and prostate cancer cells, respectively. Most increases in expression of the FAS gene are due to increased transcription,[33] although increased FAS mRNA stability, both with[107,111,117] and without[109] increased transcription, has also been reported. In one study, increased FAS synthesis was attributed to increased translation, as mRNA levels were not increased.[119]

Apart from the fetal lung, there are few reports of glucocorticoid regulation of FAS gene expression in other tissues. Corticosterone was reported to enhance the thyroid hormone-induced increase in FAS mRNA content in chick hepatocytes.[120] In rat hepatocytes, on the other hand, dexamethasone alone was reported to increase FAS gene expression but to decrease the effect of thyroid hormone.[121] Dexamethasone was reported to enhance the stimulatory effect of insulin in hepatocytes[121] but to antagonize it in adipose tissue.[115] Dexamethasone increased chloramphenicol acetyltransferase (CAT) expression in several tissues

of transgenic mice transfected with the 2.1 kb 5' flanking promoter region of the FAS gene fused with the CAT reporter gene.[122] The greatest increase was in white adipose tissue, followed by liver, and the increase was also apparent in the lung.[122]

The molecular mechanisms by which hormones and other factors regulate transcription of the FAS gene have recently been investigated.[123-134] The DNA sequences (cis-acting elements) responsible for the regulatory effects of insulin,[123-127] cAMP[128,129] and nutrients[130-132] have been identified. The sequence 68 to 52 base pairs (bp) in the 5' flanking region upstream of the transcription start site (-68 bp to -52 bp) is responsive to insulin[124] and the -99 bp to -92 bp sequence to cAMP.[128] Nutrient responsive sequences are within 300-500 bp upstream or downstream of the transcription start site.[125,130,131] Information on nuclear proteins (trans-acting elements) that bind to the DNA is also becoming available.[126,129,133,134]

There is little information on the cis- and trans-acting elements responsible for glucocorticoid regulation of FAS transcription. The 5' end and flanking region of the rat,[89,135] human[136] and goose[91] FAS genes contain several sequences that may be involved in transcriptional regulation. In particular, one half of the glucocorticoid response element (GRE) consensus sequence is located at -422 bp in the rat gene[89] and there are also tentative GREs in the first intron.[89,135] Putative GREs have also been identified in the human[136] and goose[91] FAS genes. To identify functional GREs or other glucocorticoid responsive regions, we transiently transfected A549 cells with chimeric constructs consisting of serial deletions of the -1592 bp to +67 bp region of the rat FAS gene ligated to the firefly luciferase reporter gene.[137] Dexamethasone increased luciferase gene expression in response to all constructs as much as 2-fold. The full effect of the hormone was retained in response to the -33 bp to +56 bp fragment, suggesting that this region has an important role in glucocorticoid stimulation of FAS gene expression.[138] Further studies are required to identify the precise DNA sequence responsible for the glucocorticoid effect, as well as trans-acting factors that bind to it.

Future Directions

In recent years the major emphasis in studies on surfactant biosynthesis has been on the protein components and the lipids have been less studied. That is largely due to the fact that the surfactant protein genes were cloned earlier than those involved in lipid biosynthesis. Molecular approaches to regulation of expression of the surfactant protein genes have long been feasible. However, it is important to emphasize that the lipids, particularly PC and DSPC, are the major components of surfactant and are critical for its function in reducing surface tension at the alveolar surface (see chapter 1). A complete understanding of the mechanisms regulating synthesis of surfactant lipids is essential in order to prevent and/or treat RDS and other lung diseases resulting from surfactant deficiency.

Much is known about regulation of PC biosynthesis, particularly in the developing fetal lung. However a number of outstanding questions remain to be answered. It is clear that fatty acid biosynthesis has a critical role in the glucocorticoid regulation of CYT activity. However, the mechanism by which CYT activity is regulated and whether it is activated by free fatty acids, lipids into which they become incorporated or possibly other fatty acid metabolites needs to be established. Do other hormones and/or agents used in the prevention of RDS stimulate CYT activity in the same manner as glucocorticoids? Do such agents increase expression of the CYT gene? How is expression of the CYT gene regulated during normal development? Do fatty acids have a role in regulation of CYT in adult lung and, if so, is expression of the FAS gene involved in the process? How is FAS gene transcription stimulated by glucocorticoids? What cis-active regions of the gene are involved and do transactive proteins other than the glucocorticoid receptor play a role? How is FAS mRNA stability increased by glucocorticoids? How important is de novo fatty acid synthesis in the lung

versus supply by the blood in the provision of substrate for surfactant biosynthesis? Are enzymes other than CYT involved in the regulation of surfactant synthesis? Addressing those and related questions will lead to a more comprehensive understanding of the regulation of surfactant phospholipid biosynthesis.

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CHAPTER 3

The Surfactant Protein A Gene and Its Regulation

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Surfactant protein (SP)-A, the major pulmonary surfactant-associated protein, is a sialoglycoprotein ($M_r \approx 29\text{-}36{,}000$) synthesized primarily in alveolar type II cells.[1] A number of functions have been ascribed to it. SP-A binds strongly to surfactant glycerophospholipids[2] and acts in the presence of calcium and SP-B to promote the structural transformation of the lamellar body to tubular myelin.[3,4] SP-A also acts in a cooperative and calcium-dependent manner with the hydrophobic surfactant-associated proteins SP-B and SP-C to promote the rapid formation of phospholipid surface films and thus facilitates the reduction of alveolar surface tension[5] (see chapter 1). Through calcium-dependent binding to specific high affinity receptors on the surface of type II cells, SP-A has been reported to mediate reutilization of secreted surfactant components[6,7] (see chapter 8), and to inhibit surfactant secretion.[8,9] Secreted SP-A also may serve a bacteriocidal function in lung by facilitating bacterial killing through its binding to and activation of alveolar macrophages[10-12] (see chapter 9).

In mice rendered SP-A deficient by targeted gene disruption, secreted surfactant contains decreased numbers of tubular myelin figures, and at low surfactant concentrations, minimum surface tensions are elevated as compared to surfactant of wild type animals.[13] Despite these differences, perinatal survival of SP-A -/- mice is similar to wild type. Upon infection with group B streptococcus by tracheal instillation, SP-A -/- mice have increased pulmonary infiltration of the bacteria, with dissemination to the spleen and decreased association of the bacteria with macrophages as compared to wild type mice.[14]

SP-A gene transcription is initiated in fetal lung after ~70% of gestation is completed, and reaches maximal levels just prior to birth.[15] Temporal regulation of SP-A gene expression is more closely associated with the induction of surfactant glycerophospholipid synthesis and appearance of identifiable type II cells than is the developmental regulation of genes encoding SP-B, SP-C and SP-D. Studies from a number of laboratories suggest that SP-A gene expression in fetal lung is under multifactorial control; we have observed that glucocorticoids and agents that increase cyclic AMP (cAMP) play important roles in its regulation.[16-18] In this chapter, we will first briefly consider SP-A structure, followed by a more comprehensive review of the SP-A genes and the mechanisms of regulation.

Structure of SP-A

The primary structure of SP-A is highly conserved among species and is comprised of 247-248 amino acids (see ref. 1 for review). A hydrophobic amino-terminal signal sequence

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is presumably cleaved upon insertion of the protein into the lumen of the endoplasmic reticulum. However, in attempts to obtain the sequence of the amino-terminus of rabbit SP-A, we were unable to obtain meaningful data, suggesting that the amino-terminus could be blocked. Floros and colleagues[19] also suggested that the signal peptide may not be cleaved. SP-A has four distinct structural domains; a short amino-terminal domain, a collagen-like sequence comprised of 24 Gly-X-Y repeats (where Y is frequently proline) that is interrupted once at its mid-position,[19] a largely hydrophobic neck region and a carboxyterminal carbohydrate recognition domain (CRD) that comprises approximately one-half of the protein.[20] The CRD is highly similar to the carboxyterminal globular domains of a number of mammalian C-type lectins, such as mannose-binding protein which also has an amino-terminal collagen-like region.[21] Depending on the species, there are one or two consensus sites for N-linked oligosaccharide addition; the site that is conserved in SP-A of all species is present within the CRD. The other glycosylation site is present at the extreme amino-terminus. As in the case of collagen, the Gly-X-Y repeats of SP-A form a triple helix. Post-translational modifications of SP-A include potential cleavage of the signal peptide, formation of interchain disulfide bonds at the amino-terminus, hydroxylation of proline residues within the collagen-like domain and addition of N-linked oligosaccharide sidechains containing sialic acid residues. Within the alveolus, SP-A exists as an octadecamer comprised of six triple helical structures (18 polypeptide chains) with a molecular weight of approximately 700,000.[22] This high molecular weight aggregate of SP-A, which is due both to sulfhydryl- and nonsulfhydryl-dependent interactions, has been likened to a flower bouquet.

Structure of the SP-A Gene

SP-A exists as a single-copy gene in rabbits,[23,24] rats[25] and mice.[26] In contrast, humans possess two transcribed SP-A genes (SP-*A1* and SP-*A2*)[19,20,27,28] and an SP-A pseudogene in their genomes.[29] Those three genes, together with the genes encoding SP-D and the mannose-binding protein C-type lectins, are localized in the same region of human chromosome 10.[30] By use of primer extension and reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of 5'-cDNA ends (RACE), we found that a major difference between the two human SP-A (hSP-A) genes lies in their exon-intron organization; *h*SP-*A2* mRNA transcripts are always comprised of sequences contained within 6 exons, while the majority of transcripts of the *hSP-A1* gene are comprised of sequences contained within 5 exons.[28] The unique exon of the *h*SP-*A2* gene (exon II of VI) is a 30 base pair (bp) sequence which encodes additional 5'-untranslated region of the mRNA.

The baboon, like the human, also contains two highly similar SP-A genes in its genome.[31] This finding suggests that duplication of the SP-A gene occurred more than 26.5 million years ago, prior to the time that ancestral catarrhini diverged into the old world monkeys (baboons and macaques) on the one hand and into the gibbons, great apes and man on the other.[32] The SP-A proteins encoded by the two baboon SP-A genes are 97.2% identical at the amino acid level (242 out of 249 amino acids are identical). This represents a higher degree of similarity than that which exists between the two human SP-A proteins (95.6%; 238 out of 249 amino acids are identical). Since the platyrrhini, or new world monkeys, and the catarrhini diverged from ancestral anthropoids more than 40 million years ago,[32] it will be of interest to determine whether new world monkeys such as marmosets, owl, wooly and squirrel monkeys[33] possess one or two SP-A genes. We named the two baboon genes baboon SP-*A1* (*bSP-A1*) and *bSP-A2*, because by comparison of 5'-flanking and intronic sequences with the human SP-A genes, one is more similar to *hSP-A1* and the other to *hSP-A2*. SP-A mRNA transcripts encoded by the *b*SP-*A1* gene are comprised of sequences contained within either 5 or 6 exons. However, the mRNA transcripts encoded by the *b*SP-*A2* gene are comprised of sequences contained within only 5 exons. This is in con-

trast to the human, in which mRNA transcripts of the *h*SP-*A1* gene are encoded by sequences contained within 5 exons whereas mRNA transcripts of the *h*SP-*A2* gene are encoded by sequences present in 6 exons.[28]

Regulation of SP-A Gene Expression in Fetal Lung

Tissue and Cellular Localization of Expression

Expression of the SP-A gene is essentially lung-specific.[23] However, low levels of SP-A expression have been reported in intestinal epithelium[34] and in a phospholipid-rich layer recovered from rat and human colon.[35] The results of in situ hybridization studies indicate that SP-A gene expression within the lung occurs primarily in alveolar type II cells,[36] and to a lesser extent in nonciliated bronchiolar epithelial (Clara) cells of proximal and distal airways.[37-38] In second trimester human fetal lung, SP-A and its mRNA also have been detected in nonmucous tracheal glands, in bronchial glands and in isolated cells of conducting airway epithelium.[39]

Developmental Regulation

Expression of the SP-A gene is developmentally regulated in fetal lung tissue. SP-A gene expression is initiated after ~75-80% of gestation is completed in all mammalian species thus far studied; in this respect, the developmental induction of SP-A expression is more closely linked to surfactant glycerophospholipid synthesis than is expression of any of the genes encoding the other surfactant-associated proteins. SP-A gene transcription is first discernible in fetal rabbit lung tissue on day 24 of gestation and reaches maximum levels by day 28.[40] This increase in SP-A gene transcription is associated with the appearance of SP-A and its mRNA on day 26,[16,40,41] just prior to the time at which augmented surfactant glycerophospholipid synthesis occurs. In situ hybridization analysis of fetal rabbit lung tissue at different developmental stages revealed that SP-A mRNA is first detectable in type II cells on day 26 of the 31 day gestation period; by day 31, SP-A mRNA transcripts are also detectable in Clara cells.[37,38] In rats, SP-A and SP-A mRNA are first detectable on day 18 of gestation (term = day 22); the levels increase markedly through day 21 to approximately 50% of adult levels, decline moderately during the first week of life and then increase to adult levels by day 28.[42] In human fetal lung tissue, SP-A and its mRNA are undetectable at 16-20 weeks of gestation.[43,44] Immunoreactive SP-A is detectable in amniotic fluid at 30 weeks gestation and increases further during development in association with the increase in the ratio of lecithin to sphingomyelin (L/S).[45]

Organ culture provides a convenient model system for study of developmental and hormonal regulation of SP-A gene expression in fetal lung; the preservation of tissue architecture and appropriate cellular interactions appear to be essential for the initiation and maintenance of type II pneumonocyte differentiation.[46,47] Lung explants from midgestation human abortuses[48,49] and 19- or 21-day fetal rabbits[50] differentiate spontaneously when placed in organ culture in serum-free, defined medium. Before culture, the tissue is comprised of small ducts surrounded by abundant connective tissue; the ductular epithelial cells are columnar, filled with cytoplasmic glycogen, and contain no lamellar bodies. Within 4 days of organ culture, the ducts enlarge, the amount of connective tissue decreases and the epithelium differentiates into recognizable type II cells containing lamellar bodies.[48] These changes in morphology are associated with an induction of SP-A mRNA and protein which are undetectable in the fetal lung prior to culture.[16,41] cAMP analogs enhance the rate of enlargement of the prealveolar ducts and of type II cell differentiation in midgestation human fetal lung explants.[17] cAMP also increases expression of the SP-A gene in rabbit, baboon and human fetal lung tissue in organ culture (see below).

Multifactorial Regulation

SP-A gene expression in fetal lung is regulated by a number of hormones and other factors including glucocorticoids, agents that increase cellular levels of cAMP, retinoids, insulin, growth factors, cytokines and activators of protein kinase C (PKC). Glucocorticoids and other hormones and factors that increase intracellular levels of cAMP have marked effects on SP-A gene expression in lung explants of the rabbit, baboon and second trimester human fetus.

Retinoids

Retinoids are well recognized as embryonic morphogens and as regulators of epithelial cell differentiation. In cultured human fetal lung, retinoic acid causes a dose-dependent decrease in SP-A mRNA levels, as well as a decrease in epithelium volume density and a corresponding increase in volume density of connective tissue. In contrast, retinoic acid causes a dose-dependent increase in SP-B mRNA levels although it inhibits SP-C gene expression, but only at the highest concentrations tested.[51] In cultured lung buds from 13.5-day gestation rat embryos, retinoic acid at high concentrations ($\geq 10^{-6}$ M) interferes with formation of the distal epithelial buds and promotes the development of tubules with characteristics of proximal airways.[52] In the same system, retinoic acid causes a dose-dependent decrease in the mRNAs encoding SP-A, SP-B and SP-C. Interestingly, expression of retinoic acid receptor-β is restricted to the trachea and larger proximal airways in the early mouse embryo.[53] This likely contributes to retinoid promotion of proximal airway development at the expense of branching and differentiation of distal epithelial buds and expression of surfactant protein genes.

Insulin

Although respiratory distress syndrome (RDS) is primarily associated with premature birth, term infants of mothers with certain forms of diabetes also manifest an increased incidence of the condition.[54] It has been suggested that the fetal hyperinsulinemia associated with maternal diabetes[55] exerts a deleterious effect on lung development. The observation that the incidence of RDS is increased in newborn infants of diabetic mothers, despite amniotic fluid L/S ratios indicative of fetal lung maturity, is suggestive that a surfactant component other than phosphatidylcholine (PC) is affected. It has been found that surfactant phosphatidylglycerol (PG) is markedly decreased in aminiotic fluid from diabetic pregnancies;[56] however, PG does not seem to serve an important role in surfactant function.[57] In two independent studies, SP-A levels in amniotic fluid from diabetic women were significantly reduced compared to gestational age-matched nondiabetic subjects.[45,58] In a third study, there was no difference between diabetic and nondiabetic subjects in amniotic fluid SP-A levels.[59] It was suggested that lung maturation and SP-A production in the infants of diabetic mothers in that study may be due to improved metabolic control.[59]

In midgestation human fetal lung in organ culture, concentrations of insulin as low as 2.5 ng/ml cause a marked inhibition of immunoreactive SP-A accumulation.[44] The effect of insulin is dose-dependent and apparent within 48 hours.[44] Insulin also causes a dose-dependent inhibition of SP-A mRNA accumulation in the same system.[60,61] On the other hand, only a high concentration of insulin (2.5 μg/ml) inhibits SP-B mRNA accumulation and insulin has no effect on SP-C mRNA content.[60]

In a rat model of streptozotocin-induced diabetes, the levels of SP-A and its mRNA are decreased in lung tissues of 18-21 day fetuses as compared to lungs of fetuses of nondiabetic mothers; in neonates, however, differences in SP-A expression are no longer observed.[62] Insulin treatment of the diabetic mothers during the last 5-10 days of pregnancy increases fetal lung SP-A mRNA levels as compared to the nontreated diabetic group; however, fetal

lung SP-A mRNA levels in the insulin-treated diabetic group remain lower than those of age-matched controls.[63] On the other hand, when diabetic rats are treated with insulin for the entire pregnancy, there is an inhibitory effect on fetal lung SP-A mRNA levels.[63] Dexamethasone treatment of streptozotocin-induced diabetic and control pregnant rats increases the levels of SP-A, SP-B and SP-C mRNA in lung tissues of 18-20 day fetuses.[64]

Growth factors and cytokines

A number of growth factors and cytokines regulate SP-A gene expression in fetal lung. In midgestation human fetal lung in culture, epidermal growth factor (EGF) causes a dose-dependent induction of SP-A synthesis and mRNA levels.[65] The stimulatory effect of EGF is antagonized by coincubation with transforming growth factor-β (TGF-β).[65] TGF-β_1, -β_2, and -β_3 are equally effective in inhibiting SP-A expression in NCI-H441-4 lung adenocarcinoma cells.[66] In embryonic mouse lung buds in culture, EGF stimulates branching morphogenesis and DNA synthesis,[67,68] and increases choline incorporation into disaturated PC and SP-C gene expression.[68] On the other hand, treatment of mouse embryonic lung buds with either the EGF receptor-specific tyrosine kinase antagonist tyrphostin[67] or an antisense oligonucleotide directed against EGF[69] inhibits branching morphogenesis and DNA synthesis. Mice homozygous for targeted deletion of the EGF receptor gene were created by three different research groups.[70-72] Depending upon their genetic background, mice in two of the studies develop respiratory failure at birth.[70,71] The respiratory failure is associated with a decrease in branching morphogenesis and reduced numbers of alveoli and differentiated type II cells.[73]

As mentioned above, members of the TGF-β family antagonize the stimulatory effects of EGF on SP-A gene expression. TGF-β also opposes the effects of EGF on branching morphogenesis in embryonic lung. In transgenic mice carrying a fusion gene in which a constitutively active form of TGF-β1 is placed under control of the SP-C promoter, there is perinatal mortality. Lung morphogenesis is arrested in these transgenic mice at the late pseudoglandular phase of development with decreased acinar bud formation and reduced expression of Clara cell secretory protein γ and proSP-C.[74] In mouse embryonic lung buds, the inhibitory effect of TGF-β1 on branching morphogenesis is associated with inhibition of N-*myc* expression.[75] This finding is of interest, since N-*myc* gene targeted mice also have defects in lung development.[76]

Tumor necrosis factor-α (TNF-α) and phorbol esters inhibit SP-A transcription in pulmonary adenocarcinoma cell lines.[77-79] The inhibition is blocked by actinomycin D, suggesting an indirect role for these factors in the regulation of SP-A gene transcription.[78] It should be noted that, whereas phorbol esters activate PKC, the effects of TNF-α are mediated by two different cell surface receptors coupled to several signal transduction pathways, including activation of PKC, sphingomyelinase and protease cascades.[80] TNF-α induction of apoptosis is mediated by induction of protease cascades. It is not known if one or more of these mechanisms mediate the pronounced inhibitory effects of TNF-α on SP-A expression; however TNF-α inhibition of SP-A expression in H441-4 lung adenocarcinoma cells occurs without effects on cell growth or viability.[78]

Interferon-γ (IFN-γ) causes a dose-dependent induction of SP-A and its mRNA in midgestation human fetal lung in culture. After 3 days of culture, IFN-γ and dexamethasone synergistically increase SP-A content. In contrast, IFN-γ has no effect on SP-B or SP-C expression.[81] It has been suggested that this cytokine may increase SP-A synthesis as part of the immune response to pulmonary infection.[81] Interleukin-1 (IL-1) is an example of another inflammatory cytokine produced by activated macrophages that induces SP-A expression in fetal lung. When IL-1α is injected into the amniotic sacs of fetal rabbits, it causes a dose-dependent increase in SP-A mRNA levels in fetal lung tissue and an increase in com-

pliance of lungs of prematurely delivered pups.[82] Treatment of fetal lung explants with IL-1α also increases SP-A expression.[83]

Keratinocyte growth factor/fibroblast growth factor-7

In type II cells in primary culture, maintenance of cellular differentiation, surfactant glycerophospholipid biosynthesis and SP-A, SP-B and SP-C gene expression are dependent on extracellular matrix (ECM) interactions,[84,85] cell shape[84] and type II cell-fibroblast interactions.[86] When adult rat type II cells are cultured on ECM in the absence of added fibroblasts, keratinocyte growth factor (KGF)/fibroblast growth factor-7 (FGF-7), which is produced by lung fibroblasts, markedly induces SP-A and SP-B mRNA and surfactant glycerophospholipid synthesis.[87] In mesenchyme-free embryonic lung epithelial cell cultures, KGF stimulates epithelial proliferation, resulting in cyst-like structures and enhanced expression of SP-A and SP-B mRNAs throughout the explants.[88] In contrast, acidic FGF (FGF-1) enhances epithelial proliferation resulting in bud formation (branching) without the formation of cysts. Effects of FGF-1 on epithelial cell differentiation are restricted to SP-B mRNA induction, which is evident only at the distal tips.[88]

Agents that increase intracellular levels of cAMP

The levels of SP-A and its mRNA in cultured fetal rabbit,[16,23] human[17] and baboon[89] lung are markedly induced by cAMP analogs. In contrast, the rat and mouse SP-A genes are essentially unaffected by cAMP treatment.[90] The lack of response of the rat and mouse SP-A genes to cAMP appears to be due to species-specific sequence differences within gene regulatory regions. This is because cAMP markedly induces promoter activity of the rabbit and human SP-A genes in transfected rat type II cells in primary culture, while expression of the endogenous SP-A gene in the rat cells is unaffected by cAMP.[91-93]

The inductive effects of cAMP on SP-A mRNA levels in rabbit[40] and human[94,95] fetal lung in culture are associated with comparable increases in the rate of SP-A gene transcription. As discussed previously, the human genome contains two genes, *hSP-A1* and *hSP-A2*, which are highly similar in their protein coding sequences, but which differ in their intron-exon organization and in the sequences of the 5'-flanking and 3'-untranslated regions.[28] A combination of primer extension and RNA Northern blotting were used to analyze the regulation of expression of the human SP-*A1* and SP-*A2* genes. Whereas *SP-A2* mRNA transcripts are present at levels approximately twice those of *SP-A1* in human adult lung tissue, only *SP-A1* mRNA transcripts were detectable in the lungs of a 28-week prematurely born infant. In human fetal lung explants cultured in the presence of dibutyryl cAMP (Bt_2cAMP), SP-*A2* gene expression is induced ~11-fold, whereas that of SP-A1 is increased only 2-fold.[96] These studies suggest that SP-*A2* is the more highly regulated gene during fetal development and is more sensitive to the effects of cAMP than SP-*A1*.

The β-adrenergic agonist terbutaline increases the levels of immunoreactive SP-A in human fetal lung in culture.[17] This suggests that catecholamines, acting through β-adrenergic receptors and cAMP, may stimulate SP-A gene expression in fetal lung. β-Adrenergic receptors have been identified in fetal lung tissues,[97-99] and their concentration, as well as the responsiveness of adenylate cyclase to catecholamines, increases in fetal rabbit lung tissue with advancing gestational age.[100] The findings that norepinephrine levels in human fetal plasma increase during late gestation,[101] and that administration of β-adrenergic agonists as tocolytic agents to women in preterm labor results in a decreased incidence of RDS in their prematurely born infants,[102-104] are further suggestive of a role for the adrenergic system in fetal lung maturation and surfactant synthesis.

The lung actively synthesizes and degrades prostaglandins and other eicosanoids.[105] Prostaglandins have been reported to have a role in surfactant secretion in adult type II

cells[106] and in the synthesis of surfactant glycerophospholipids in fetal lung tissue.[107] Human fetal lung in organ culture produces large quantities of prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ and thromboxane A_2.[108] Treatment of the human fetal lung explants with the cyclooxygenase inhibitor indomethacin markedly reduces cAMP formation and blocks the spontaneous induction of SP-A gene expression. Indomethacin also significantly inhibits the spontaneous increase in alveolar lumenal volume density and reduces the number of lamellar bodies in type II cells.[108] The findings that treatment of the cultured fetal lung tissue with PGE_2 increases cAMP formation and that PGE_2 or Bt_2cAMP overcomes the inhibitory effect of indomethacin on SP-A gene expression suggest that endogenous prostaglandins, acting through cAMP, may serve a role in the spontaneous differentiation of midgestation human fetal lung in culture.[108]

Spontaneous differentiation of cultured human fetal lung explants and cAMP induction of SP-A gene expression are dependent on the amount of atmospheric oxygen in the culture environment.[109] When fetal lung explants are cultured in an atmosphere containing 1% oxygen (as compared to the standard 20%), they fail to differentiate and no induction of SP-A gene expression is apparent. Furthermore, Bt_2cAMP induction of morphologic differentiation and SP-A gene expression are not apparent in 1% oxygen. These inhibitory effects of low oxygen are reversed when the tissue is transferred to a 20% oxygen environment. The effects of atmospheric oxygen on SP-A gene expression and on morphologic development are concentration-dependent; the stimulatory effects of cAMP are observed only at oxygen concentrations ≥10%.[109] These findings suggest that oxygen plays a permissive role in the spontaneous morphological and biochemical differentiation of human fetal lung in culture. Furthermore, the increase in oxygen delivery to presumptive alveolar epithelial cells that occurs with increased vascularization of the developing lung may serve to increase responsiveness to intracellular cAMP, produced by a number of regulatory factors including catecholamines and prostaglandins.

Glucocorticoids

Glucocorticoids have complex actions on SP-A gene expression in fetal lung tissues that appear to be species-specific and dependent on developmental stage. In fetal rabbit lung, glucocorticoids have both inhibitory and stimulatory effects on SP-A gene expression that appear to be related to the state of differentiation of the fetal lung tissue. Treatment of lung explants from 21 day fetal rabbits with cortisol or dexamethasone (10^{-7} M) causes an acute (6-24 hours) inhibition of SP-A gene transcription and reduces the magnitude of the stimulatory effect of Bt_2cAMP.[40] However, after 48-72 hours incubation, a stimulatory effect of glucocorticoid is observed and there is an additive effect with Bt_2cAMP on SP-A gene transcription.[40] To date, we have no explanation for these paradoxical effects of glucocorticoids; however, they may be related to changes in chromatin structure accompanying cellular differentiation which could render glucocorticoid-response elements (GREs) accessible to trans-acting factors (e.g., the glucocorticoid receptor).

Glucocorticoids have complex actions on SP-A expression at both the transcriptional and post-transcriptional levels.[94,95] In midgestation human fetal lung in organ culture, glucocorticoids exert both stimulatory and inhibitory effects on the levels of SP-A and its mRNA that are both dose- and time-dependent.[18,43,94,110,111] Dexamethasone stimulates SP-A gene transcription in a dose-dependent manner, with maximum stimulation at concentrations of 10^{-8}-10^{-7} M. The stimulatory effect of dexamethasone on SP-A transcription is synergistic with that of Bt_2cAMP.[94,95] On the other hand, dexamethasone markedly decreases the levels of SP-A mRNA and reduces the magnitude of the stimulatory effect of Bt_2cAMP.[18] The inhibitory effects of dexamethasone are due to reduced SP-A mRNA stability. SP-A mRNA half-life is estimated to be 11.4 hours in control human fetal lung explants and is

reduced >60%, to 5 hours, by dexamethasone (10^{-7} M).[95] The inhibition is dose-dependent, completely reversible and blocked by the glucocorticoid receptor antagonist RU486.[95] In contrast to the above findings, Iannuzzi et al[112] reported that dexamethasone inhibits SP-A gene transcription in the same human lung model; however, they also reported the inhibitory effect on mRNA stability.

A combination of primer extension and RNA northern blotting were used to analyze glucocorticoid effects on regulation of the *hSP-A1* and *hSP-A2* genes. Dexamethasone markedly reduces the level of *hSP-A2* mRNA, whereas the level of *hSP-A1* mRNA is largely unaffected by the hormone.[96] Therefore, *hSP-A2* is more highly regulated than *hSP-A1* during development and by cAMP and glucocorticoids.

In lung explants from 90, 125 and 140-day gestation fetal baboons (term = 184 days), dexamethasone causes a dose-dependent inhibition of SP-A mRNA levels and antagonizes the stimulatory effect of Bt_2cAMP.[89] The stimulatory effects of Bt_2cAMP and inhibitory effects of dexamethasone in the 92-140 day fetal baboon lung are very similar to those in lung explants of midgestation human abortuses. In contrast, SP-A mRNA is present at relatively high levels in lung tissues of 160 and 174 day fetal baboons prior to culture and remains essentially unchanged by incubation for 5 days in control medium. The stimulatory effect of Bt_2cAMP and the inhibitory effect of dexamethasone on SP-A mRNA levels are relatively modest in lung explants from 160 and 174 day fetal baboons compared to those from 92, 125 and 140 day fetuses. These findings suggest that with increased lung maturation and the developmental induction of SP-A gene expression, there is a decrease in the response of the fetal lung to the stimulatory effects of cAMP and the inhibitory effects of glucocorticoids on SP-A gene expression.[89]

In vivo studies suggest that maternal administration of synthetic glucocorticoids enhances SP-A gene expression in lung tissues of fetal rabbits[113,114] and rats.[115,116] Differences in the findings of venous studies may be due to developmental changes in the sensitivity of the SP-A gene to glucocorticoids. In rats, dexamethasone appears to have its greatest effect on stimulation of SP-A gene expression during the glandular phase of lung development.[115] In situ hybridization studies on glucocorticoid regulation of surfactant protein expression in fetal rabbits showed that, whereas SP-A mRNA is localized primarily to lung type II cells of fetuses from saline treated or uninjected does, SP-A mRNA transcripts are readily detected both in type II cells and bronchiolar epithelial cells from those of betamethasone-injected animals.[114]

There is little question that glucocorticoid treatment of fetal lung in vivo or in culture regulates SP-A gene expression. However, the physiological role of endogenous glucocorticoids in the regulation of SP-A gene expression in fetal lung must be carefully considered in view of findings of apparently normal levels of SP-A mRNA in lungs of mice homozygous for targeted disruption of the glucocorticoid receptor (GR) gene.[117] GR -/- mice die several hours after birth as a result of respiratory failure caused by underdeveloped and atelectatic lungs.[117] The lack of GR was suggested to impair development of the terminal bronchioles and alveoli beyond 15.5 days gestation. Of note, however, is the finding of comparable numbers of alveolar type II cells and apparently normal levels of mRNA encoding SP-A, SP-B and SP-C in lungs of newborn GR -/- mice compared to those of heterozygous or wild type animals.[117] It was suggested that the respiratory failure of the GR -/- neonates is not due to inadequate development of the surfactant system but rather to decreased expression of the glucocorticoid-responsive amiloride-sensitive epithelial Na^+ channel, normally induced in mouse alveolar epithelium after 17 days gestation. The absence of expression of this channel in alveolar epithelium of mice with a targeted disruption of its α-subunit results in neonatal RDS and death due to defective lung liquid clearance.[118] It is not known if surfactant glycerophospholipid synthesis or composition are altered in the lungs of the GR -/- mice, so

it is possible that the perinatal mortality in the mutant mice is due, in part, to inadequate surfactant lipid synthesis and/or secretion.

Analysis of Genomic Elements Involved in the Regulation of SPA Gene Expression

As discussed above, expression of the rabbit SP-A and human SP-A2 genes is most highly regulated at the transcriptional level by cAMP and glucocorticoids. To functionally define the cis-acting elements required for cAMP and glucocorticoid regulation of SP-A promoter activity, we constructed fusion genes comprised of various amounts of 5'-flanking DNA from the rabbit and human SP-A genes linked to the human growth hormone (hGH) structural gene, as reporter, and transfected them into cultured type II cells. Because of the lung type II cell-specific nature of SP-A gene expression, we reasoned that the transfections should be carried out using type II cells that maintain their phenotypic properties, particularly SP-A gene expression. To accomplish this, we devised a method for primary monolayer culture of rat, rabbit, mouse and human type II cells.[85] The type II cells are isolated by collagenase digestion of midgestation human, 18-day fetal rat, 16-day fetal mouse or 23-day fetal rabbit lung explants that have been maintained for several days in organ culture in medium containing Bt_2cAMP (10^{-3} M). The cell suspension is enriched in type II cells by incubation with DEAE-dextran (human cells) or by a 'panning' method (rat, mouse and rabbit cells) and plated on dishes coated with ECM prepared from Madin-Darby canine kidney cells. The isolated cells contain osmiophilic lamellar inclusions with the ultrastructural characteristics of lamellar bodies and continue to express the SP-A gene at elevated levels for up to three weeks in culture.[85] Since these cells are resistant to conventional methods of DNA transfection, the fusion genes are incorporated into the replication-defective human adenovirus vector, Ad5,[91] and introduced into the type II cells by infection. This results in highly efficient and reproducible transfection of fusion gene constructs into primary cultures of type II cells. SP-A promoter activity is analyzed by radioimmunoassay of hGH protein secreted into the culture medium over each 24 hour period for 5 days (Fig. 3.1).

Regulatory Elements of the Rabbit SP-A Gene

To begin to functionally define the genomic regions that regulate rabbit SP-A promoter activity, we constructed fusion genes comprised of -1766, -991, -378 and -47 bp of DNA flanking the 5'-end of the rabbit SP-A gene, the transcription initiation site and 20 bp of exon I linked to the hGH structural gene. In type II cells transfected with $SP\text{-}A_{-1766}$:hGH and $SP\text{-}A_{-991}$:hGH fusion genes, hGH production is induced ~40- and 20-fold, respectively, by Bt_2cAMP (Fig. 3.2). In cells transfected with the $SP\text{-}A_{-378}$:hGH fusion gene, basal levels of expression are reduced by >50%, as compared to $SP\text{-}A_{-991}$:hGH; however, Bt_2cAMP increases hGH production 11-fold. In type II cells transfected with the $SP\text{-}A_{-47}$:hGH fusion gene, basal levels of hGH production are essentially undetectable and Bt_2cAMP has no stimulatory effect.[91] Dexamethasone (10^{-7} M) has little effect when added alone but it inhibits cAMP-induced expression of the $SP\text{-}A_{-1766}$:hGH, $SP\text{-}A_{-991}$:hGH and $SP\text{-}A_{-378}$:hGH fusion genes by 65% (Fig. 3.2). This inhibitory effect of dexamethasone is unexpected because, as discussed previously, dexamethasone increases transcription of the endogenous SP-A gene in rabbit lung explants and has an additive stimulatory effect with Bt_2cAMP.[46] Dexamethasone inhibition of cAMP-induced $SP\text{-}A_{-1766}$:hGH expression in transfected type II cells is concentration-dependent, with half-maximal inhibition at 8×10^{-10} M (Fig. 3.3A), similar to the equilibrium dissociation constant for binding of dexamethasone to the GR. This inhibitory effect of dexamethasone is blocked by the GR antagonist RU486 (Fig. 3.3B),[91] further suggesting that the GR antagonizes cAMP induction of SP-A:hGH expression in transfected type II cells.

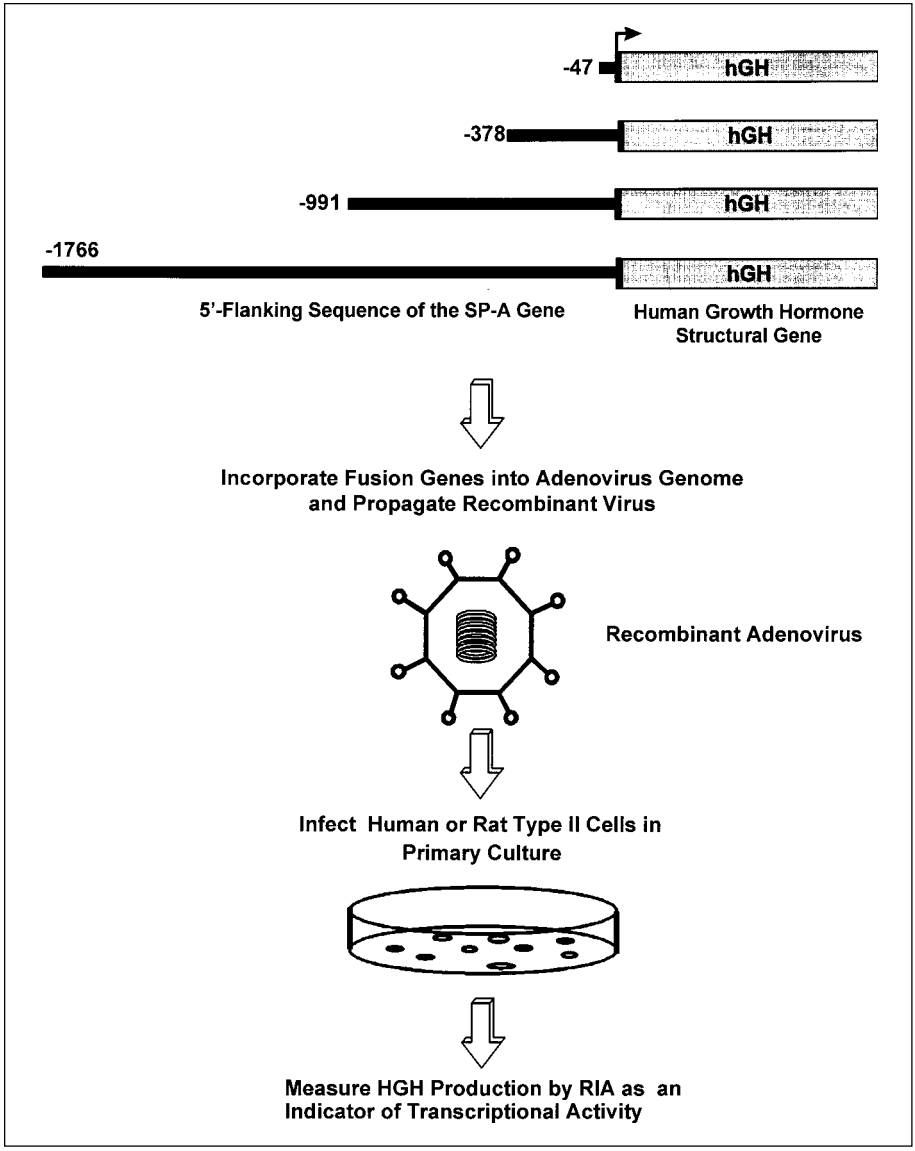

Fig. 3.1. Construction of recombinant adenoviruses containing SP-A:hGH fusion genes and transfection of type II cells in primary culture. Fusion genes comprised of various amounts of 5'-flanking DNA and 20 bp of the first exon of the rabbit or human SP-A genes linked to the hGH structural gene as reporter (SP-A:hGH) were constructed. The fusion genes were incorporated into the genome of the replication-defective human adenovirus, Ad5, and introduced into primary cultures of human or rat type II cells by infection. Fusion gene expression was analyzed by radioimmunoassay of hGH secreted into the culture medium.

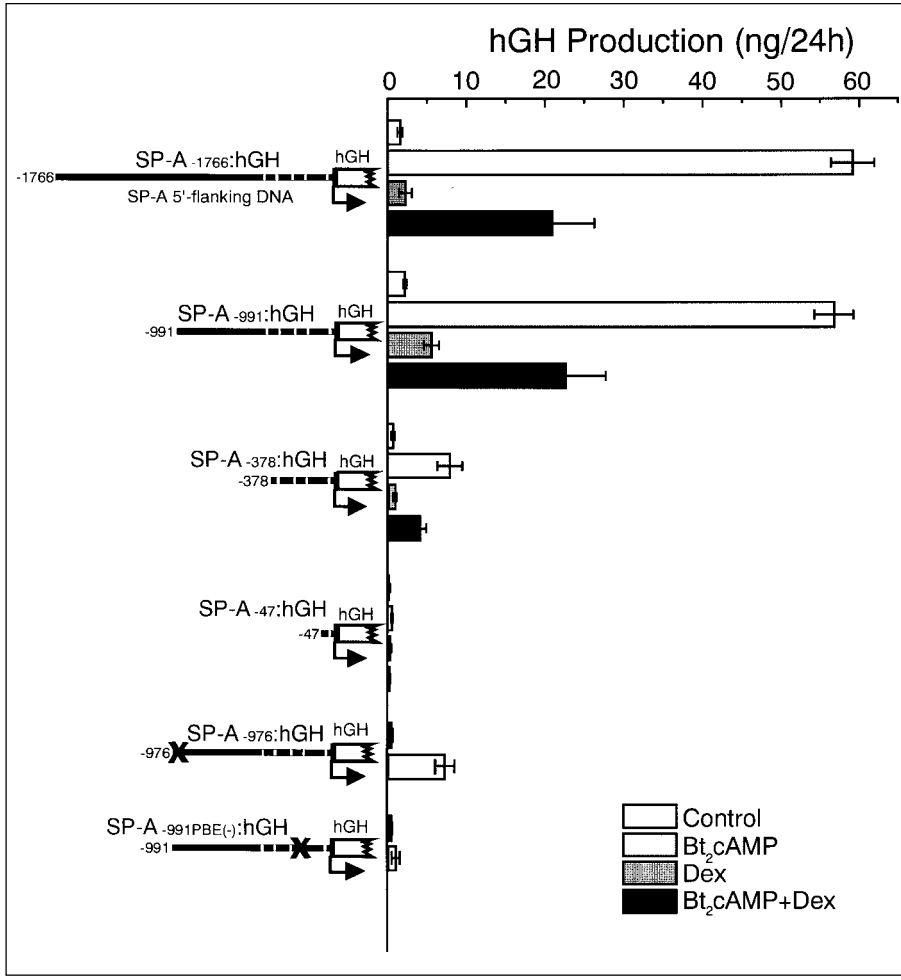

Fig. 3.2. Effects of cAMP and dexamethasone on expression of SP-A:hGH fusion genes in rat type II cells. The type II cells were transfected with SP-A:hGH fusion genes containing 1766, 991, 378 or 47 bp of 5'-flanking sequence of the rabbit SP-A gene. After transfection, the cells were cultured in serum-free medium in the absence (Control) or presence of 10^{-3} M Bt_2cAMP, 10^{-7} M dexamethasone (Dex) or Bt_2cAMP + Dex. Expression of SP-A_{-976}:hGH, which lacks the DBE (distal binding element), and a SP-$A_{-991(PBE-)}$:hGH fusion gene containing a mutation in the PBE (proximal binding element) were also analyzed in type II cells cultured with and without Bt_2cAMP. Culture media were harvested and replaced daily. Shown are the levels of hGH that accumulated in the medium over a 24 hour period between days 4 and 5 of culture. Values are means ± SEM from three independent experiments conducted in triplicate. Adapted with permission from Alcorn JL, Gao E, Chen Q et al. Primary cell culture of human type II pneumonocytes: maintenance of a differentiated phenotype and transfection with recombinant adenoviruses. Mol Endocrinol 1993; 7:1072-1085 (© The Endocrine Society) and Gao E, Alcorn JL, Mendelson CR. Identification of enhancers in the 5'-flanking region of the rabbit surfactant protein A (SP-A) gene and characterization of their binding proteins. J Biol Chem 1993; 268:19697-19709.

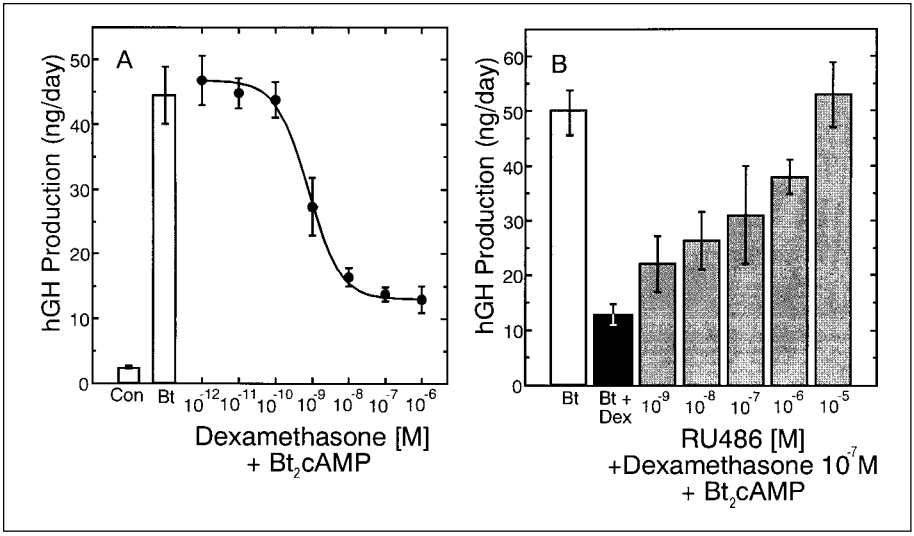

Fig. 3.3. Effects of dexamethasone and the glucocorticoid receptor antagonist, RU486, on expression of SP-A$_{-1766}$:hGH fusion genes in type II cells. (A) Rat type II cells transfected with SP-A$_{-1766}$:hGH cultured in the absence (Con) or presence of Bt$_2$cAMP (Bt) alone and in combination with dexamethasone (10^{-12}-10^{-6} M). (B) Rat type II cells transfected with SP-A$_{-1766}$:hGH cultured with Bt$_2$cAMP alone and in combination with dexamethasone and in the absence or presence of RU486 (10^{-9}-10^{-5} M). Shown are the levels of hGH secreted into the culture medium between days 4 and 5 of culture. Values are means ± SEM from two independent experiments performed in triplicate. Reproduced with permission from Alcorn JL, Gao E, Chen Q et al. Primary cell culture of human type II pneumonocytes: Maintenance of a differentiated phenotype and transfection with recombinant adenoviruses. Mol Endocrinol 1993; 7:1072-1085, © The Endocrine Society.

The mechanism(s) whereby glucocorticoids inhibit cAMP induction of SP-A promoter activity has not been determined. We have reasoned that our fusion gene constructs may lack a functional GRE, a palindrome of the sequence TGTTCT with a three nucleotide spacer (AGAACAnnnTGTTCT).[119] Based on sequence analysis of the rabbit SP-A gene and surrounding genomic regions, we were unable to find a palindromic GRE within 3.0 kb of 5'-flanking DNA or within the structural gene; however, we found two sequences with homology to GRE half-sites at -150 and -190 bp and two other GRE half-sites within the first intron. It is evident from the data described above that the GRE half-sites at -150 and -190 bp do not function as stimulatory GREs. To determine whether one or both of the GRE-like sequences within the first intron could serve as a functional GRE, an SP-A:hGH fusion gene containing 991 bp of 5'-flanking DNA, the first exon, the first intron and part of the second exon of the rabbit SP-A gene linked to hGH (SP-A$_{-991+670}$:hGH) was constructed and transfected into rat type II cells. Again, dexamethasone caused a marked inhibition of cAMP-induced SP-A$_{-991+670}$:hGH expression.[91] These findings suggest that the stimulatory effect of glucocorticoids on expression of the endogenous SP-A gene may be mediated by sequences that lie far upstream, within the SP-A structural gene downstream of the first exon or within the 3'-flanking sequence. As a consequence of the perceived complexity of glucocorticoid regulation of SP-A gene expression, we have subsequently focused on defining the mechanisms whereby cAMP regulates SP-A gene expression in type II cells.

The pronounced stimulatory effect of cAMP on expression of the SP-A$_{-1766}$:hGH fusion gene is apparent in both rat and human type II cells in primary culture. In two lung adenocarcinoma cell lines of presumed type II cell origin, NCI-H358 and A549, which do not express SP-A, there are relatively high levels of basal expression but little or no stimulation in response to cAMP. In primary cultures of cAMP-responsive ovarian granulosa and thecal cells and in the cAMP-responsive adrenal cell line Y1, basal expression is barely detectable and again there is no cAMP effect.[91] These findings suggest that cAMP regulation of SP-A gene expression requires the interaction of type II cell-specific transcription factors with tissue-specific enhancers. These cell-specific transcription factors may be reduced in the lung adenocarcinoma cell lines and absent in the ovarian and adrenal Y1 cells. Alternatively, H358 and A549 cells may be deficient in some component of the cAMP response pathway.

Electrophoretic mobility shift assays (EMSA) revealed that rabbit lung nuclear proteins bind to several elements within the 5'-flanking region of the rabbit SP-A gene; two of these, termed distal binding element (DBE, -986 to -977 bp) and proximal binding element (PBE, -87 to -70 bp), have related core E-box-like sequences (DBE: CACGTG; PBE: CTCGTG).[120] To assess the functional role of the DBE and PBE in the regulation of SP-A promoter activity, SP-A:hGH fusion genes containing deletions or mutations in these regions were introduced into rat type II cells in primary culture. The levels of basal and cAMP-induced expression of SP-A$_{-976}$:hGH fusion genes, which just lack sequences containing the DBE, are markedly reduced to levels that are comparable to those observed with SP-A$_{-378}$:hGH fusion genes (Fig. 3.2). The finding that expression of SP-A$_{-976}$:hGH is stimulated ~15-fold by Bt_2cAMP treatment (compared to a 22-fold stimulation of SP-A$_{-991}$:hGH) suggests that the DBE serves a more important role as a general rather than a specific enhancer of cAMP regulated expression. In type II cells transfected with SP-A$_{-991}$:hGH fusion genes containing a mutation of the PBE sequence, basal expression is reduced to levels comparable to those of cells transfected with SP-A$_{-47}$:hGH, and Bt_2cAMP has essentially no stimulatory effect (Fig. 3.2).[120] The EMSA and type II cell transfection data suggest that the DBE and PBE act as enhancers that interact with the same or related trans-acting factors and serve important roles in type II cell-specific, cAMP-mediated regulation of SP-A gene expression.[120] It is apparent that the PBE serves a more critical role in basal and cAMP regulation of SP-A promoter activity than does the DBE. Whether this is due to its proximity to the promoter or to the of PBE-bound transcription factors interaction with other transcription factors bound to adjacent response elements remains to be determined.

The above studies indicate the presence of an enhancer element(s) between -378 and -991 bp that increases basal levels of expression and of an element(s) between -47 and -378 bp that is essential for cAMP induction of SP-A promoter activity in type II cells.[91] Sequence comparison of this region with the binding site consensus sequences of known transcription factors identified a cis-acting element at -261 bp with sequence similarity to a cAMP-response element (CRE), which is termed CRE$_{SP\text{-}A}$ (TGACCTCA). CRE$_{SP\text{-}A}$ differs by one nucleotide from the palindromic consensus CRE (CRE$_{pal}$, TGACGTCA) which is known to bind the cAMP-response element-binding protein (CREB) as a homodimer.[121] CREB activates transcription of target genes, such as somatostatin, subsequent to cAMP-dependent protein kinase (protein kinase A, PKA) catalyzed phosphorylation at serine$_{133}$.[122] CREB belongs to a superfamily of transcription factors, the basic-leucine zipper (bZIP) family, whose members all contain leucine zipper dimerization domains and have significant sequence similarity in both their DNA-binding and leucine zipper domains. CRE$_{pal}$ is also recognized by several related members of the bZIP superfamily, namely activating transcription factor-1 (ATF-1) and cAMP-response element modulator (CREM), which are also cAMP-responsive transcription factors.[122] To determine the functional role of CRE$_{SP\text{-}A}$ in cAMP

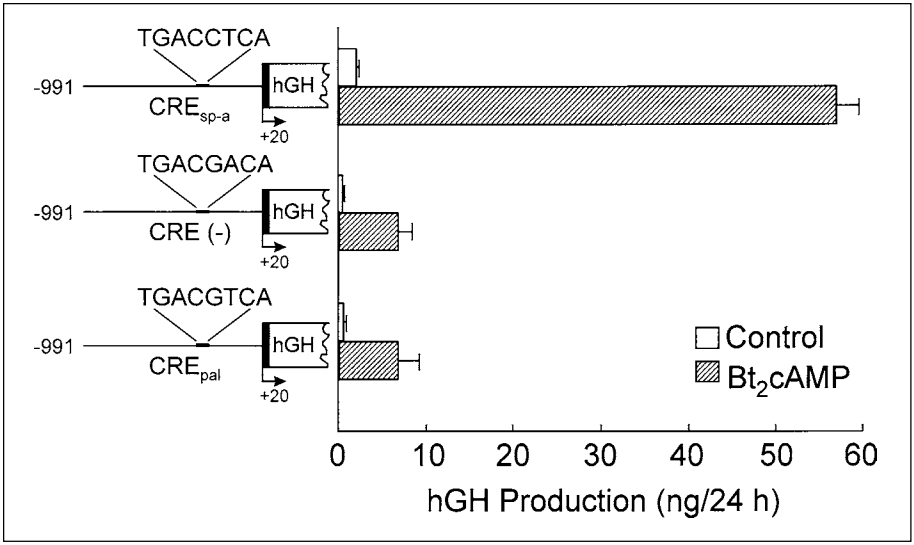

Fig. 3.4. Effects of mutations in CRE_{SP-A} on basal and cAMP-induced expression of SP-A_{-991}:hGH fusion genes in type II cells. Rat type II cells were transfected with SP-A_{-991}:hGH containing the wild type CRE (TGACCTCA), CRE^- (TGACGACA) or CRE_{pal} (TGACGTCA) and cultured in the absence (Control) or presence of Bt_2cAMP for 5 days. Shown are the levels of hGH secreted into the medium over a 24 hour period between days 4 and 5 of incubation. Values are means ± SEM from three independent experiments conducted in triplicate. Reproduced with permission from Michael LF, Alcorn JL, Gao E et al. Characterization of the cyclic adenosine 3',5'-monophosphate response element of the rabbit surfactant protein-A gene: Evidence for transactivators distinct from CREB/ATF family members. Mol Endocrinol 1996; 10:159-170, © The Endocrine Society.

regulation of SP-A promoter activity, CRE_{SP-A} in SP-A:hGH fusion genes containing 991 bp of 5'-flanking DNA and 20 bp of exon I from the rabbit SP-A gene was mutated either to a sequence known to weakly support cAMP induction of gene expression (TGACGACA; CRE^-)[123] or to the CRE_{pal} sequence (TGACGTCA).[92] In type II cells transfected with SP-A_{-991}:hGH fusion genes containing the wild type CRE (CRE_{SP-A}), Bt_2cAMP increases reporter gene expression 22-fold compared to transfected cells maintained in control medium (Fig. 3.4). By contrast, in cells transfected with the fusion gene containing the CRE^- sequence, there is a pronounced decrease in basal and cAMP-regulated expression. Surprisingly, in cells transfected with the fusion gene containing CRE_{pal}, basal and cAMP-induced hGH production are also markedly reduced compared to cells transfected with the wild type construct (Fig. 3.4).[92] Together, these findings indicate that cAMP stimulation of SP-A gene transcription is mediated, in part, through this CRE-like sequence and that transacting factors other than CREB homodimers may interact with this element.

Regulatory Elements of the Human SP-A2 Gene

Studies to define the cis-acting elements of the *hSP-A2* gene involved in cAMP-regulated expression in type II cells showed that as little as -296 bp of *h*SP-*A2* 5'-flanking sequence is sufficient to direct high basal and cAMP-inducible expression in type II cells (Fig. 3.5A) but not in other cell types (Fig. 3.5B).[93] This region contains a CRE-like se-

quence (TGACCTTA) at -242 bp which differs by two nucleotides from CRE_{pal} (TGACGTCA) and by one nucleotide from the CRE-like sequence of the rabbit SP-A gene (TGACCTCA). On comparison of the promoter region of *h*SP-*A2* with that of *hSP-A1*[124] and of baboon (Mendelson CR, unpublished observations), rabbit,[24] rat[125] and mouse[26] SP-A genes, it becomes evident that a sequence similar to $CRE_{SP\text{-}A}$ is highly conserved, particularly within the first six residues, TGACCT (Fig. 3.6). Furthermore, with the exception of the rat SP-A gene 5'-flanking region, the CRE-like sequence is located within 261 bp upstream of the transcription initiation sites of all of the SP-A genes thus far characterized. The finding that mutation of $CRE_{SP\text{-}A}$ in the SP-$A2_{-296}$:hGH fusion gene results in a marked reduction in basal and cAMP-induced hGH expression (Fig. 3.5A) suggests that $CRE_{SP\text{-}A}$ serves an important role in the regulation of *SP-A2* promoter activity.[93]

Another highly conserved sequence localized at -61 bp in the 5'-flanking region of the *hSP-A2* gene contains the core sequence GGGGTGGGG ($GT_{SP\text{-}A}$) (Fig. 3.6). To examine the role of the $GT_{SP\text{-}A}$ in regulation of promoter activity and in response to cAMP, we used site-directed mutagenesis to alter 5 bases of the core sequence within the SP-$A2_{-296}$:hGH fusion gene.[126] SP-$A2_{-296}$:hGH fusion genes containing the wild type and mutated $GT_{SP\text{-}A}$ were introduced into primary cultures of human type II cells. Mutagenesis of $GT_{SP\text{-}A}$ within the -296 bp fusion gene dramatically reduces basal expression (by >90%) and abolishes that induced by cAMP (Fig. 3.5A). Expression of SP-$A2_{-296GTmut}$:hGH is similar to that of the minimal promoter construct, SP-$A2_{-47}$:hGH, which lacks the $GT_{SP\text{-}A}$ sequence and includes only the TATA motif. Basal expression of the SP-$A2_{-62}$:hGH construct, which just includes $GT_{SP\text{-}A}$, is >3-fold higher than the -47 bp minimal promoter construct (Fig. 3.5A). These findings indicate that $GT_{SP\text{-}A}$ is also essential for elevated levels of basal and cAMP-induced SP-*A2* promoter activity. It should be noted that in H441 and A549 cells, in contrast to primary cultures of human fetal type II cells, cAMP does not induce SP-A2:hGH fusion gene expression, although basal expression of SP-$A2_{-296}$:hGH fusion genes is detectable in both cell lines (Fig. 3.5B).[93] We also examined the role of the $GT_{SP\text{-}A}$ in expression of SP-A2:hGH fusion genes in the lung adenocarcinoma cell lines. Mutagenesis of the $GT_{SP\text{-}A}$ in the SP-$A2_{-296}$:hGH fusion gene results in a similar fold reduction of basal expression (>90%) in A549 and H441cells as in primary cultures of type II cells.

In studies to define cis-acting elements within the 5'-flanking sequence of the mouse SP-A gene that are required for expression in lung type II cells, a region between -255 and -57 bp containing four binding sites for thyroid transcription factor-1 (TTF-1) was found to mediate expression in a mouse lung epithelial cell line but not in other cell types.[127] TTF-1 is a homeodomain transcription factor expressed selectively in developing thyroid, diencephalon and lung epithelium from the earliest stages of organogenesis.[128] Mice rendered TTF-1 deficient by gene targeting lack thyroid, anterior pituitary and lung parenchyma.[129] By mutagenesis, it was found that the TTF-1 sites in the mouse SP-A gene 5'-flanking sequence are required for expression of the SP-A promoter constructs in the transfected mouse lung epithelial cells.[127] We have identified three TTF-1-binding elements (TBE) in the 5'-flanking region of the baboon *SP-A2* gene that serve important roles in basal expression and in cAMP induction of SP-A promoter activity. One of these elements, TBE1 at -172 bp, is highly conserved with respect to position and sequence in all of the SP-A genes thus far characterized (Fig. 3.6). Mutagenesis of TBE1 has a more pronounced effect on reduction of basal and cAMP-induced expression than mutagenesis of either TBE2 or TBE3 (Li J, Gao E, Mendelson CR, unpublished observations).

The transfection studies suggest that basal and cAMP induction of SP-A promoter activity in type II cells is the result of the cooperative interaction of transcription factors bound to at least four response elements which lie within 400 bp upstream of the SP-A gene

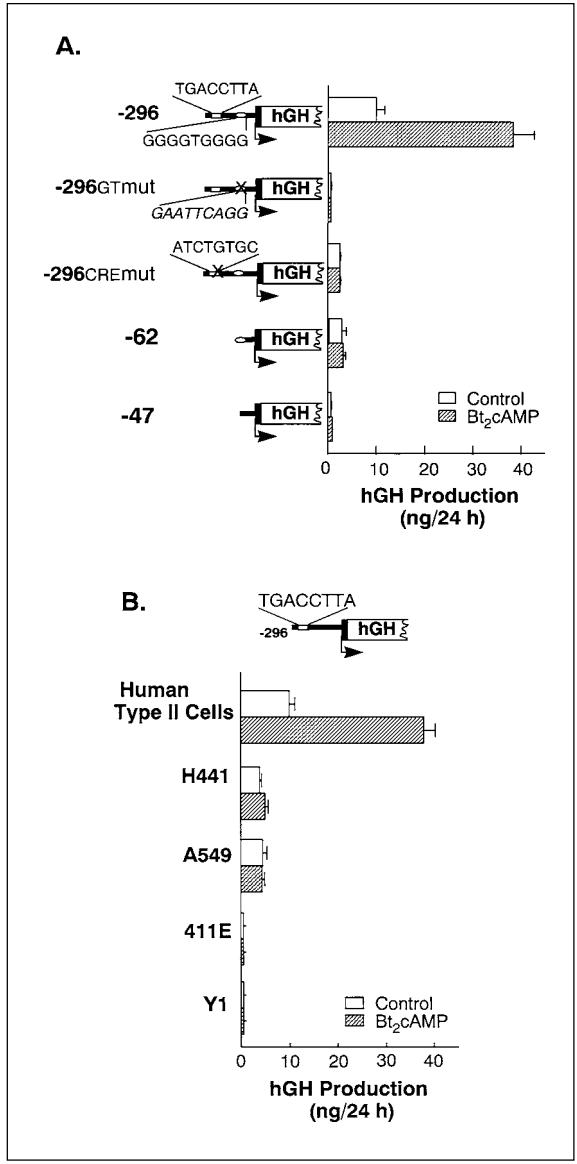

Fig. 3.5. Expression of SP-A2:hGH fusion genes in human type II cells in primary culture and other cell types and the effects of mutagenesis of $CRE_{SP\text{-}A}$ and the GT-box ($GT_{SP\text{-}A}$) on basal and cAMP-induced expression. (A) Human type II cells transfected with SP-A2:hGH fusion genes comprised of 296, 62 and 47 bp of 5'-flanking sequence of the human *SP-A2* gene or with $SP\text{-}A_{\text{-}296}$:hGH containing mutations in $CRE_{SP\text{-}A}$ or $GT_{SP\text{-}A}$ were cultured for 5 days in the absence (Control) or presence of Bt_2cAMP. (B) Primary cultures of human type II cells as well as the human lung adenocarcinoma cell lines H441 (Clara cell origin) and A549 (type II cell origin), the rat hepatoma cell line 4IIE and the mouse adrenocortical tumor cell line Y1 were transfected with $SP\text{-}A2_{\text{-}296}$:hGH and cultured with or without Bt_2cAMP for 5 days. Shown are the levels of hGH that accumulated in the medium over a 24 hour period between days 4 and 5 of incubation. Values are means ± SEM from two independent experiments conducted in triplicate. Reproduced with permission from Young PP, Mendelson CR. A CRE-like element plays an essential role in cAMP regulation of human SP-A2 gene in alveolar type II cells. Am J Physiol 1996; 271:L287-L299.

transcription initiation site. These elements include a CRE-like element, several TTF-binding elements, an E-box-like sequence and a GT-box. The CRE-like element, TTF-1 sites and GT-box are highly conserved in sequence and position in nearly all of the SP-A genes thus far studied. Although the sequence of the PBE motif characterized in the rabbit SP-A gene 5'-flanking region does not appear to be highly conserved, we are investigating the possibility that a related sequence in this region serves an analogous function in the SP-A genes of other species.

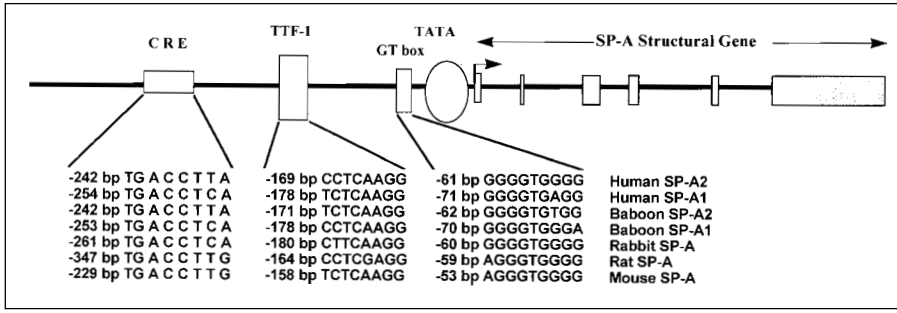

Fig. 3.6. Schematic diagram of the SP-A structural gene and 5'-flanking region containing response elements conserved among various species. Open boxes denote positions of the CRE, TTF-1 and GT-box elements. The oval represents the TATA box and the arrow denotes the start of transcription. Sequences and positions of these elements relative to the transcription initiation sites of the SP-A genes of various species are shown. Adapted with permission from Young PP, Mendelson CR. A GT box element is essential for basal and cyclic adenosine 3',5'-monophosphate regulation of the human surfactant protein A2 gene in alveolar type II cells: Evidence for the binding of lung nuclear proteins distinct from Sp1. Mol Endocrinol 1997; 11:1082-1093, © The Endocrine Society.

Isolation and Characterization of Transcription Factors Involved in Lung-Specific and Developmental Regulation of SP-A Gene Expression

Transcription Factors that Bind to the Distal and Proximal Binding Elements in the 5'-Flanking Region of the Rabbit SP-A Gene

As discussed above, in type II cells transfected with SP-A:hGH fusion genes, mutagenesis of the DBE or PBE results in a marked reduction of basal and cAMP-stimulated fusion gene expression.[120] Rabbit lung nuclear proteins bind specifically to the DBE and PBE in EMSA. Binding activity is enriched in type II cells compared to whole lung tissue. DNaseI footprinting indicated that lung nuclear proteins protect the palindromic sequence CCCACGTGGG in the DBE. The underlined core sequence is an E-box motif; a similar sequence (CCCTCGTG) is present within the PBE. Both elements compete for binding to the same size species of nuclear proteins, of M_r ~69,000, 45,000 and 22,000.[120]

To characterize transcription factors that bind to these E-box motifs, the PBE was used to screen a rabbit fetal lung cDNA expression library; an cDNA insert was isolated that is very similar in sequence to human upstream stimulatory factor 1 (USF1), a basic helix-loop-helix-zipper transcription factor.[130] Two isoforms of rabbit USF1 (rUSF1a and 1b) mRNAs were identified in fetal rabbit lung and other tissues by RT-PCR. The level of rUSF1 mRNA reaches a peak in fetal rabbit lung at 23 days gestation, in concert with the time of initiation of SP-A gene transcription.[130] Binding complexes of nuclear proteins obtained from fetal rabbit lung tissue and isolated type II cells with the DBE and PBE are supershifted by addition of anti-rUSF1 immunoglobulin G (IgG). USF1-binding activity is highly enriched in type II cells as compared with lung fibroblasts (Fig. 3.7). Overexpression of rUSF1s in A549 cells positively regulates SP-A promoter activity of cotransfected reporter gene constructs (Fig. 3.8).[130] These findings suggest that rUSF1, which binds to DBE and PBE, may serve a key role in the regulation of SP-A gene expression in type II cells.

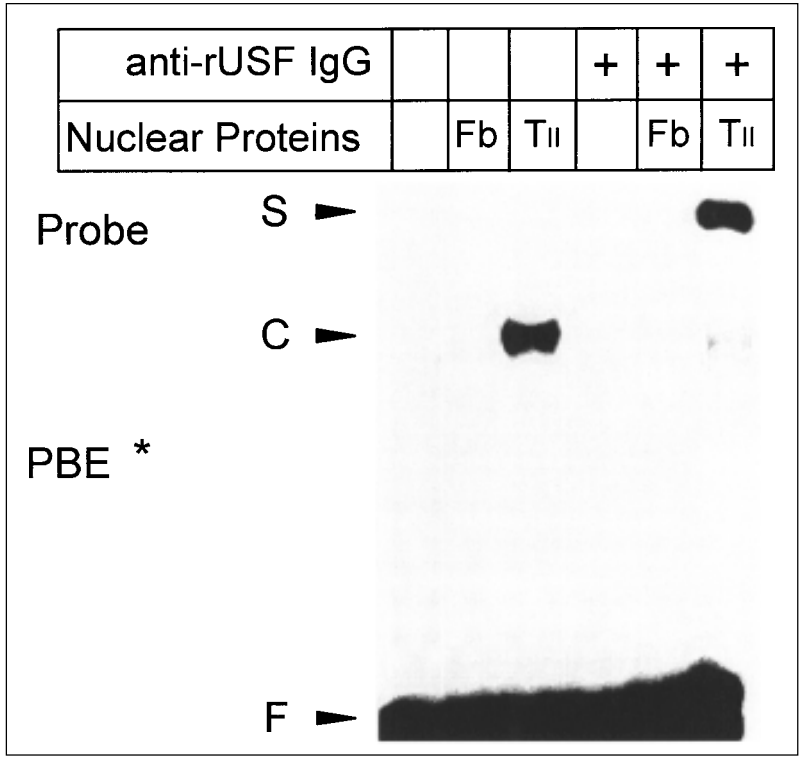

Fig. 3.7. USF1 binding activity in type II cell and fibroblast nuclear proteins. ^{32}P-labeled PBE was incubated with nuclear proteins from rabbit type II cells (TII) and lung fibroblasts (Fb), then incubated in the absence or presence of anti-USF IgG and analyzed by EMSA. F: free probe; C: DNA-protein complexes; S: supershift of DNA-protein complexes with anti-USF1 IgG. Adapted with permission from Gao E, Alcorn JL, Mendelson CR. The basic helix-loop-helix-zipper transcription factor USF1 regulates expression of the surfactant protein-A (SP-A) gene. J Biol Chem 1997; 272:23398-23406.

Rabbit Transcription Factors that Bind to $CRE_{SP\text{-}A}$

As discussed above, mutagenesis of $CRE_{SP\text{-}A}$ (TGACCTCA) to the palindromic sequence CRE_{pal} (TGACGTCA), known to bind the transcription factor CREB as a homodimer, in SP-$A_{\text{-}991}$:hGH fusion genes results in a marked decrease in basal and cAMP-induced expression of SP-A promoter activity.[92] The findings of competitive EMSA using fetal rabbit lung nuclear extracts suggests that different protein complexes bind $CRE_{SP\text{-}A}$ and CRE_{pal}. UV crosslinking analysis showed that a ~43 kDa protein complex interacts both with $CRE_{SP\text{-}A}$ and CRE_{pal}; however, purified CREB is ineffective in binding $CRE_{SP\text{-}A}$ but does bind CRE_{pal}. In EMSA using fetal rabbit lung nuclear proteins, antibodies directed against CREB, CREM, and ATF-1 fail to supershift the complex of proteins bound to $CRE_{SP\text{-}A}$ whereas they do supershift those bound to CRE_{pal}.[92] Moreover, in competition EMSA using radiolabeled $CRE_{SP\text{-}A}$ and fetal rabbit lung nuclear proteins, a purified bZIP polypeptide fails to compete for binding. In contrast, the bZIP polypeptide competes effectively with CRE_{pal}. This finding suggests that leucine zipper transcription factors do not bind $CRE_{SP\text{-}A}$. Additionally,

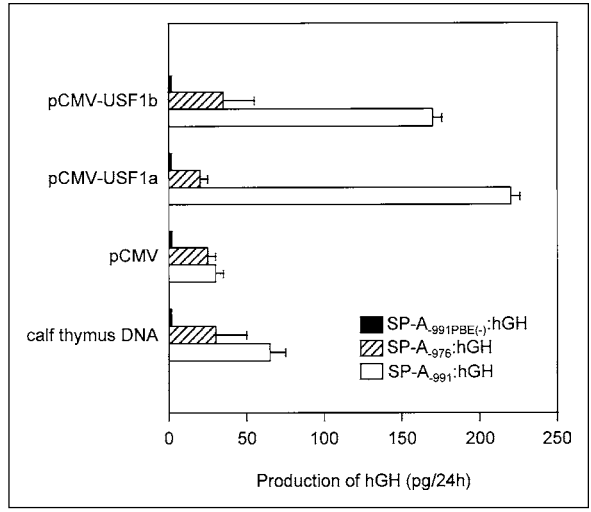

Fig. 3.8. Effect of cotransfection with USF1a or USF1b on expression of rabbit SP-A:hGH fusion genes in lung adenocarcinoma cells. SP-A_{-991}:hGH, SP-A_{-976}:hGH (DBE deleted) and SP-$A_{-991(PBE-)}$:hGH (PBE mutated) were cotransfected into A549 cells with cytomegalovirus (CMV) expression vectors containing rabbit USF1b (pCMV-USF1b), USF1a (pCMV-USF1a), empty vector (pCMV) or calf thymus DNA. Shown are the levels of hGH that accumulated in the culture medium over a 24 hour period. Data are means ± SEM of three determinations from a representative of three experiments. Reproduced with permission from Gao E, Alcorn JL, Mendelson CR. The basic helix-loop-helix-zipper transcription factor USF1 regulates expression of the surfactant protein-A (SP-A) gene. J Biol Chem 1997; 272:23398-23406.

expression of a $CRE_{SP\text{-}A}$-containing histidine 3 (HIS3) fusion gene in yeast is unaffected by either CREB or bZIP polypeptides fused to the GAL4 activation domain. In contrast, HIS3 expression is markedly induced by both CREB and bZIP fusion proteins in a yeast strain in which the HIS3 gene is under control of CRE_{pal}.[92] By competition EMSA using mutagenized $CRE_{SP\text{-}A}$ oligonucleotides, the critical protein-binding nucleotides in $CRE_{SP\text{-}A}$ were found to constitute a hexameric element, TGACCT, which corresponds to a half-site for binding members of the nuclear receptor superfamily.[92] Since the TGACCT motif is present in the SP-A gene as a single site, we propose that a unique orphan member of the nuclear receptor superfamily may bind to this as a monomer element.

To identify members of the nuclear receptor superfamily expressed in type II cells, degenerate primers corresponding to two conserved segments of the DNA-binding domain shared by the GR, retinoid X receptor, retinoic acid receptor, NGFI-B and Ad4BP/SF-1 were designed. The DNA-binding domains of these receptors were chosen because they represent the three classes of receptors—homodimeric, heterodimeric and monomeric DNA-binding proteins. The primers were used in PCR with cDNA prepared from RNA isolated from rabbit type II cells cultured in the presence of Bt_2cAMP. PCR products of the predicted size of 154 bp were subcloned and sequenced. Of the putative DNA-binding domains that were sequenced, approximately 56% were homologous to various retinoic acid receptor isoforms, 8% encoded ARP1/COUP-TFII and 4% encoded each of NURR1 and Rev-ErbA-α. Interestingly, 26% of the PCR products encoded the DNA-binding domain of members of the peroxisome proliferator activated receptor (PPAR) subfamily; 38% of these were homologous to the PPARγ isoform.[131] Since type II cells actively synthesize lipids, we were intrigued to find evidence for the adipose-specific, lipid-activated PPARγ isoform in these cells.

We utilized the PPARγ DNA-binding domain to screen a rabbit type II cell cDNA library and isolated a full length cDNA encoding PPARγ1.[131] Although another PPARγ isoform, PPARγ2, is known to be highly expressed in adipocytes, only PPARγ1 is detected in rabbit

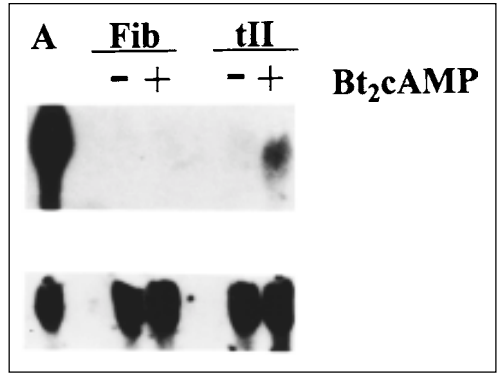

Fig. 3.9. Effect of cAMP on PPARγ mRNA content in lung fibroblasts and type II cells. Northern blot analysis of total RNA (30 μg) isolated from rabbit lung fibroblasts (Fib) and type II cells (tII) cultured in the absence or presence of Bt_2cAMP for 3 days. Rabbit adipose tissue RNA (A) was analyzed as a positive control for PPARγ expression. Full-length rabbit PPARγ cDNA was used as a probe (upper panel); loading variances were detected by probing the blot with a glyceraldehyde phosphate dehydrogenase cDNA (lower panels). Adapted with permission from Michael LF, Lazar MA, Mendelson CR. Peroxisome proliferator-activated receptor gamma 1 expression is induced during cyclic adenosine monophosphate-stimulated differentiation of alveolar type II pneumonocytes. Endocrinology 1997; 138:3695-3703, © The Endocrine Society.

type II cells by RT-PCR and library screening. Rabbit PPARγ1 has 90% nucleotide sequence identity and 95% amino acid identity to mouse PPARγ1. PPARγ1 and its mRNA are readily detected in rabbit type II cells and are induced by cAMP (Fig. 3.9). By Western blot analysis, PPARγ protein expression is induced in concert with SP-A expression during type II cell differentiation (Fig. 3.10).[131] The findings that $CRE_{SP\text{-}A}$ is unable to compete with a radiolabeled PPAR-response element (PPARE) in binding to type II cell nuclear proteins, and that antisera specific for PPARγ fail to supershift the complex of type II cell nuclear proteins bound to $CRE_{SP\text{-}A}$, indicate that $CRE_{SP\text{-}A}$ does not serve as a PPARE.[131] In view of the role of PPARγ in adipocyte differentiation and lipid homeostasis, we postulate that PPARγ induction by cAMP may play a role in the differentiation and expression of lipogenic enzymes in type II cells. Studies are ongoing to determine whether PPARγ plays a role in the regulation of SP-A gene expression.

Transcription Factors that Bind to the GT-Box of the Human SP-A2 Gene

As discussed above, mutagenesis of the GT-box at -61 bp in the 5'-flanking sequence of the human *SP-A2* gene markedly reduces basal and abolishes cAMP-induced reporter gene expression in transfected type II cells.[126] Similarly, this mutation dramatically lowers fusion gene expression in A549 and H441 cells. By EMSA, nuclear proteins isolated from primary cultures of type II cells bind the GT box as five specific complexes. In contrast, nuclear proteins isolated from lung fibroblasts display notably less binding activity. Competition and supershift EMSA indicated that the ubiquitously expressed transcription factor Sp1, a GC box binding protein of ~100 kDa, is a component of the complex of proteins that bind the GT box of SP-*A2*.[126] The finding that only two of the five GT-box binding complexes are supershifted by incubation with Sp1 antibody suggests that a factor(s) in type II cell nuclear extracts that is distinct from Sp1 also interacts with the GT box. By UV crosslinking and sodium dodecyl sulfate polyacrylamide gel electrophoresis/EMSA analysis, we have identified a ~55 kDa GT-box binding factor in type II cell nuclear proteins that preferentially binds the GT-box of *SP-A2* over the consensus Sp1 GC-box sequence. This 55 kDa factor binds the GT-box independently of Sp1.[126] Sp1 is a member of the Krüppel family of zinc finger-containing transcription factors. Several novel proteins belonging to the Krüppel family that manifest significantly higher binding activity towards the GT/CA box than does Sp1

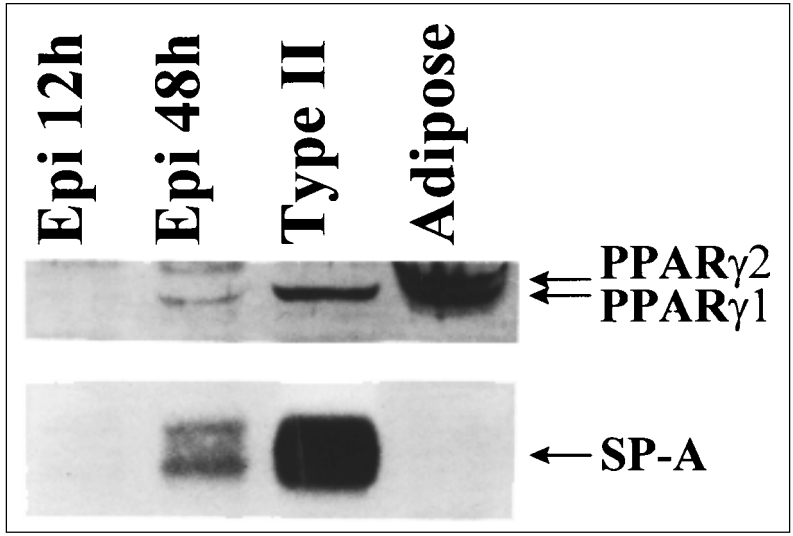

Fig. 3.10. Immunoblot analyses of PPARγ1 and SP-A in differentiating human fetal type II cells. Protein extracts (50 μg) from isolated midgestation human fetal lung epithelial cells and from differentiated type II cells isolated from cultured human fetal lung explants were analyzed by Western blotting using antisera directed against PPARγ (top panel) and SP-A (bottom panel). Proteins isolated from human adipose tissue were analyzed as a positive control for PPARγ. The lung epithelial cells differentiate into type II cells during culture. After 48 hours of culture, immunoreactive PPARγ and SP-A were detected in the primary lung epithelial cells; Higher levels of PPARγ and SP-A immunoreactivity were detected in the fully differentiated type II cells. Reproduced with permission from Michael LF, Lazar MA, Mendelson CR. Peroxisome proliferator-activated receptor gamma1 expression is induced during cyclic adenosine monophosphate-stimulated differentiation of alveolar type II pneumonocytes. Endocrinology 1997; 138:3695-3703, © The Endocrine Society.

have recently been identified.[132] We suggest that the 55 kDa factor that interacts with the GT-box of SP-*A2* may be a new member of this protein family.

Conclusions and Future Directions

SP-A, the major surfactant-associated protein, is a sialoglycoprotein that is a member of the C-type lectin family. SP-A serves a role in the formation of tubular myelin and the reduction of alveolar surface tension, in mediating surfactant uptake and reutilization by type II cells and in activation of alveolar macrophages for clearance of bacterial pathogens from the lung alveolus. The SP-A gene is expressed primarily in lung type II cells and is developmentally regulated in fetal lung in association with surfactant glycerophospholipid synthesis. SP-A gene expression is regulated by a number of hormones and factors. Positive regulators include EGF, KGF, IFN-γ, glucocorticoids and agents that increase intracellular levels of cAMP. Negative regulators also include glucocorticoids as well as insulin, TGF-β, TNF-α and PKC activators. Whether glucocorticoids are positive or negative regulators of the SP-A gene depends on the species examined, the concentration used and whether transcriptional or post-transcriptional effects are studied.

SP-A is encoded by a single copy gene in rats, mice, rabbits and dogs and by two highly similar genes in baboons and humans. Four different regulatory elements have been defined within a 400 bp region of the SP-A gene that mediate high basal and cAMP induction of promoter activity in transfected type II cells. These include a CRE-like sequence that may bind a member of the nuclear receptor superfamily ($CRE_{SP\text{-}A}$), several TTF-1 binding sites, an E-box that binds the transcription factor USF1 and a GT-box that binds Sp1 along with other proteins that may be tissue-selective members of the Krüppel family of transcription factors. Studies are in progress to identify transcription factors that bind to $CRE_{SP\text{-}A}$ and $GT_{SP\text{-}A}$ and to determine how all of these factors interact to mediate type II cell-specific, developmental and cAMP regulation of SP-A gene expression in fetal lung.

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CHAPTER 4

Cellular and Molecular Processing of Surfactant Protein B

Sui Lin and Timothy E. Weaver

Pulmonary surfactant is composed primarily of phospholipids (predominantly phosphatidylcholine and phosphatidylglycerol) and small amounts of surfactant proteins and carbohydrate.[1,2] Both the phospholipid and protein components are synthesized by the type II epithelial cell, one of two epithelial cells lining the alveolus.[3-6] Newly synthesized surfactant is stored with surfactant recycled from the airway in large secretory granules referred to as lamellar bodies.[7] Lamellar bodies are released into the airway by exocytosis[8,9] and the phospholipid and protein contents are rapidly adsorbed to the air-liquid interface of the alveolus where they form a film covering the entire surface of the alveolar epithelium.[1,2,4,5] Film formation reduces surface tension along the alveolar epithelial wall, thereby preventing alveolar collapse (atelectasis) at end expiration.[1,2,4,5] Pulmonary surfactant protein B (SP-B) is a lung-specific peptide associated with the surface film and is absolutely required for lung function at birth. Consequently the appropriate developmental regulation of SP-B expression, the proteolytic processing of the newly synthesized preproprotein to the mature peptide, and the assembly of the mature peptide with surfactant phospholipids is critical for successful adaptation to airbreathing at birth.

Structure of SP-B Gene and Proteins

SP-B, M_r approximately 8,000, was initially purified from organic solvent extracts of pulmonary surfactant isolated from alveolar lavage fluid.[10-12] cDNAs encoding human SP-B were isolated by screening a human lung cDNA library with oligonucleotide probes, based on limited amino acid sequence of purified SP-B,[13,14] or with antiserum generated against purified bovine SP-B.[15] The human SP-B cDNA was subsequently used to screen a human genomic library to isolate the SP-B gene[16] which was shown to encompass approximately 9.5 kilobases (kb) of DNA on chromosome 2.[16-18] The 11 exons of the human gene encode a mRNA of approximately 2 kb which, in turn, encodes a preproprotein of 381 amino acids consisting of a 23 residue signal peptide, a 177 residue NH_2-terminal propeptide, a 79 residue mature peptide and a 102 residue COOH-terminal propeptide (Fig. 4.1).[6,13,15,19] Processing of the preproprotein within the secretory pathway results in cotranslational removal of the signal peptide followed by proteolytic cleavage of the NH_2- and COOH-terminal propeptides to produce the bioactive mature peptide.[20-23] SP-B cDNA from human,[13-15] canine,[12] rat,[24] rabbit[25,26] and mouse[27] are highly conserved, with 80-85% similarity at the mature peptide level and approximately 67% similarity across the entire preproprotein.[19,28]

Several polymorphisms of the human SP-B gene have been reported.[16,29] One polymorphism results in an amino acid substitution of isoleucine to threonine at position 131

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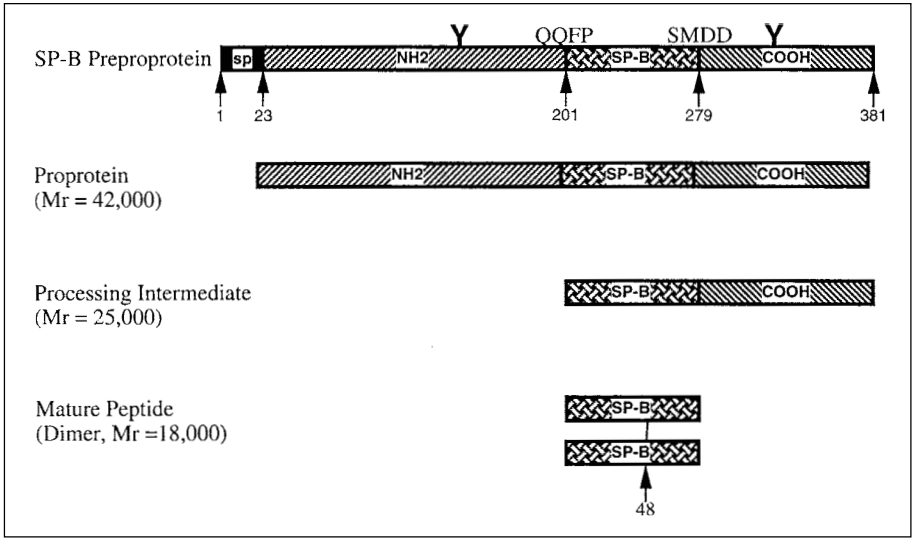

Fig. 4.1. SP-B processing. Processing of the human SP-B preproprotein includes cotranslational removal of the 23 residue signal peptide and subsequent proteolytic cleavage between gln_{200} and phe_{201} and between met_{279} and asp_{280} to produce the 79 residue mature peptide, which forms a sulfhydryl-dependent homodimer. A consensus sequence for the addition of N-linked oligosaccharide (Y) is present in the COOH-terminal propeptides of all species. A second glycosylation consensus sequence in the NH_2-terminal propeptide is the result of a polymorphism in the human gene.

in the NH_2-terminal propeptide, resulting in a potential site for the addition of complex-type asparagine-linked oligosaccharide (Asparagine-X-Serine/Threonine, where X is any amino acid except proline) at residue 129 of the NH_2-terminal propeptide.[15] A second glycosylation site is present at residue 311 in the COOH-terminal propeptide of SP-B[13,15] which is conserved among all species examined to date.[12,24-27] Although glycosylation at both sites has been detected,[20,21,30,31] the significance of this modification is not known.

The 79 residue mature peptide is positively charged and highly hydrophobic. More than 50% of SP-B is composed of hydrophobic amino acids, predominantly valine and leucine which alone account for almost one-third of all residues in the mature peptide. The mature peptide is also strongly positively charged due in part to the relatively large number of arginine residues. The charged residues and hydrophobic residues are arranged in three to four amphipathic helices such that more than half of the mature peptide is present in an α-helical conformation.[27,32,33] This structure facilitates association of SP-B with the surface film, mainly through interaction with negatively charged headgroups of phosphatidylglycerol.[32,34,35] SP-B promotes aggregation, disruption and fusion of liposomes containing negatively charged phospholipids,[34,36-39] properties that may be important in organizing newly secreted phospholipids within the alveolar hypophase.

Another significant feature of SP-B structure is the high cysteine content of both the mature peptide (7 residues) and the proprotein (25 residues).[12-16,24-27,40] The pattern of disulfide bridges in the porcine mature peptide consists of three intrachain bonds which link cysteine residues 8, 11 and 35 with residues 77, 71 and 46, respectively, resulting in a small disulfide loop within a larger loop;[41,42] the remaining cysteine at position 48 of the mature peptide participates in the formation of homodimers. Although homodimers are the pre-

dominant form of SP-B in most species,[19,41] the importance of dimerization for SP-B function is not known. The positions of the cysteines are absolutely conserved in SP-B among all species,[12-15,24-27,40,42] suggesting that disulfide bridges are critical for stabilizing SP-B conformation. This pattern of disulfide bridging and helical structure is also detected in the saposins, peptides involved in the lysosomal catabolism of glycosphingolipids.[33,43] The importance of intramolecular disulfide bonds for saposin C function was demonstrated by disruption of the first disulfide bridge which resulted in an inability to activate β-glucosidase.[43,44] Interestingly the disulfide bonding pattern typical of saposins and the mature SP-B peptide is reiterated in the NH_2- and COOH-terminal propeptides of SP-B.[45] Although the pattern of cysteine pairing in the propeptides is not known, it is likely that these residues participate in intramolecular bonds, since sulfhydryl-dependent oligomerization of the proprotein or the major processing intermediate (M_r = 25,000) are never observed.

Biosynthesis and Intracellular Trafficking of SP-B

Cellular Site of SP-B Synthesis in the Lung

The cellular localization of SP-B has been confirmed at both mRNA and protein levels by in situ hybridization and immunohistochemistry in various species including human,[22,46-49] rabbit,[50] rat[51,52] and mouse.[27,53] Human SP-B mRNA and proprotein are primarily expressed in the distal conducting and terminal airway epithelium in developing and postnatal lung,[47,49,54] similar to surfactant protein A (SP-A) expression, whereas surfactant protein C (SP-C) expression is restricted to type II cells of the alveolar epithelium.[49,51] In human fetal lung, SP-B mRNA, proprotein and mature peptide are colocalized in alveolar type II cells, whereas only SP-B mRNA and proprotein are detected in nonciliated bronchiolar epithelial cells (Clara cells).[46,47,49] Although SP-B expression increases in Clara cells and decreases in type II cells following hyperoxic lung injury,[53,55] it remains unclear if Clara cells process the proprotein and secrete mature peptide into the alveolar space in either normal or injured lung. The inability to detect the mature peptide in Clara cells of normal lung by immunocytochemistry suggests distinct functions for SP-B in alveolar and bronchiolar epithelial cells. SP-B mature peptide is also detected in alveolar macrophages of mature lung; however, neither SP-B mRNA nor SP-B proprotein or processing intermediate are detected in these cells, consistent with SP-B catabolism in this compartment.[46,47,51,52]

Intracellular Processing, Transport, Secretion and Metabolism of SP-B

SP-B is synthesized by the alveolar type II epithelial cell as a 381 amino acid preproprotein. Translocation of newly synthesized SP-B preproprotein into the endoplasmic reticulum (ER) is accompanied by the cleavage of the 23 residue signal peptide and the addition of high mannose oligosaccharide (Fig. 4.1). The 42 kDa proprotein is further cleaved between gln_{200} and phe_{201} to a 25 kDa intermediate consisting of the mature peptide and the COOH-terminal propeptide; subsequent processing between met_{279} and asp_{280} results in liberation of the 8 kDa mature peptide (Fig. 4.1).[21-23,30] The compartment in which endoproteolytic cleavage occurs has been identified by immunogold labeling of ultrathin frozen sections: SP-B proprotein is detected in the ER, Golgi and multivesicular bodies of alveolar type II cells, whereas the mature peptide is only detected in multivesicular and lamellar bodies.[56] Double immunogold labeling analyses confirmed that multivesicular bodies are the only compartment which contains both the SP-B precursor and the mature peptide, consistent with proteolytic processing in this organelle.[56] The identity of the endoprotease(s) responsible for propeptide cleavage is currently not known.

Trafficking of SP-B to the lamellar body requires the appropriate folding of the proprotein within the ER lumen and subsequent transport to the trans-Golgi network, where

the proprotein is sorted away from the constitutive secretory pathway and into the regulated secretory pathway (i.e., to the lamellar body). Given the potential of the mature SP-B to disrupt lipid membranes, the proprotein must be folded such that the mature peptide is prevented from interacting with membranes of the secretory compartment. Similar to most enzymes and peptide hormones, SP-B is synthesized as part of a larger, inactive polypeptide precursor in which the hydrophobic, mature peptide is flanked by relatively hydrophilic NH_2- and COOH-terminal propeptides. Recent studies demonstrated that the NH_2-terminal propeptide alone is both necessary and sufficient for appropriate folding and transport of the mature peptide in the secretory pathway[31] and that the combination of the NH_2-terminal propeptide and the mature peptide is absolutely required for sorting of SP-B to lamellar bodies in vivo.[57] The colocalization of SP-A, SP-B and SP-C in vesicles originating from the trans-Golgi network[58] and the targeting of SP-B to secretory granules in both endocrine and exocrine cells[57] suggest that distinct targeting determinants within each surfactant protein are recognized by a universal sorting mechanism.

The alveolar type II epithelial cell is a specialized exocrine cell which secretes pulmonary surfactant in response to a variety of extracellular stimuli[2,4,59] (see chapter 7). Similar to secretory granules of other exocrine cells, secretion of lamellar bodies is stimulated by secretagogues such as β-adrenergic agonists,[60-62] A_2 and P2 purinoceptor agonists[63,64] and 12-O-tetradecanoylphorbol-13-acetate (TPA).[60,65,66] Although the physiologically relevant stimuli are not known, it is likely that hyperventilation[67-69] and stretch[70] play a role in surfactant secretion. Following secretion, lamellar bodies rapidly transform into a lattice-like structure referred to as tubular myelin, which is likely the immediate precursor of the surface film.[8,71,72] The mechanism underlying the tubular myelin to surface film transition is not clear. Although it has been shown that both SP-B and SP-A are required for the formation of tubular myelin in vitro,[73,74] the importance of this structure is unclear, as SP-A deficient mice which lack tubular myelin have normal lung function.[75] It is clear that SP-B plays an important role in facilitating the rapid adsorption to and insertion of phospholipids into the expanding surface film[76-78] (see chapter 1). SP-B may also facilitate the removal of lipids from the monolayer during compression of the surface film.[35,78-80] It is important to note that these properties are also shared by SP-C[35,76,77] and that the individual contributions of these two peptides to the adsorption, spreading, surface tension reducing properties and turnover of surfactant phospholipids remains unclear.

The majority of phospholipid removed from the monolayer is recycled from the airspace by the type II cell.[81-84] The internalization of phospholipids by isolated type II cells and immortalized murine lung epithelial (MLE) cells is promoted by both native and synthetic SP-B peptides,[85-88] consistent with a role for SP-B in surfactant recycling. SP-B is also recycled by the type II cell,[89] but its clearance is more rapid than that of phospholipids.[90-92] Although SP-B internalization does not appear to be mediated by a receptor, this issue is not completely settled.[89,93] Following internalization, SP-B is detected in endocytic vesicles, multivesicular bodies and lamellar bodies as well as lamellar bodies undergoing exocytosis,[89] indicating that both the multivesicular body and the lamellar body communicate extensively with the endocytic pathway within the alveolar type II cell and that some fraction of secreted SP-B peptide is recycled and reutilized. A fraction of the SP-B internalized by isolated type II cells is also degraded;[94] SP-B degradation has also been detected in isolated alveolar macrophages[94] and it is likely that these two cell types represent the major sites of SP-B catabolism.

Regulation of SP-B Expression

Expression of SP-B During Lung Development

The ontogeny of SP-B has been studied at both the mRNA and protein levels in human,[48,49,95,96] rabbit,[25,26,50,97,98] rat[99-101] and mouse lung.[27] The levels of SP-B mRNA and protein increase during fetal lung development in all species examined to date. SP-B mRNA is detected as early as 12 weeks gestation in human fetal lung and increases to 50% of the amount in adult lung by the end of the second trimester.[95] Expression of SP-B and SP-C genes is initiated at an earlier stage of gestation than SP-A,[50,95,102,103] consistent with independent regulation of these genes during fetal and postnatal development. SP-B proprotein is detected as early as 15 weeks gestation;[49] however, significant amounts of mature SP-B are not detected until late in the second trimester, consistent with the onset of lamellar body morphogenesis at approximately 24 weeks gestation.[96] Synthesis and secretion of SP-B dramatically increase during approximately the last 20% of gestation, as indicated by the detection of SP-B in amniotic fluid at approximately 30 weeks gestation.[104,105] SP-B mRNA and protein peak immediately prior to birth, reaching levels that exceed those in adult lung.[25,26,99,106] This phenomenon likely reflects increased transcription of the SP-B gene as well as an increase in the number of SP-B expressing cells and correlates with improved surface properties of surfactant and overall lung maturity.[96,107] Genetic ablation of the SP-B gene in transgenic mice results in atelectasis and the lack of lamellar bodies and tubular myelin in the lungs of newborn animals,[108] supporting a role for SP-B in lamellar body biogenesis as well as tubular myelin formation; however, overall lung development (airway branching, number of type II cells, etc.) is not impaired in SP-B deficient mice.

Basal, Tissue/Cell-Specific and Developmental Regulation of SP-B Expression

The modulation of gene expression is dependent upon specific interactions of transcription factors with regulatory elements which reside most frequently in the flanking regions of promoters.[109,110] Lung-specific expression of SP-B results from the binding of specific transcription factors, in particular combinations at the 5'-flanking region of the human[111-113] and murine[114] SP-B promoter. Two cis-acting elements have been mapped to the proximal region (nucleotide positions -73 to -111 bp, relative to the transcription start site) and the distal region (-439 to -331 bp) in the 5'-flanking sequence of the human SP-B promoter by DNase I hypersensitivity and DNase I footprinting analyses.[111] The proximal element is sufficient to direct lung-specific expression in human lung adenocarcinoma (H441) cells in vitro[111] and in transgenic mice (Bohinski R and Whitsett JA, unpublished observations); a complex binding site, consisting of two sites for thyroid transcription factor 1 (TTF-1) and one site for hepatocyte nuclear factor 3α (HNF-3α), resides in this proximal region of the SP-B promoter.[112] Binding of TTF-1 and HNF-3α results in significant enhancement of SP-B promoter function[112,113] and the stimulatory effect of these two factors is synergistic.[112] Mutation of TTF-1 and HNF-3α binding sites in this region abolishes binding of both transcription factors and significantly diminishes transcriptional activity of the SP-B promoter.[112] The enhancer function of the distal region of the SP-B promoter was established by its ability to activate the SV40 promoter in both forward and reverse orientations.[113] Similar to the proximal region, three clustered TTF-1 binding sites were identified in this region.[113] Disruption of an individual TTF-1 binding site in this region results in either elimination or impairment of transcription, indicating that TTF-1 binding is absolutely required for enhancer activity.[113] Overall, these results suggest that TTF-1 and HNF-3α are required for both basal and cell-specific expression of SP-B.

Expression of TTF-1, a homeodomain transcription factor of the Nkx2.1 gene family, is limited to developing thyroid, brain and lung.[115] Immunohistochemical studies demon-

strated that TTF-1 is expressed at high levels in distal epithelial cells of the branching airway during lung development[115-118] and that its expression in the alveolar type II cell precedes and subsequently overlaps with expression of the SP-B proprotein and mature peptide.[118] Such data further support a regulatory role for TTF-1 in the developmental and lung-specific expression of SP-B. Disruption of the mouse TTF-1 gene results in severe pulmonary hypoplasia,[119] consistent with a key role for TTF-1 in lung epithelial morphogenesis. As for TTF-1, expression of HNF-3α overlaps with SP-B expression,[120] consistent with its proposed role in mediating developmental and lung-specific expression of SP-B. HNF-3α has also been shown to activate transcription of TTF-1 luciferase in MLE cells in vitro[121] and to have an earlier developmental onset than TTF-1,[112] suggesting that HNF-3α may be an important early determinant of epithelial cell differentiation. Interestingly, TTF-1 has recently been shown to bind regulatory elements within the HNF-3α promoter, suggesting that crosstalk between these two transcription factors may play an important role in the regulation of SP-B expression in developing lung.[122] However, both TTF-1 and HNF-3α are detected in tissues other than lung,[115,123] and TTF-1 is required for expression of Clara cell secretory protein (CCSP),[112,124] SP-A[112,125] and SP-C,[112,126] indicating that these two factors are not sufficient for cell-specific expression of SP-B. Recently, Margana and Boggaram reported that the ubiquitous nuclear proteins Sp1 and Sp3 are also necessary for maintenance of SP-B promoter activity in H441 cells;[127] however, as for TTF-1, Sp1 and Sp3 may also play a role in transcription of the CCSP and HNF-3α genes.[122,124] Overall, the regulation of SP-B gene transcription is very complex and the cis- and trans-acting elements that direct basal, cell-specific and developmental expression of SP-B are incompletely understood.

Modulation of SP-B Expression by Hormones, Cytokines and Growth Factors

Expression of SP-B mRNA and protein are rapidly induced in explant culture and are detected as early as 12 hours when fetal lung tissue is cultured in the absence of hormones.[20,95] Expression of the 42 kDa SP-B proprotein and 25 kDa processing intermediate, as well as the 18 kDa dimer form of the mature peptide are detected, indicating that processing enzymes are also expressed in explant culture.[20,22] Taken together, these data suggest that type II cell maturation proceeds rapidly when fetal lung tissue is removed from an inhibitory in vivo environment. A number of hormones, cytokines and growth factors alter the time course and level of SP-B expression in explant culture. In contrast, several agents, including interleukin-1 and interferon-γ,[128,129] alter expression of SP-A and/or SP-C without any effect on that of SP-B.

Positive Regulation of SP-B Expression

Glucocorticoids

Maternal administration of glucocorticoids has been shown to enhance fetal lung maturation and to stimulate synthesis of surfactant phospholipids[130,131] (see chapter 2). The effects of glucocorticoid treatment on SP-B expression have been studied, mainly in explants of fetal human,[95,96,132,133] rat[134] and rabbit[26,106,135] lung as well as in H441 cells.[136-138] In explant culture, the levels of SP-B mRNA increase approximately 2- to 3-fold in the absence of exogenous hormones and SP-B expression is further enhanced by addition of dexamethasone to the culture medium.[95,96,133,134] Glucocorticoid effects are receptor-mediated and occur in a dose- and time-dependent manner.[20,95,106,132-134] Increased SP-B mRNA levels are detected after 10 hours exposure to dexamethasone and reach maximal levels (approximately 4-fold) by 24-48 hours; half maximal stimulation occurs at a concentration of 1 nM dexamethasone.[95] Increased mRNA levels are completely reversed within 48 hours of removing hormone from the culture medium.[95] SP-B mRNA is also induced by natural corti-

costeroids but not by sex steroids.[95] Similar effects of glucocorticoids on SP-B mRNA expression are seen in explants of fetal rabbit[26,106,135] and rat[134] lung although the kinetics of induction are different among these species. Dexamethasone also enhances SP-B mRNA and protein expression in H441 cells in a dose-responsive fashion and removal of the hormone results in decreased SP-B mRNA levels.[136-138] The stimulatory effects of glucocorticoids on SP-B expression seen in vitro also occur in fetal lung in vivo following maternal administration of dexamethasone.[100,131,139]

Although glucocorticoid treatment increases SP-B expression in vivo and in vitro, the molecular mechanism underlying this effect is not clear. Both actinomycin D and 5,6-dichloro-1-β-ribofuranyosyl benzimidazole, inhibitors of RNA synthesis, block dexamethasone-induced accumulation of SP-B mRNA,[26,132,133,138] consistent with an effect of glucocorticoids on SP-B transcription. Nuclear run-on experiments demonstrate that dexamethasone treatment results in either a 2- to 3-fold increase[26,132,138] or no detectable effect[106] on SP-B gene transcription. In the studies in which glucocorticoid treatment is associated with an increased rate of transcription, the magnitude of the increase is consistently less than the overall increase in mRNA level, suggesting that mRNA stability also contributes to glucocorticoid-induced accumulation of SP-B mRNA. Direct evidence for this hypothesis is provided by studies of SP-B mRNA half life, which demonstrate that dexamethasone significantly increases SP-B mRNA stability.[26,132] Collectively, the results of these studies suggest that the stimulatory effects of glucocorticoid on SP-B expression in vitro are mediated by an increase in the transcription rate of the SP-B gene and increased stability of its mRNA.[26,132,138]

A number of questions remain regarding the mechanism whereby glucocorticoids stimulate SP-B gene transcription and the importance of this effect in vivo. The relative contributions of increased rate of transcription (an increase in the number of transcripts from each SP-B gene) and increased number of type II cells (an increase in the number of SP-B genes available for transcription) to the overall increase in the number of SP-B mRNA transcripts following glucocorticoid treatment remain unresolved. It is also not clear if glucocorticoids act directly or indirectly on the SP-B gene; to date no functional glucocorticoid response element has been identified within or flanking the SP-B gene. Finally, there are some questions as to the role of endogenous glucocorticoids in the initiation and maintenance of SP-B expression. Adrenalectomy does not alter SP-B mRNA levels, suggesting a minor role for glucocorticoids in the maintenance of SP-B expression.[140] Targeted disruption of the mouse glucocorticoid receptor gene results in acute respiratory distress and death within a few hours of birth; however, SP-B expression appears to be unaffected.[141] In summary, although exogenous glucocorticoids stimulate SP-B expression, there is relatively little evidence that endogenous glucocorticoids play an important role in the developmental or basal regulation of SP-B expression or that glucocorticoids directly bind elements within or around the SP-B gene.

Cyclic AMP

Compared to glucocorticoids, SP-B mRNA levels are enhanced to a lesser extent by cyclic AMP (cAMP) and its analogs in cultured explants of fetal human,[20,95] rat[134] and rabbit[26] lung. Levels of SP-B mRNA are also elevated by agents such as terbutaline, forskolin and prostaglandins that increase intracellular cAMP concentration.[95,142] In combination experiments, treatment of rat fetal lung explant cultures with dibutyryl cAMP and dexamethasone results in an additive effect on the levels of SP-B mRNA expression, whereas the effect of a combination of 8-bromo cAMP and dexamethasone is not significantly different from that of dexamethasone alone.[134] Dibutyryl cAMP increases both transcription and

overall SP-B mRNA levels by two to 3-fold without any effect on mRNA stability, suggesting that cAMP modulates SP-B expression primarily at the level of gene transcription.[26]

The sequence from -218 to +41 bp in the 5'-flanking region of the human SP-B gene shows markedly increased reporter gene expression in response to cAMP-dependent protein kinase (protein kinase A, PKA); however, the absence of a canonical cAMP response element within this region of the SP-B promoter suggests that the effect of cAMP is indirect. Consistent with this hypothesis, the catalytic subunit of PKA (cat-β) phosphorylates TTF-1 at thr_9 and phosphorylation at this position correlates directly with increased transcription from the SP-B promoter.[143] Overall, the results of these studies suggest that cAMP is likely an important regulator of SP-B expression, although its precise role in basal, developmental or cell-specific expression remains to be elucidated.

Keratinocyte growth factor (KGF) and retinoic acid

KGF and retinoic acid may play important roles in lung development. KGF is a potent mitogen for alveolar type II cells in vivo[144] and in vitro[145,146] and treatment of isolated type II cells with KGF results in a 2- to 3-fold increase in SP-B mRNA.[144,146] A similar response is detected following treatment with acidic fibroblast growth factor which also binds the KGF receptor. The effect of retinoic acid on SP-B expression is less clear with both an increase[147,148] and a decrease[149] in SP-B mRNA reported in fetal lung explants. The molecular mechanisms underlying altered SP-B expression are not known and the roles of KGF and retinoic acid in type II cell differentiation and airway branching morphogenesis in vivo remain to be established.

Negative Regulation of SP-B Expression

There is increasing evidence that lowered levels of SP-B contribute to the pathogenesis of various lung diseases. Decreased levels of SP-B are associated with increased risk of respiratory distress syndrome (RDS) in premature infants[105] and of acute RDS in adults.[150] Low levels of SP-B were also associated with chronic lung disease in a human patient[151] and SP-B levels of less than 1% failed to rescue SP-B knockout mice;[152] further, mice hemizygous for the SP-B gene (i.e., having only one functional SP-B allele) exhibit decreased lung compliance and airtrapping.[153]

At least two agents decrease SP-B mRNA levels, tumor necrosis factor-α and TPA. The inhibitory effect of these agents is mediated by both transcriptional and post-transcriptional mechanisms.[154-156] Reduced levels of SP-B mRNA in H441 cells are primarily related to decreased mRNA stability, which is conferred by an element within the 3'-untranslated region of human SP-B mRNA.[157] The mechanism leading to decreased transcription of the SP-B gene is less clear. The human SP-B gene contains an AP-1 element downstream from the transcription initiation site (+15 to +31 bp) which is likely required for basal transcription,[158] but there is no evidence that TPA acts through this element. In contrast, TPA strongly inhibits transcription from the upstream promoter region (-208 to -54 bp);[159] however, this region does not contain an AP-1 element, suggesting that a novel element mediates the effect or, more likely, that TPA acts indirectly through some other transcription factor, such as TTF-1, that binds this sequence. Further studies leading to the identification of cis- and trans-acting factors that negatively regulate SP-B expression may provide insight into the molecular mechanisms underlying SP-B deficiency associated with various pathologic conditions.

Function of SP-B

Surfactant deficiency results in compromised gas exchange (i.e., poor oxygenation) that frequently requires respiratory support. The most common cause of surfactant defi-

ciency in neonates is premature birth, which is often accompanied by RDS. Administration of surfactant lipid extracts containing SP-B and SP-C to newborn infants suffering from RDS dramatically improves oxygenation, thereby reducing the need for respiratory support.[160-162] Surfactant replacement preparations that do not include SP-B appear to be less effective in vitro and in vivo[163,164] and the addition of SP-B significantly improves such preparations.[165]

The importance of SP-B for surfactant function is underscored by the lethal phenotype associated with genetic deficiency of SP-B. Several infants have been identified in which a substitution of GAA for C in exon four of the SP-B gene leads to the introduction of a premature termination codon for translation, resulting in a complete absence of SP-B mRNA and protein.[166] These infants are generally full-term but rapidly develop serve RDS and ultimately succumb despite intensive respiratory support.[166,167] Genetic ablation of the SP-B gene by homologous recombination in transgenic mice results in similar perinatal respiratory failure associated with atelectasis and alterations in the routing, storage and function of surfactant phospholipids and proteins.[108] This disorder is also associated with the aberrant processing and secretion of an immature SP-C peptide which may contribute to respiratory dysfunction.[167,168] Collectively, these results demonstrate that SP-B is required for intracellular organization of surfactant phospholipids, proteolytic processing of the SP-C proprotein and optimal surface properties of alveolar surfactant. The mechanism(s) by which SP-B facilitates these processes and the structural domains underlying each of these functions are currently not well understood.

Intracellular Functions of SP-B

Administration of surfactant replacement preparations containing SP-B failed to reverse progressive respiratory decline in an infant with inherited SP-B deficiency, suggesting that intracellular SP-B plays a critical role in surfactant homeostasis.[169] Recent studies by Akinbi et al[152] have provided some insight into the potential intracellular functions of SP-B. The absence of SP-B in SP-B knockout mice is accompanied by a complete lack of typical lamellar bodies and the occurrence of composite bodies containing multivesicular bodies and loosely organized multilamellar membranes.[108] Expression of an SP-B construct encoding the NH_2-terminal propeptide and mature peptide ($SP\text{-}B_{\Delta C}$, in which the sequence encoding the 102 amino acid COOH-terminal propeptide is deleted) in SP-B knockout mice results in the formation of lamellar bodies consisting of tightly organized multilamellated membranes similar to those in wild type littermates; further, these mice are all viable, indicating that $SP\text{-}B_{\Delta C}$ restores the surface properties of alveolar surfactant. These results suggest that the mature SP-B peptide and/or the NH_2-terminal propeptide play an important role in lamellar body morphogenesis. However, despite the normal concentric arrangement of membranes in lamellar bodies in rescued SP-B knockout animals, these inclusions are often dramatically enlarged, resulting in a significant increase in the intracellular surfactant pool. Surfactant synthesis and secretion are only marginally affected in these animals and the alveolar surfactant pool size and phospholipid composition are normal. Taken together, these results suggest that the COOH-terminal propeptide of SP-B may play a role in the metabolism of surfactant phospholipids internalized from the airway. Finally, expression of $SP\text{-}B_{\Delta C}$ in SP-B knockout mice largely corrects misprocessing of the SP-C proprotein, suggesting that the COOH-terminal propeptide is not required for this function. Overall, the expression of various mutated or deleted SP-B proteins in the knockout background provides a powerful experimental paradigm that should facilitate systematic structure/function analyses of SP-B within the context of the intact animal.

The Anti-Atelectatic Properties of SP-B

Results of a number of studies suggest that the mature SP-B peptide enhances formation, maintenance and turnover of the surfactant monolayer, thereby promoting alveolar stability during respiration (see chapter 1). Alveolar surface tension at equilibrium is about 25 mN/m and is reduced to near zero at end expiration in order to oppose the forces created by decreased alveolar radius.[5] The reduction in surface tension is primarily related to the phospholipid components of surfactant; however, in the absence of SP-B and SP-C the adsorption and spreading of phospholipids is relatively poor,[170,171] resulting in elevated surface tension at low lung volumes and increased resistance to inflation. Purified SP-B dramatically enhances the formation of phospholipid films in vitro.[12,164,172,173] SP-B promotes the formation of a stable surface film by facilitating the insertion and removal of phospholipid molecules from the monolayer during alveolar expansion and contraction.[35,76-80] This property is likely related to the interaction of the positively charged SP-B peptide with negatively charged phosphatidylglycerol molecules.[32,34,35] SP-B may also contribute to stabilization of the surface film during lung injury: The influx of serum proteins into the alveolar space markedly inhibits surface activity and this inhibition is partially overcome by SP-B.[164,174] Finally, in addition to promoting alveolar stability, SP-B may play a role in maintaining the patency of terminal airways. Heterozygous SP-B +/- mice have a 50% reduction in mature SP-B peptide and normal levels of SP-C.[153] Inflation/deflation curves in these mice showed significantly increased residual volumes relative to wild type littermates, likely related to small airway collapse at low deflation pressures.[153] Whether this outcome is the result of decreased SP-B in type II cells or Clara cells remains unclear.

The Role of SP-B in Phospholipid Recycling

The removal of phospholipid molecules from the surfactant monolayer results in vesicles which have low surface activity and are relatively depleted in surfactant proteins.[175,176] Most of this form of surfactant phospholipid is taken up by type II cells for reutilization.[83,84] Native and synthetic SP-B peptides enhance the uptake of phospholipid by isolated type II cells and fibroblasts;[7,88] however, uptake stimulated by SP-B is significantly less than that stimulated by SP-C and the latter uptake is reduced in the presence of SP-B.[88] Overall, the precise role of SP-B in phospholipid uptake in vitro and the physiological relevance of these findings remain unclear.

Future Directions

Although it is now clear that SP-B is absolutely required for lung function, many questions remain regarding the regulation of SP-B expression, the precise intracellular and extracellular functions of SP-B and the mechanisms by which these functions are executed. The recent generation of cell lines with type II cell characteristics[177] should expedite the identification of transcription factors and their cognate recognition sequences that modulate basal and cell-specific expression of SP-B, while the use of transgenic mice, although expensive and time-consuming, should facilitate analyses of the developmental regulation of SP-B expression. The availability of lung cell-specific promoters (such as CCSP and SP-C) and the SP-B knockout mouse[108] will permit replacement of wild type SP-B in type II cells and/or Clara cells with selectively mutated forms of SP-B, as recently described.[152] This strategy should provide new insight into the various functions of SP-B and facilitate the identification of structural domains underlying these functions.

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Chapter 5

Molecular Processing and Cellular Metabolism of Surfactant Protein C

Michael F. Beers

The pioneering observations of Pattle[1] and Clements[2] over 40 years ago provided the initial convincing evidence for the presence of a pulmonary "surfactant" (surface active agent) and related the pressure-volume properties of the lung to the surface tension-surface area behavior of the alveolar lining material. Based upon a voluminous literature on the composition, structure and function of lung surfactant which has accumulated since its discovery in pulmonary edema fluid, lung slices and alveolar lavage, we have come to recognize the diversity of structure, function and metabolism of individual surfactant components. Following the initial description of the effect of surfactant on alveolar surface tension, it was soon established that phospholipids, particularly phosphatidylcholines, make up the major mass of surfactant.[3-4] Early research initiatives focused primarily on the description of the composition, biophysical properties and metabolism of these phospholipids and these are extensively reviewed in other chapters of this volume.

The cell-free, lipid-rich pellet obtained by differential centrifugation of bronchoalveolar lavage fluid from a variety of species is composed of 90% lipids and 10% proteins.[5-7] Much of the protein fraction in isolated surfactant consists of serum proteins.[8-10] However, over the past 25 years we have come to recognize a small but important group of surfactant specific proteins which have been shown to be essential for normal surfactant biophysical function and metabolism. In 1973, King and colleagues working in Clements' laboratory reported the isolation of a unique glycoprotein from the surface active material obtained from canine lung lavage (variably termed SP26-35 or SAP-A).[11] After the initial characterization of what is now known as surfactant protein A (SP-A), two other smaller and more hydrophobic surfactant associated proteins were soon described. In 1979, Phizackerly reported on a family of proteins obtained from organic extracts of surfactant fractions of pig lung lavage and lung homogenate.[12] In 1984, Claypool and colleagues described a small, hydrophobic peptide obtained from an ethanol-diethylether extraction of purified rat surfactant which their group termed Apo-Et.[13] Similar peptides (3.5-6.0 kDa, reduced) were subsequently isolated from human (SP-Val, SAP-6),[14-16] bovine,[16] porcine[17] and canine (SP5)[18] sources. The cDNA sequence for these proteins as well as rat, rabbit, murine and mink surfactant protein C (SP-C) have been determined.[19-23] A consensus conference in 1988 unified the nomenclature for surfactant proteins and designated the lowest molecular weight surfactant protein as SP-C.[24]

The alveolar form of SP-C recovered in lung lavage from a variety of species is a 3.5-3.7 kDa lung-specific peptide whose primary sequence represents one of the most hydrophobic proteins known.[25] Purified SP-C, when reconstituted with surfactant phospho-

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lipid components, has been shown both in vitro and in vivo to enhance the biophysical surface activity of these lipid mixtures.[26-28] This protein is also an active component of some clinical surfactant preparations.[29] See chapter 1 for a detailed discussion of the biophysical properties of SP-C and its interactions with other surfactant proteins.

This chapter will review the molecular and cellular mechanisms underlying the expression and metabolic life cycle of SP-C. Early studies of SP-C were concerned with isolation and biochemical characterization of SP-C protein and of the cloning and tissue specific expression of the SP-C gene. In the decade since its isolation and cloning, attention has focused upon the important regulatory elements of SP-C gene expression, the synthesis and post-translational processing of SP-C and the use of SP-C promoters for the direction of lung-specific expression of a variety of genes in transgenic mouse models. Each of these topics will be discussed in the context of both developmental and adult models. In addition, the unique structural properties of SP-C will also be highlighted, and the abnormalities in post-translational processing of SP-C recently underscored by the syndrome of congenital surfactant protein B (SP-B) deficiency will be reviewed.

Structure and Properties of SP-C

The structure and properties of SP-C render it markedly different from either SP-B or the more hydrophilic SP-A and surfactant protein D (SP-D). Differences in structure, post-translational modifications and functional properties between SP-C and the other surfactant proteins are summarized in Table 5.1.

Primary Sequence

The alveolar form of SP-C ("mature" SP-C; SP-$C_{3.7}$) isolated from lung lavage fractions of various species is composed of 33-35 amino acids.[14-23] Among species, the majority of amino acid residues in the SP-C primary sequence are highly conserved (Fig. 5.1). SP-C is a predominantly hydrophobic molecule due to a high content of Val, Ile and Leu (~60-65% of the primary sequence).[25] Although most of the amino acids comprising SP-C are apolar, the molecule does contain a degree of amphipathicity. The amino terminus contains positively charged, hydrophilic residues, while the center of the peptide is made up of a 23 amino acid, polyvaline, hydrophobic domain.

Based upon amino acid sequencing of purified preparations, the NH_2-terminus of most species of SP-C contains a fair degree of heterogeneity, with the presence of N, N-1, and N-2 forms.[16,30] The presence of truncated forms is probably due to variability of N-terminal proteolysis during synthetic processing from a larger proprotein precursor (see Metabolic Life Cycle of SP-C, below).

Homology of the SP-C sequence with other proteins has not been widely reported. One report has suggested a minor sequence similarity between a region of 5 amino acids in the N-terminal portion of mature SP-C (residues 3-7), the C-terminus of SP-B and the primordial nonheme containing oxygen binding proteins hemerythrin and myohemerythrin.[31] While functional similarities for these sets of proteins are not obvious, the authors proposed that these sequences function as adhesive patches and could provide a means of sticking SP-C and SP-B together (i.e., biochemical velcro®).[31]

By sodium dodecyl sulfate polyacrylamide gel electrophoresis, the relative molecular mass (M_r) of SP-C ranges from 3,500 to 9,000.[15-18,32-33] Part of this variability is related to analytical difficulties imposed by the molecule's intrinsic properties (see Special Analytical Problems in the Quantitation and Characterization of SP-C, below). However, under nonreducing conditions, the presence of oligomers has been consistently documented in electrophoretic analyses of SP-C from nearly all species but in the presence of reducing agents (β-mercaptoethanol or dithiothreitol), the higher molecular weight forms are

Table 5.1. Comparison of structure and function of SP-C with that of other surfactant proteins

Property	Hydrophobic		Hydrophilic	
	SP-C	SP-B	SP-A	SP-D
Structure				
M_r (x 10^{-3}) reduced (nonreduced)	3.7 (6-7)	9 (18)	36 (72-700)	43 (130)
Primary translation product, kDa	21	42	26	39
Homology	None*	Prosaposin	C1q+;C-Lectins+;Collagen+	C1q+;Collagen+;C-Lectins+
α–helix	Yes	No	No	No
Membrane integration	Yes	No	No	No
Post-translational processing				
Signal peptide	No	Yes	Yes	No
Propeptide cleavage (no.)	Yes (2)	Yes (3)	No	No
Glycosylation	No	Proprotein only	Yes (N-linked)	Yes (to lysine)
Palmitoylation	Yes	No	No	No
Functions∞				
Phospholipid adsorption	Yes (<SP-B)	Yes (>SP-C)	± (Needs SP-B)	No
Tubular myelin	No	Yes	Yes	No
Resistance to inactivation	Yes (<SP-B, >SP-A)	Yes	Yes	No
Monolayer formation	Yes (>SP-B)	Yes	No	No
Vesicle fusion	Yes	Yes	No	No
Host defense	No	No	Yes	Yes
Regulation of lipid secretion	No	No	Yes (↑)	Yes
Regulation of lipid uptake	±	±	Yes (↓)	No

*Minor amino acid sequence homology with hemerythrin and myohemerythrin.
+ Structural homology only
∞ Biophysical functions reviewed in more detail in chapter 1

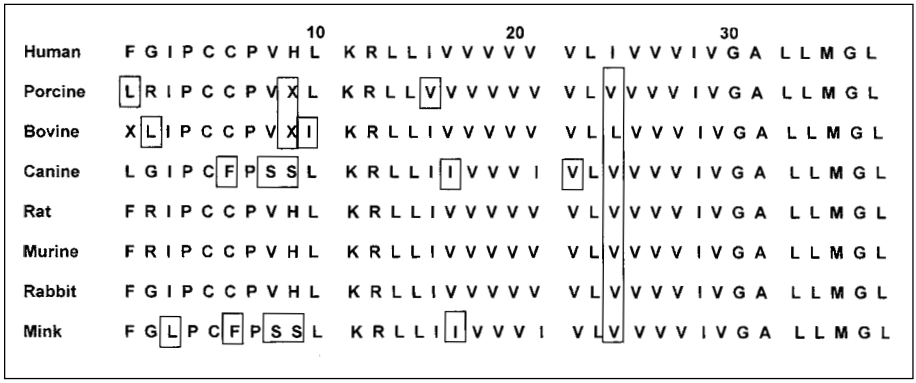

Fig. 5.1. Complete amino acid sequence of the mature SP-C peptide. Deduced amino acid sequence of SP-$C_{3.7}$ from 8 mammalian species (from refs.14-23). Boxed regions indicate nonhomologous amino acid substitutions (compared to the human sequence). Alternative NH_2-termini determined from direct amino acid sequencing data appear shaded (Direct NH_2-terminal sequence data for rat, murine, rabbit and mink SP-C are not available).

converted to a low molecular weight form. A naturally occurring dimeric form of SP-C (SP-C_2) has been isolated from bovine and canine lung lavage.[32-33] Both monomeric and dimeric forms appear to exhibit biophysical activity; however, there are differences in calcium requirements and efficiency of monolayer insertion. The relative amounts of each SP-C form in the alveolus and the physiological significance of SP-C_2 remain to be established.

Secondary Structure

From the cDNA sequence, modeling of the secondary structure of monomeric SP-C had indicated that the polyvaline stretch contained in the primary sequence of the protein is highly α-helical and capable of spanning phospholipid bilayers in the liquid crystalline phase.[34] The remainder of the secondary structure was predicted to be in a mainly random coil conformation.

The existence of the valyl-rich α-helix was confirmed by analyses of the secondary structure of SP-C from several species, using a variety of biophysical techniques including Fourier transform infrared spectroscopy (FT-IR),[32,35-36] circular dichroism (CD)[33] and ^2H and ^1H nuclear magnetic resonance (NMR).[37-38] FT-IR measurements of bovine SP-C in dipalmitoyl phosphatidylcholine (DPPC) films has shown that the long axis of the α-helix of mature SP-C associates with lipid layers in an orientation parallel to lipid acyl chains.[35-36] CD studies of monolayers of lipid and canine SP-C at an air-liquid interface measured at two different surface pressures demonstrated a similar secondary structure.[33] A ^1H-NMR structure determination of native porcine SP-C dissolved in apolar solvent mixtures demonstrated an outstandingly regular helical structure between amino acid positions 9 and 34.[37] ^2H-NMR of selectively deuterated SP-C also indicated the presence of an α-helix with the N-terminal dodecapeptide segment of SP-C calculated to be in proximity to the lipid bilayer.[38] Taken together, the biophysical measurements indicate that a significant portion of the secondary structure of monomeric SP-C (46-90%) is an α-helix. Such a stretch would encompass 13-25 of the total residues, resulting in a helical length of 27-37 Å. This is clearly sufficient to achieve membrane spanning orientation of most lipid bilayers.

Despite the unequivocal presence of an α-helix, there is some variability in the estimates of the amounts of random coil and β-sheet structure in monomeric SP-C. Variations

in these determinations made by the techniques detailed above appear to be dependent upon differences in the solvent and lipid environments used. Additional differences in these secondary structures exist for dimeric SP-C_2 (versus monomeric forms), but further characterization is required.[32-33] Neither X-ray diffraction measurements nor a crystal structure determination of SP-C or SP-C_2 have been accomplished to date.

Palmitoylation

In addition to the extreme hydrophobicity conveyed by its primary sequence, mature SP-C is known to be a true lipopeptide, i.e., it contains covalently linked lipid residues. In this regard, SP-C is unique among surfactant proteins. Two groups have separately shown that monomeric SP-C isolated from the surfactant of five mammalian species exists in a palmitoylated form with a palmitoyl thioester linkage to either one serine or two cysteine residues near the N-terminus (Fig. 5.2).[39-41] Greater than 90% of natural bovine, canine and human SP-$C_{3.7}$ is found in the palmitoylated form. Using mass spectroscopy and high pressure liquid chromatography (HPLC) analysis, treatment of isolated alveolar SP-C with either hydroxylamine or KOH has been reported to result in cleavage of covalently linked palmitic acid groups. In vitro palmitoylation of recombinantly synthesized SP-C has also been described.[40] On a molar basis, the number of palmitate residues is proportional to the number of cysteine residues in the primary sequence. With the exception of canine and mink SP-C, all other species contain a double palmitoylation motif at residues cys_5-cys_6 of the mature molecule. Monopalmitoylation of dog and mink SP-C probably occurs at cys_5. Although intracellular pools of SP-$C_{3.7}$ have not been analyzed directly for the presence of covalent lipid, palmitoylation of SP-C is most likely an early post-translational modification which occurs in the Golgi (see Metabolic Life Cycle of SP-C, below).

The addition of palmitic acid to the SP-C molecule represents a novel and potentially important feature. Covalent modifications of proteins with fatty acids are associated with enhancement of protein-membrane interactions.[42-43] Structurally, the covalent palmitoylation modification converts the relatively hydrophilic N-terminal region of mature SP-C into a more hydrophobic domain, and palmitoylation of an a priori hydrophobic protein is not unprecedented.[44] The exact biological role of SP-C palmitoylation is as yet unknown. Based upon a review of the literature, potential functions for a palmitoylated SP-C can be categorized based upon either recent data on the effect of palmitoylation of SP-C in surfactant biophysics[27-28,43-45] or extrapolation from the known functions of palmitoylation of other proteins in other biological systems (reviewed in refs. 42-43). From these studies, two major possibilities exist:

1. Palmitoylation directly contributes to the structural and/or biophysical properties of SP-C;
2. Palmitoylation functions in a regulatory role affecting a nonsurfactant function of SP-C, such as its intra-/extra-cellular metabolism.

The effect of palmitoylation on SP-C structure and function has been partially addressed. Using natural and recombinant proteins and utilizing biochemical and biophysical techniques, several groups have identified structural and physical differences between palmitoylated and nonpalmitoylated SP-C.[27-28,35,43-46] Palmitoylation of SP-C further increases both its hydrophobicity and α-helical content.[35,45] The effect of acylation on surfactant biophysical functions mediated by SP-C is dependent on the methodology utilized. By Wilhelmy balance measurements, acylated SP-C is more efficient at promotion of minimal surface tension by phospholipid mixtures than the deacylated form. Using a captive bubble surfactometer, Qanbar and Possmayer recently demonstrated that SP-C palmitoylation does not influence overall surface tension reduction during quasistatic expansion and compression, but acylation does appear to enhance its effect on lipid respreading and film stability.[28]

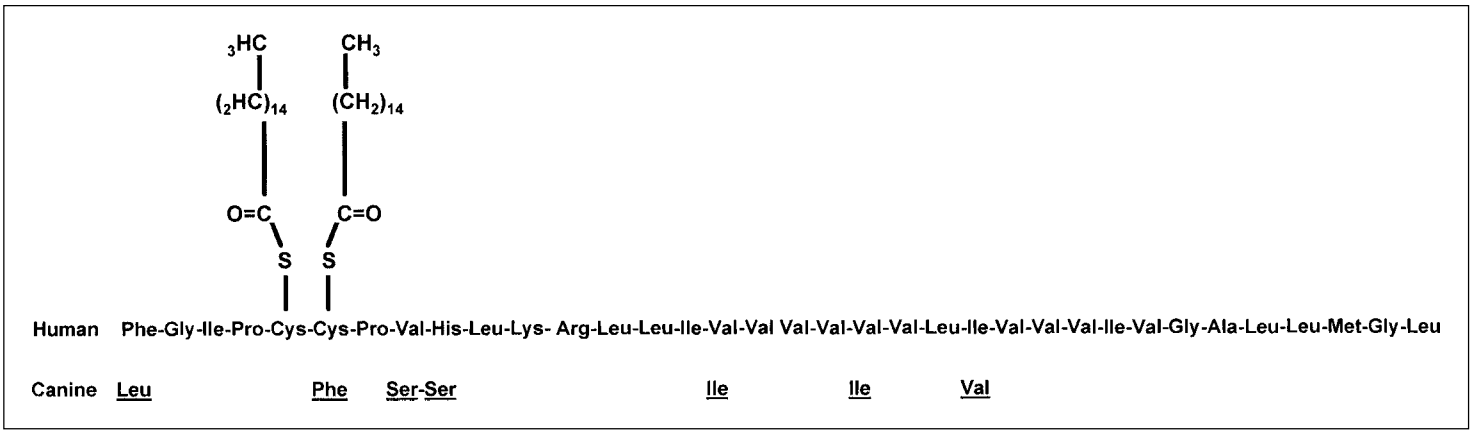

Fig. 5.2. Structure of intact SP-C. The amino acid sequence of the human form is shown. Residues which differ from the canine molecule are indicated below the human sequence. In the human form, two palmitoylation sites occur at cys_5 and cys_6. The canine species contains a single palmitic acid residue which could be covalently bound at either cys_5, ser_8 or ser_9.

In addition to a potential role in the augmentation of biophysical function, palmitoylation of SP-C could also play a regulatory role in either the post-translational processing, intracellular targeting or secretion of newly synthesized SP-C. The properties of some well-characterized palmitoylated proteins are listed in Table 5.2. From other experimental models, the palmitoylation of proteins plays important regulatory roles in the cell biology of their metabolism and/or function and in the mediation of cell physiological events. Some of the nonsurfactant, biological functions of palmitoylation include:

1. Enhancement of membrane association and/or protein function;
2. Functional modification of existing transmembrane proteins; and
3. Regulation of intracellular protein and receptor trafficking.

Enhancement of membrane association and/or protein function

Studies of nonglycosylated-palmitoylated membrane proteins such as myelin proteolipid protein,[47] $p21_{ras}$[48] and ankryrin[49] suggest that acylation is a prerequisite for membrane association. Mutational analysis of reversible palmitoylation of G-protein α subunits in COS cells has been implicated in the regulation of G_α localization and function.[50] Covalent acylation of apolipoprotein A1 before secretion appears to enhance its interactions with lipids.[51]

Functional modification of existing transmembrane proteins

Acylation is important for normal signal transduction by G-protein coupled receptors. Site directed mutagenesis of the β-receptor inhibited GTP-modulated high-affinity agonist binding.[52] The serotonin $5\text{-}HT_{1B}$ receptor also contains palmitate residues in a structurally similar position.[53]

Regulation of intracellular protein and receptor trafficking

Palmitoylation of the transferrin receptor is associated with an inhibition in the rate of ligand-receptor endocytosis.[54] Addition of the palmitoylated N-terminus of GAP-43 to chloramphenicol acetyl transferase (CAT) can direct the translocation of CAT to the plasma membrane and growth cones of nerve cells.[55]

Though structural and biochemical analyses have documented the presence of palmitate residues on SP-C, the kinetics of incorporation, sites of acylation and/or deacylation, enzymes involved and effects of palmitate on SP-C synthetic processing and/or secretion have not yet been fully delineated. Biosynthesis of palmitoylated SP-C precursors has been demonstrated in murine fetal lung and Chinese hamster ovary (CHO) cells transfected with a human SP-C cDNA.[56] Addition of cerulenin, an inhibitor of palmitate incorporation, to type II cells blocked the normal post-translational proteolytic processing profile of the SP-C proprotein.[57] It is therefore plausible to consider an expanded function for palmitoylation of SP-C beyond biophysical modification to include a role in membrane-association and/or targeting of SP-C to specific intracellular sites during synthetic processing. Additional studies utilizing metabolic inhibition and site-directed mutagenesis will be required to fully define these effects.

Isolation of SP-C

The isolation and characterization of SP-C from lung lavage, purified surfactant, lung tissue homogenate and amniotic fluid has both exploited and been victimized by the apolar properties of the molecule. Attempting to separate SP-C from both phospholipids and other surfactant proteins is often a tedious task requiring multiple step procedures.

Most methods currently in use utilize at least a three step process. An organic solvent extract of surfactant or lung tissue (using either 1-butanol[18,58] or chloroform-methanol[15]) is used to pool phospholipids and hydrophobic proteins (SP-B, SP-C). In the second step,

Table 5.2. Examples of palmitoylated proteins in eukaryotic cells

Protein (Ref.)	Source	Cellular Location	Function of Palmitate Group
Myelin proteolipid protein (47)	Myelin sheath	Plasma membrane	Targets MLP to growth cones
$p21_{ras}$ (48)	Human cell lines	Plasma membrane	Targets p21 to plasma membrane
Ankyrin (49)	Erythrocytes	Cytoskeleton	Site-specific targeting
G_{α} protein (50)	COS cells	Plasma membrane	Cellular localization
Apolipoprotein A-1 (51)	Liver cell line (HEP G2)	Secreted	Lipid interactions/lipoprotein metabolism
β-adrenergic receptor (52)	Muscle cell line	Plasma membrane	G protein coupling/agonist binding
Serotinin 5HT-1B (53)	Sf9 cells	Plasma membrane	? —see β receptor
Transferrin receptor (54)	Human T cell line	Plasma membrane	Receptor cycling
GAP 43-CAT chimera (55)	Neuronal cell lines	Plasma membrane/growth cone	Directs targeting
SP-C (27-28)	Type II cells/surfactant	LB/secreted	Biophysical function
ProSP-C (57)	Type II cells	Exocytic pathway	? intracellular targeting

LB = lamellar body
CAT= chloramphenicol acetyl transferase

delipidation of the hydrophobic proteins is effected by performing liquid chromatography with either silicic acid or Sephadex LH-20.[13,18,59] Alternatively, HPLC with Lipidex columns has been used.[15] Finally, SP-C is separated from the delipidated mixture of hydrophobic proteins by LH-60 chromatography.[13,15,18,59] While currently still available, the continued production and distribution of Sephadex LH-60 is in doubt (Haagsman HP, personal communication). Alternatively, purification utilizing isocratic HPLC or gradient reversed phase HPLC[15,60-61] has been performed, but the overall yield appears somewhat less and could be limiting for certain types of studies.

As might be ascertained from a review of these techniques, the purity of many SP-C preparations is potentially compromised by other surfactant components which exhibit overlapping solubility properties. Contamination of the final product with phospholipids[62] or surfactant protein B[25] can occur.

Tissue Content of SP-C

The amount of SP-C in surfactant has not been precisely quantified, but estimates range from 2% to 4% of total protein based on indirect methods.[25,63] In addition to lavage fractions and purified surfactant preparations, SP-$C_{3.7}$ has been found in type II cell specific storage organelles (lamellar bodies).[12,63] SP-C content of lamellar bodies has been measured indirectly by fluorescamine and comprises up to 22% of lamellar body protein.[63] This, however, may be an overestimate (see Special Analytical Problems In The Quantitation And Characterization of SP-C, below). The quantitation or characterization of SP-C in commercially available natural surfactant preparations or cell fractions has not been reported.

Special Analytical Problems in the Quantitation and Characterization of SP-C

The reliable analysis of SP-C in purified protein preparations or in isolated lung fractions has proven to be extremely difficult because of the molecule's preferential solubility in organic solvents, the presence of a rather large continuous hydrophobic segment and the occurrence of NH_2-truncated forms. Given its unusual properties, it is not surprising that there are many problems in detecting SP-$C_{3.7}$ by general protein methods. Poor solubility in aqueous solutions, lipid/detergent interference, a low molecular weight, propensity for aggregation, oligomer formation and poor staining properties on acrylamide gels are but a few of the problems which can combine to create havoc with quantitation of absolute protein content, electrophoretic analysis and N-terminal sequence determination.[25,30] Additionally, unlike the other surfactant proteins against which antibodies have been raised and for which enzyme-linked immunosorbent assay (ELISA) assays are available, attempts at producing monospecific antisera have not been successful, making SP-C specific detection and quantitation in surfactant and tissue fractions more problematic.

Quantitation of the protein content of isolated SP-C preparations can be affected by contaminating phospholipids. Protein quantitation using fluorescamine dye is often an overestimate of true content due to contaminating phosphatidylethanolamine.[64] Likewise, the Lowry assay[65] underestimates protein content because SP-C lacks tyrosine residues required for signal development by this colorimetric assay. SP-C incompletely binds Coomassie blue dye[66] so that protein quantitation by the Bradford assay[67] also underestimates true content. Even amino acid composition determination, often a 'gold standard' measurement, underestimates protein content due to difficulty with complete hydrolysis of the polyvaline-leucine region. Johansson and colleagues have shown marked variability in amino acid composition of SP-C depending upon the conditions of hydrolysis.[16,30] Acid hydrolysis of SP-C peptides requires both prolonged incubation time (>24 h) and elevated temperatures (>150°C) for complete recovery of aliphatic branched chain amino acids but at the expense

of isoleucine recovery.[16] The routine hydrolytic analysis of protein samples using extended hydrolysis conditions (3 days) is impractical. At present, standardization of a colorimetric protein assay (Lowry or Bradford) with either synthetic peptide standards or purified natural SP-C simultaneously quantitated by amino acid composition under routine (24 hours at 110°C) and extended (150°C for 72 hours) conditions seems to offer the best current approach for precise quantitation of isolated SP-C content.

Two methods for determination of SP-C content in pulmonary surfactant preparations have recently been described. One procedure for simultaneous quantitation of SP-C and SP-B is based on the method for purification of these proteins described by Curstedt and co-workers.[15] After butanol extraction, the mixture of phospholipid, SP-B and SP-C is fractionated by HPLC on Sephadex LH-60 with an isocratic elution buffer containing dichloromethane and methanol.[68] Factors limiting the widespread application of this technique include prolonged run lengths (70 minutes total) and a broad elution peak for SP-C (eluting at approximately 35 minutes), making large scale sample analyses difficult. The molar extinction coefficient of SP-C (1.92 x 10^4/M/cm at 228 nm) also contributes to a relatively high limit of detection (4 μg SP-C).

A chemical method for the detection and quantification of SP-C in surfactant based on the release of free sulfhydryl groups during depalmitoylation has been described.[69] Surfactant is reacted with ^{14}C-iodoacetamide in the presence of triethylamine, a deacylating agent. By attack of the palmitic acid-SP-C thioester linkages and subsequent replacement of palmitate with iodoacetamide, the resultant ^{14}C-product can be visualized on fluorograms following sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) separation. The reaction is stoichiometric and quantitation can be achieved by direct scintillation counting and comparison to a standard curve generated with purified SP-C. While the assay is sensitive to nanogram levels (~100 ng), the application of this method appears limited to quantitation of SP-C in surfactant and as an adjunct in following the fate of SP-C during purification.

Early interpretation of electrophoretic profiles of SP-C yielded variability in assignment of accurate M_r due to the molecule's tendency to aggregate in aqueous solution (even in the presence of detergent), rendering gels laden with multimers as well as extra bands from phospholipid. Other problems include smearing and distortion of bands below 10 kDa with use of the Laemmli method for protein separation[70] and the variability of staining SP-C with both Coomassie blue and silver stains.[66] At present, the use of Tricine SDS/PAGE[71] and a modified double staining protocol for gels with both Coomassie and silver stains[66] appears to offer good electrophoretic resolution.

SP-C, as well as SP-B, has N-terminal heterogeneity. The variability of the amino terminal residue of SP-C due to truncated (N-1, N-2) forms, as well as similarity of the SP-C amino terminus (Fig. 5.1) with that of the SP-B NH_2-terminus (phe-pro-ile-pro for human SP-B[72]), often leads to multiple amino acid assignments at each cycle of sequencing (if contaminating SP-B is present). Interpretation of N-terminal amino acid sequence analysis must therefore be done cautiously.

Finally, the use of immunotechniques for the analysis of mature SP-C is hampered by the lack of monospecific antibody probes. The production of specific antisera (polyclonal or monoclonal) using purified mature SP-C has not been reported to date. However, antiserum 559, which recognizes the mature SP-B peptide, is a polyclonal antibody generated against organic solvent extracts of bovine surfactant containing both SP-B and SP-C; it detects mature SP-C only after electrophoresis in the presence of 2-mercaptoethanol and at very low dilutions (1:50) of the antibody.[73] Based upon published studies, the threshold of antiserum 559 appears to be ~1-2 μg SP-C. Using enhanced chemilumnescence, we have noticed a similar crossreactivity with a polyclonal SP-B antibody previously produced[74]

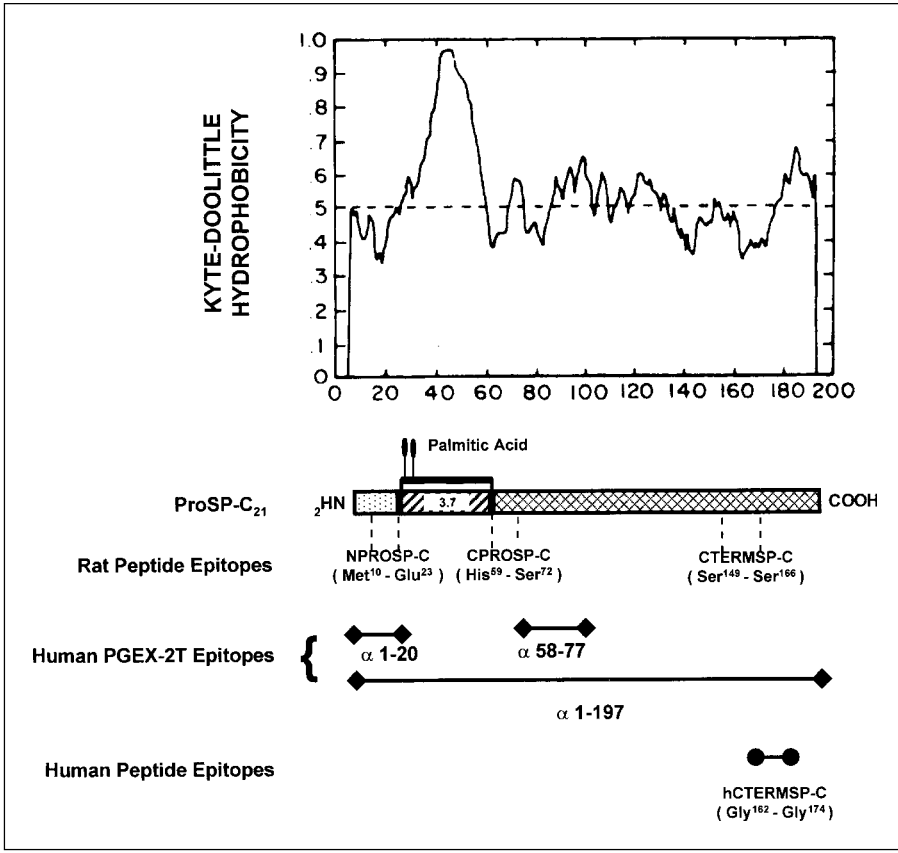

Fig. 5.3. Epitope diagram of monospecific proSP-C antibodies. A Kyte-Doolittle hydrophobicity plot of the entire SP-C propeptide (based on data from ref. 25) is superimposed over a linearized schematic diagram of the full length proSP-C sequence (proSP-C_{21}). Values are obtained from averages over 11 amino acid residues, and values >0.5 are considered hydrophobic. The mature SP-C peptide (residues 24-59) represents the most hydrophobic portion of the molecule. The locations recognized by epitope specific antisera against rat epitopes produced using synthetic peptides,[77-78] human epitopes using recombinantly produced portions of proSP-C[57,121] or human epitopes using synthetic peptides[135] are shown.

using a similar bovine proteolipid preparation containing both hydrophobic proteins (Survanta®; Abbott Pharmaceuticals, Chicago, IL) (Beers MF, unpublished observations).

Traditionally, the antigenic regions within a protein molecule are characterized by areas of high local hydropathicity (low hydrophobicity), greater mobility, a tendency to form reverse turns (hairpins) and surface exposure.[75-76] Figure 5.3 illustrates a hydrophobicity plot for the proSP-C molecule. The sequence of the mature SP-C peptide (residues 24-59) contains extremely hydrophobic residues (Kyte-Doolittle index >0.5). Thus the poor antigenicity of mature SP-C is probably related to the hydrophobic nature of the peptide, its avidity for lipid membranes and its rigid secondary structure (α-helix).

The low titer and high affinity for SP-B renders the currently available SP-B/SP-C antisera discussed above impractical for immunocytochemical localization, ELISA analysis of

SP-C content in lung tissue or immunoprecipitation analysis of SP-C metabolism. Alternative approaches have exploited the hydrophilicity of the SP-C propeptide regions (Fig. 5.3). Resulting antisera produced using either synthetic peptides or recombinant protein containing sequences of the proSP-C flanking domains have been used for immunocytochemical localization of proSP-C and for metabolic labeling studies of proSP-C synthesis and post-translational processing (detailed in Metabolic Life Cycle of SP-C, below).[56,57,77-80]

Genomic Structure and Tissue Expression of SP-C mRNA

The SP-C Gene

The human SP-C (*hSP-C*) gene is localized on the short arm of chromosome 8 (distinct from SP-A and SP-B).[14] The gene is organized into 6 exons encompassing ~2.7-3.0 kb (Fig. 5.4). The organization of the mouse SP-C (*muSP-C*) gene is similar but contains an insert of 553 bp in the first intron,[20] and the gene is localized to chromosome 14.[81] In both genes, there is a small (25 bp) 5' untranslated region on exon 1 with part of exon 5 and all of exon 6 encoding for the 3'-untranslated region. Restriction mapping of human genomic DNA indicates the presence of two highly homologous genes which may be alleles.[82] Sequence comparison reveals that the nucleotide sequences of the two genes differ at only 19 sites in exon 6, indicating that the translated regions (exons 1-5) are identical.

Two classes of *hSP-C* cDNAs encoding for SP-C proproteins with slightly different amino acid sequences have been characterized.[14,18,82] Both are ~0.9 kb in length. Since deletions are not detected in genomic DNA, the different cDNAs are the result of alternate splice sites. Differential splicing of the primary transcript from either *hSP-C* gene at the 5' end of exon 5 results in an 18 bp deletion, producing an mRNA encoding a protein reduced in size by 6 amino acids. Another minor SP-C mRNA species contains an 8 bp deletion at the 3' end of exon 5 which does not alter the size of the encoded protein. Alternative splicing of the rabbit SP-C gene (single gene) near the 3' end of exon 5 has been reported by two different groups to produce either a 27 bp[21] or 31 bp[22] mRNA polymorphism. As for *hSP-C*, these variants occur in the 3' untranslated region and the amino acid sequence of translated protein is unaffected. The *muSP-C* gene encodes a single mRNA of 0.8 kb.[20] Although the cDNA sequence of rat SP-C has been determined, its genomic DNA structure is undefined.

Tissue and Cell Specific Expression of SP-C mRNA

In the adult, Northern and/or reverse transcriptase-polymerase chain reaction (RT-PCR) analyses of human, rat, mouse and rabbit tissues established that SP-C mRNA expression is limited exclusively to the lung.[14,19-20,22] In the rat lung, message expression is markedly enriched in freshly isolated type II cells versus lung homogenate.[19] In situ hybridization studies in mouse, rabbit, rat and humans have demonstrated that expression of SP-C mRNA is limited to type II cells.[83-87] In contrast, SP-A and SP-B mRNA are present in Clara cells in addition to type II cells.[83-84,87]

SP-C Promoter

Of particular interest are the findings that the 5' upstream flanking regions of both the human and murine SP-C genes (0-3.7 kb for human; 0-4.7 kb for mouse) appear to contain the complete promoter elements for the control of lung specific expression observed in the in situ hybridization studies.[88-89] In addition to the coding regions, the 5' flanking region of both *hSP-C* and *muSP-C* have been isolated and sequenced.[14,20,83] The compactness of the coding region and promoter elements has been exploited in transgenic animal models to investigate cis-acting elements regulating gene expression in specific tissues. Transgenic mice bearing a chimeric gene containing 3.7 kb of the 5' sequence of *hSP-C* and the bacterial

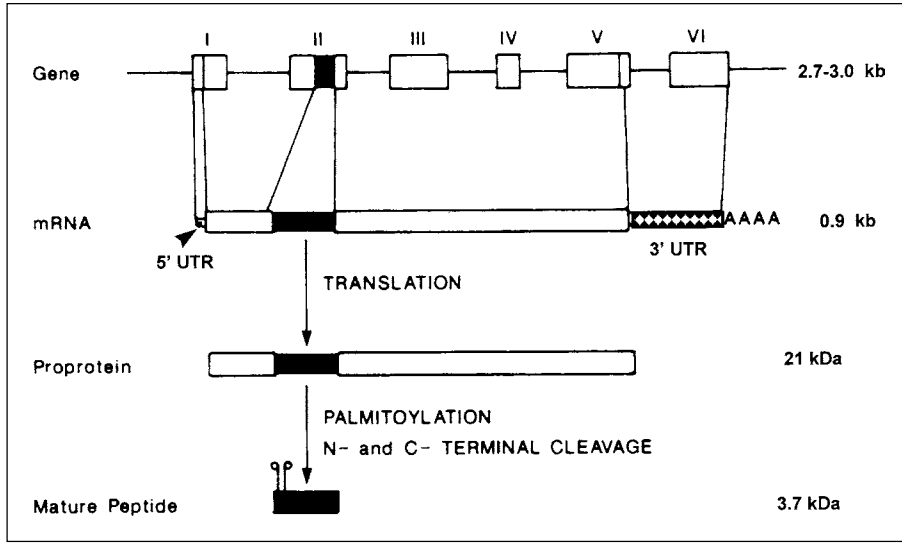

Fig. 5.4. Structure of the human SP-C gene and derivation of the mature SP-C peptide. Production of alveolar SP-$C_{3.7}$ begins with transcription and splicing of the 6 exons of the SP-C gene to yield a 0.9 kb mRNA. Not shown are alternative splicing motifs discussed in the text. Full length SP-C mRNA contains small 5' and larger 3' untranslated regions (UTR). The primary translation product of SP-C is a 21 kDa propeptide which then undergoes palmitoylation followed by C- and N-terminal cleavage of propeptide flanking domains to yield the mature 3.7 kDa peptide.

diphtheria toxin had distal respiratory cell ablation and respiratory failure in newborn pups, demonstrating the lung specific expression conferred by this segment of the SP-C gene, but the precise cells expressing the diphtheria toxin transgene were not discernible.[88] Subsequently, constructs containing the same 3.7 kb upstream fragment of *hSP-C* and the chloramphenicol acetyltransferase (CAT) reporter gene resulted in developmentally regulated, lung specific and glucocorticoid responsive CAT mRNA expression in both type II cells and bronchiolar cells, expression being greater in Clara cells than in type II cells.[83,89] Parallel experiments with a 4.8 kb 5' segment of the *muSP-C* promoter yielded similar expression patterns.[90] These results indicate that this 5' flanking domain is sufficient to drive lung specific expression of SP-C, but that other factors (either additional cis-elements elsewhere in the gene or trans-acting factors) may be required to produce the fidelity of type II cell expression seen for native SP-C.[90]

Further characterization of cis-acting elements in the *muSP-C* gene has been performed. The activation of the *muSP-C* promoter was analyzed in immortalized mouse lung epithelial cells (MLE-15 cell line, discussed below) and HeLa cells after cotransfection with a vector expressing thyroid transcription factor 1 (TTF-1). TTF-1 is a homeodomain-containing transcription factor the expression of which parallels that of SP-C and that activates transcription of SP-A and SP-B.[91-92] TTF-1 transactivated muSP-C-CAT constructs containing -13 to -320 bp of the SP-C 5' flanking region. Essential cis-acting elements are functionally localized to between -320 and -180 bp. TTF activation is abolished by site-directed mutagenesis of two consensus TTF sites located between -197 and -158 bp. In addition to TTF sites, the region -197 to -158 bp contains enhancer activity in both MLE and HeLa cells. Recently, lung specific enhancer activity has been found at approximately -320 bp of *hSP-C*

(Glasser SW, personal communication). This work supports a model whereby TTF-1 is necessary but not sufficient for the regulation of SP-C transcription. It is likely that unique combinations of TTF-1 with other factors specific to lung, as well as variations in TTF binding affinities to DNA sequences are required for cell-, organ- and developmental-specific expression of SP-C

The SP-C Promoter and Transgenic Mouse Models

The lung specificity of the 3.7 kb *hSP-C* promoter region has been exploited for the creation of new models of disease through selective targeting of chimeric transgenes expressing a variety of protein products.[88,89,93-104] Table 5.3 summarizes the results of the transgenic mouse models produced to date. Four major categories of hSP-C-transgenic models are presently in use:

1. Targeted overexpression of protein products;
2. Targeted expression of cytokines and messengers;
3. Targeted dominant negatives;
4. Targeted replacement of deficient proteins.

Targeted overexpression of protein products

Overexpression of manganese superoxide dismutase confers additional protection from oxidant injury in airway epithelial cells.[103] Lung specific expression of the large T-antigen chimeric gene leads to postnatal development of pulmonary adenocarcinoma in young mice (4-6 months of age).[93] By limiting dilution, multiple clonal lines of murine pulmonary epithelial cells have been produced—the MLE cells.[94-95] These cell lines are phenotypically diverse, expressing a mosaic of surfactant mRNAs and morphologic features of type II cells (microvilli, multivesicular bodies, lamellar bodies). Some lines, such as MLE-15, have been used to study regulation of surfactant protein gene transcription.

Targeted expression of cytokines and messengers

Other models expressing cytokine transgenes (transforming growth factor (TGF)-β1, TGF-α, tumor necrosis factor-α (TNF-α) and keratinocyte growth factor) or signaling factors (bone morphogenic protein-4) develop either arrested lung morphogenesis during development or postnatal pulmonary fibrosis.

Targeted dominant negatives

Using the *huSP-C* promoter, the targeted expression of a dominant negative fibroblast growth factor (FGF) receptor in transgenic mice results in complete inhibition of branching morphogenesis and epithelial cell differentiation, thus establishing a specific role for FGFs in normal mammalian lung development.

Targeted replacement of deficient proteins

Expression of a construct consisting of 3.7 *huSP-C* and the granulocyte-macrophage colony stimulating factor (GM-CSF) gene in GM-CSF null background mice leads to a reversal of the alveolar proteinosis previously described in the original knockout animal.[104] Human SP-$C_{3.7}$ has also been used to target human cystic fibrosis transmembrane conductance regulator (CFTR) to the distal respiratory epithelium of normal mice and has been proposed as a method of restoring gene function to CFTR deficient animals.

As can be ascertained from this list, the use of the human SP-C promoter in combination with transgenic technology represents an important experimental paradigm for the advancement of our understanding of pulmonary cell biology.

Table 5.3. Use of 3.7hSP-C promoter for lung specific expression of chimeric transgenes

Chimeric Transgene	Result	Ref.
hSP-C-Diphtheria Toxin	Obliteration of distal respiratory epithelium	88
hSP-C-CAT	Definition of promoter driven developmental pattern of CAT expression	89
hSP-C-Large T Antigen	a) Model of lung adenocarcinoma b) Production of immortalized lung epithelial cell lines	93 94, 95
hSP-C-MnSOD	Conference of protection from oxidant injury	103
hSP-C-TGF-β_1	Arrested lung morphogenesis and epithelial differentiation	96
hSP-C-KGF	Disrupted branching of morphogenesis	97
hSP-C-TGF-α	Pulmonary fibrosis	98
hSP-C-TNF-α	Fibrosing alveolitis	99
hSP-C-bmp-4	Inhibition in differentiated type II cells/disrupted morphogenesis	100
hSP-C-FGF#	Inhibition of all branching morphogenesis	101
hSP-C-hCFTR	Lung specific human CFTR expression	102
hSP-C-GM-CSF	Restoration of surfactant homeostasis+	104

KGF = keratinocyte growth factor; bmp-4 = bone morphogenic protein; MnSOD = manganese superoxide dismutase; CFTR = cystic fibrosis transmembrane conductance regulator. Other abbreviations as in the text.
# Targeted dominant negative of abnormal FGF receptor
+ Transgene expression in GM-CSF-/- background

Metabolic Life-Cycle of SP-C Protein

Figure 5.4 schematically depicts the gene structure for human SP-C and the derivation of the active peptide. The full length SP-C mRNA (0.9 kb), produced by splicing of multiple exons, yields a primary translation product 197 amino acids in length. In vitro translation of human lung RNA produces SP-C primary translation products of M_r 21,000-22,000.[14] Similar sized products have been detected in primary cultures of rat type II cells[57,79,105-106] and produced in CHO cells transfected with a human SP-C cDNA.[56] Like SP-B, mature SP-C is contained within the sequence of a larger precursor proprotein (proSP-C). The predominant form of SP-C isolated from extracellular surfactant is composed of 35 amino acids extending from residues 24 to 59 of the proprotein. Unlike SP-A and SP-B, the NH_2-terminal flanking region does not contain a classic 'signal sequence' and there are no sites for asparagine-linked glycosylation (Table 5.1).[3,12,113] Nonetheless, SP-C must undergo proteolytic processing for production of the 35 amino acid form secreted into the airways. Generation of mature SP-C from the larger proSP-C precursor involves removal of 23-25 residues of N-terminal peptide, cleavage of 133-139 residues of C-terminus and post-translational addition of covalent palmitic acid.[25] Mature SP-C, packaged with phospholipid in the lamellar body, is secreted into the air space and, in order to maintain normal homeostasis, must then be cleared.

Immunocytochemical Localization of SP-C Synthesis

While SP-A and SP-B peptides have been localized immunocytochemically to granular inclusions within the apical cytoplasm of type II cells,[87,107-110] actual immunocytochemical localization of the mature SP-C peptide has not been reported due to unavailability of monospecific SP-$C_{3.7}$ antisera. We and others have used immunocytochemistry with monospecific antisera directed against portions of proSP-C to characterize expression of the precursor molecule.[57,77-79,105] In the adult rat lung, SP-C propeptides can be localized intracellularly to alveolar epithelial cells which also show binding to *Maclura pomifera* antigen (i.e., type II cells).[77] Fluorescence immunochemical staining of isolated rat type II cells shows a subcellular distribution of proSP-C peptides in endoplasmic reticulum (ER), Golgi and highly acidic phospholipid enriched cytoplasmic organelles (i.e., lamellar bodies).[79,105] Ultrastructural localization of proSP-C expression in the rat using immunogold electron microscopy has demonstrated the presence of proSP-C peptides in the ER, Golgi and transport vesicles of the late secretory pathway (multivesicular bodies), but ultrathin cryomicrotomy failed to detect proSP-C in lamellar bodies.[57,80] However, in sections prepared in the latter manner, lamellar bodies also failed to show preservation of the classic lamellated morphology of vesicular surfactant lipid, raising the issue of phospholipid and lipid-associated proSP-C extraction during fixation.

Synthesis, Post-translational Processing and Intracellular Targeting of proSP-C

Details of the proteolytic processing pathway are partially elucidated. In addition to problems generating antisera against mature SP-C, the complete characterization of the post-translational processing pathway for proSP-C is hampered by the lack of a cultured cell line which exhibits stable expression and complete processing of proSP-C to SP-$C_{3.7}$. Alveolar type II cells isolated from rats and placed in primary culture on tissue culture plastic exhibit rapid loss of both SP-C mRNA[111] and proprotein expression.[79] To circumvent these technical difficulties, epitope-specific antisera against proSP-C have been used to study proSP-C synthetic processing in surrogate models (e.g., isolated perfused adult rat lung, fresh rat type II cells, murine fetal lung, cultured human fetal lung, transfected cell

lines and in vitro translation systems) which mimic some or most aspects of in vivo type II cell function and proSP-C synthetic processing.

The production and characterization of several epitope-specific polyclonal antibodies directed against rat proSP-C using synthetic peptides corresponding to spatially distinct regions of the proSP-C primary sequence have been reported by our group[77,78] and others.[56,57] A schematic diagram of the published proSP-C antisera is shown in Figure 5.3. Using these reagents, analyses using the experimental models mentioned above have begun to dissect the post-translational processing pathways. Progress to date has occurred in five major areas:

1. Palmitoylation;
2. Post-translational proteolysis;
3. Membrane association and orientation;
4. Cis-acting elements of proSP-C sequence regulating intracellular trafficking;
5. Trans-acting factors affecting proSP-C processing.

Palmitoylation

^{3}H-Palmitic acid labeling of CHO cells stably transfected with a full-length *hSP-C* resulted in palmitoylation of proSP-C_{21} and appearance of a 26 kDa *hSP-C* intermediate.[56] A 21.5 kDa form has been found in rat type II cells.[57] In both systems, addition of cerulenin, an inhibitor of fatty acid incorporation, blocked the appearance of higher molecular weight forms. In the case of human SP-C, the change in M_r (5 kDa) is larger than would be expected by the addition of two palmitic acid residues alone and the authors have raised the possibility of a second palmitoylation-dependent modification.

Post-translational proteolysis

In our laboratory, immunoprecipitation with epitope-specific antisera of homogenates from a perfused rat lung preparation and lysates from freshly isolated type II cells labeled with ^{35}S-methionine/cysteine identified the ^{35}S-labeled 21 kDa SP-C primary translation product.[78-79] Pulse-chase experiments demonstrated processing of proSP-C_{21} through 16 kDa and 6 kDa intermediate forms (proSP-C_{16}, proSP-C_6) by cleavage of C-terminal propeptide domains which was blocked by brefeldin A (BFA). In the rat type II cells, the presence of these intermediates in pulse-chase profiles preceded the appearance of a mature SP-$C_{3.7}$ band detected by Tricine SDS/PAGE of organic extracts of the cell lysates.[105] Inhibition of proSP-C proteolysis by BFA also inhibited production of mature SP-C. Vorbroker et al reported similar findings using proSP-C antisera and fresh rat type II cells.[57] Although the sizes of the proSP-C intermediates identified were different, they confirmed the early cleavage of C-terminal domains and mimicked the BFA effect on post-translational proteolysis through the use of low temperature (20°C) to completely block cleavage of proSP-C. The effects of BFA and temperature observed in these two reports indicate that all post-translational proteolysis occurs distal to the trans-Golgi network.

Membrane association and orientation

Using membrane association and protease digestion assays, Keller et al demonstrated by in vitro translation of an epitope tagged proSP-C mRNA in the presence of canine pancreatic microsomal vesicles that proSP-C_{21} is a type II bitopic membrane protein (= COOH in the ER lumen) with the sequence corresponding to mature SP-C within the propeptide acting as the membrane anchoring domain.[112] Vorbroker et al[56] showed that membrane fractions from CHO cells expressing transfected SP-C cDNA contain proSP-C_{21} which is resistant to $NaCO_3$ extraction, confirming integral membrane association of proSP-C. However, protease protection assays of native human proSP-C and pancreatic

microsomes indicated that proSP-C_{21} may be a type III protein (= NH_2 in ER lumen).[57] A preliminary report using $NaCO_3$ extraction of membrane fractions from rat type II cells, lamellar bodies and microsomes has also found integral membrane association of proSP-C_{21} and proSP-C_6.[113] The exact orientation of membrane associated proSP-C intermediates in native type II cell membranes remains to be clarified.

Cis-acting elements of proSP-C sequence regulating intracellular trafficking

Cis-acting elements within the proSP-C sequence have also been proposed to direct its intracellular trafficking. Although a classic signal sequence is lacking, either the mature SP-C sequence (phe_{24}-his_{59}), palmitoylated moieties or domains flanking the mature SP-C sequence in proSP-C_{21} could play a role in its intracellular targeting and processing. In CHO cells transfected with truncated human SP-C constructs, immunofluoresence localization has shown that intracellular targeting of proSP-C to cytoplasmic vesicular structures is dependent upon the last 22 amino acids (C-terminus) of proSP-C_{21} (= gly_{176}-ile_{197}).[114] A similar deletion of the C-terminus of rat proSP-C blocks the normal targeting of wild type proSP-C to cytoplasmic vesicles in A549 cells and results in inhibition of proteolysis (Beers MF, unpublished observations). Between species, there is 82% amino acid homology for this region. Other proSP-C domains which have a high degree of homology include met_1-arg_{21} (96%) and met_{60}-gly_{74} (93%); their role in targeting has not been fully characterized. Intracellular trafficking of site-directed mutants deleted for either the mature SP-C region or the palmitoylated cysteine residues (cys_{28}; cys_{29}) has not been characterized.

Trans-acting factors affecting proSP-C processing

In addition to cis-acting elements contained within the proSP-C sequence, it is likely that other cell related (trans-acting) factors are also required for proper SP-C synthesis and processing. The complete proteolytic processing pattern of proSP-C appears to be lung epithelial cell specific. Expression of recombinant human proSP-C_{21} protein in CHO cells results in generation of SP-C_{21} and a single intermediate.[56] Similarly, proSP-C expressed in mammary tissue via a cell-specific promoter is secreted as a partially processed proSP-C intermediate in the transgenic milk.[115] In preliminary studies, transfection of rat SP-C into A549 cells resulted in a processing profile identical to that in rat type II cells; two nonlung epithelial cell lines transfected with the same construct failed to process translated proSP-C_{21}.[116]

In addition to effects of BFA and temperature, post-translational proteolysis of proSP-C is also inhibited by disruption of the progressive acidification normally present in organellar compartments along the exocytic pathway.[105] The membrane permeable weak base, methylamine, as well as the lysosomotropic agent chloroquine, the proton ionophore monensin, and bafilomycin A_1, a specific vacuolar H^+-ATPase inhibitor, each disrupted proSP-C proteolysis in a similar manner leading to inhibition of SP-$C_{3.7}$ formation. These results are consistent with similar effects of these reagents on processing of prohormones by neuroendocrine cells.[117-118] At present, none of the proteases which process SP-C are currently defined, nor is the effect of pH on actual targeting known.

The results detailed above can be integrated into a working model for SP-C synthetic processing. As Figure 5.5 demonstrates, the post-translational processing of proSP-C is complex and requires several intracellular compartments. Following synthesis of proSP-C_{21} in a microsomal (ER) compartment, it is most likely that palmitoylation takes place post-translationally (independent of de novo protein synthesis) in the Golgi. This event is followed by proteolysis of palmitoylated proSP-C (proSP-$C_{21, 21.5, 24 \text{ or } 26}$) which entails initial cleavage of C-terminal flanking domains in acidic intracellular organellar compartments. Final cleavage of the N-terminal flanking domains occurs later in the secretory pathway.

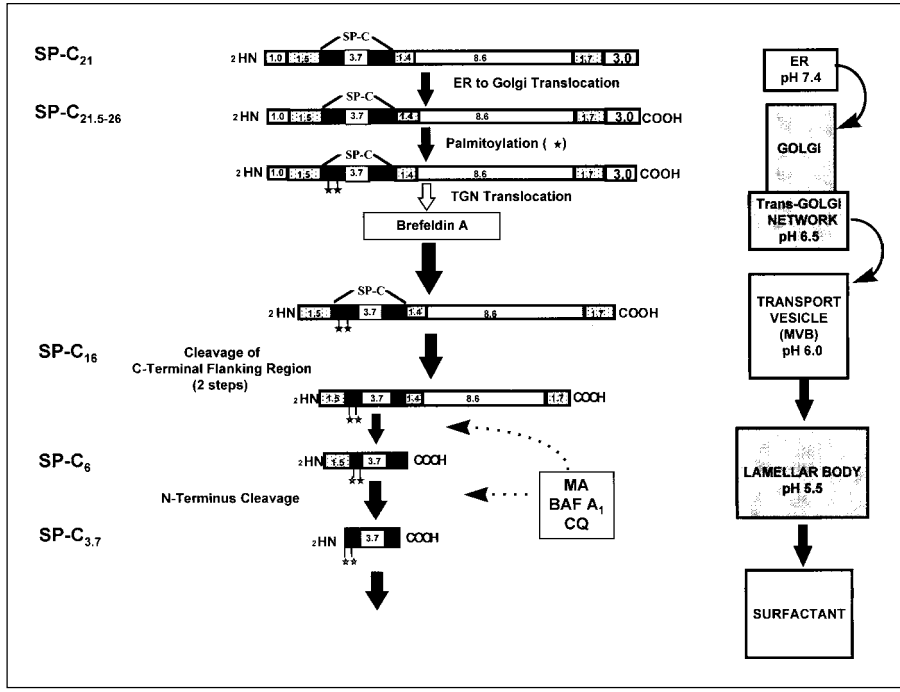

Fig. 5.5. Schematic diagram of SP-C synthetic processing pathway in alveolar type II cells. Diagrammatic representation of proSP-C processing pathway in the adult rat depicting the major proteolytic steps and the intracellular compartments involved in post-translational processing (based on data from refs. 56, 57, 78,79 and 105). (Left) The M_r of the major intermediates are given. (Right) The major subcellular compartments involved in processing and the intraorganellar pH along the exocytic pathway (based upon data in refs. 105 and 117), showing progressive acidification of the secretory pathway, is schematically illustrated. Inhibition of proSP-C processing steps can be accomplished using: (1) The acidotropic agents methylamine (MA) and chloroquine (CQ), both of which neutralize the organellar pH of all acidic compartments; (2) Bafilomycin (BAF A_1), which blocks vesicular ATPase-mediated proton translocation; (3) Brefeldin A, which completely blocks proSP-C processing by inhibition of Golgi-budded vesicle formation.

The data published to date also indicate that all proteolytic processing of proSP-C_{21} to mature SP-$C_{3.7}$ occurs distal to the trans-Golgi network.

Based upon currently available data, the most likely sites for proteolytic processing of proSP-C along the secretory pathway include the multivesicular body and the lamellar body. Immunogold electron microscopy studies identified proSP-C within multivesicular bodies of human lung tissue,[80] and a highly purified lamellar body fraction from rat lung is enriched in proSP-$C_{6\text{-}10}$.[78-79] In addition, ultrastructural analysis of lungs from infants with congenital SP-B deficiency has shown disruption of normal vesicle formation, the presence of morphologically abnormal lamellar bodies, aberrant SP-C processing resulting in the accumulation of proSP-C intermediates and a lack of detectable mature SP-$C_{3.7}$.[119-121] Taken together, evidence to date indicates that delivery of proSP-C to compartments of the late secretory pathway, which include the multivesicular body and lamellar body, is important

for proper processing of proSP-C to mature SP-C. Additional morphological and biochemical characterization are required to more accurately define the processing compartments.

Secretion and Clearance of SP-C

From biochemical analysis of lamellar bodies and surfactant, it is likely that SP-$C_{3.7}$ is stored in the lamellar bodies of type II cells along with synthesized surfactant phospholipid prior to release to the alveolar space.[5,63] The effect of the known phospholipid secretagogues (see chapter 7) on the kinetics of SP-C secretion is undefined but it is likely that if SP-C is cosecreted with the phospholipid, then these same signal transduction mechanisms would play a part.

Once secreted into the alveolar space, the fate of SP-C in the adult lung is relatively unknown. A significant body of data exists to support the notion of reuptake and reutilization of phospholipids, SP-A and SP-B by type II pneumocytes.[122-126] Morphological and ^{125}I-binding studies in isolated type II cells confirm saturable binding, internalization, and appearance in endosomes of both SP-A and SP-B.

Based on limited studies to date, reuptake of SP-C by type II cells also occurs.[127-129] Biosynthesized, radiolabeled SP-C instilled into lungs of neonatal rabbits accumulates in lamellar body-enriched fractions in a time dependent manner.[127] Cell association of ^{125}I-SP-C with isolated type II cells occurs in a concentration-, time- and temperature-dependent manner but is not saturable up to 150 μg/ml.[129] In animal models, the time course for clearance of SP-C and of simultaneously instilled lipid (labeled DPPC) is similar and the internalized SP-C is not degraded.[128,129]

Developmental Expression and Metabolism of SP-C

Temporal and Spatial Pattern of Expression

Expression of SP-C mRNA in developing human lung occurs early in the second trimester and at an equivalent gestational age in other species.[83,86,130-132] Using Northern blot analysis, SP-C mRNA can be detected in human fetal lung tissue as early as 13 weeks gestation but remain at 5-10% of adult levels until very late in the second trimester, reaching 15% of adult levels by 24-25 weeks.[131] Compared to SP-B, the levels of expression of SP-C mRNA are relatively lower (as a percentage of term lung) and the increase at the end of the second trimester lags slightly behind that of SP-B mRNA. The presence of detectable message for both hydrophobic proteins precedes the differentiation of type II cells, synthesis of surfactant phospholipids, production of SP-A and appearance of most lamellar bodies, i.e., before complete phenotypic differentiation of the type II cell.[131,133-134]

Using in situ hybridization, the expression of SP-C mRNA is seen in air-space epithelial cells prior to formation of alveoli in both the mouse and rabbit. Localization of SP-C mRNA by in situ hybridization and SP-C proprotein by immunocytochemistry with polyclonal antiserum generated against proSP-C showed expression of both mRNA and proprotein exclusively in airway epithelial cells of early second trimester human fetal lung at ~14-16 weeks.[86,88,132,135] After 24 weeks, SP-C mRNA and precursor protein are detected in type II cells and then increase with advancing gestational age.[132]

Despite the presence of detectable message and proprotein, the fetal lung appears incapable of fully processing synthesized proSP-C. Immunoprecipitation of ^{35}S-labeled murine fetal lung explants demonstrated synthesis of proSP-C_{21} and an ~24 kDa intermediate, but failed to detect the presence of lower molecular weight intermediates (indicative of proteolysis).[56] Similarly, by Western blotting and immunoprecipitation of second trimester human fetal lung tissue with an epitope-specific proSP-C antiserum directed against the

NH_2 terminus (anti-NPROSP-C), proSP-C_{21} is detected as early as week 12. However, as for the murine model, lower molecular weight forms are not detected up to week 24.[135]

Hormonal and Explant Culture Modulation of SP-C Expression

Maturation of the pulmonary surfactant system is well known to be under hormonal regulation. Clinically, antenatal glucocorticoids are administered as early as 24 weeks gestation to mothers at risk for premature delivery.[136] In several animal models, administration of antenatal steroids markedly affects both lung maturation and expression of SP-C.[137-139] In vivo experiments in fetal rabbits demonstrated increased expression of SP-C mRNA in lung tissue after maternal administration of glucocorticoids; however some of this effect could be attributed to a stress response.[137] Increases in SP-C mRNA have also been observed after in vivo antenatal administration of dexamethasone to rats.[138-139]

The culture of explanted human fetal lung tissue stimulates morphological maturation and surfactant production.[133-134,140] Second trimester human lung tissue placed in explant culture in defined medium exhibits morphogenic changes including increased airspace size, precocious cytodifferentiation of airway epithelial cells, lamellar body formation and accompanying increases in steady state levels of SP-A and SP-B mRNA.[131,133,141] In contrast, SP-C mRNA levels remain low (<10% of adult levels).[131] However, addition of glucocorticoids (10-100 nM dexamethasone) results in a ~20- to 30-fold increase in SP-C mRNA content,[131] induction of a further increase (2- to 3-fold) in SP-B mRNA,[131,141] additional increases in lamellar body number[133-134,140] and induction of lipogenic enzymes (see chapter 2). In fetal rat lung, explant culture in the presence of dexamethasone initiates similar changes in SP-C message expression.[142]

The mechanism of corticosteroid induction of human SP-C mRNA levels has been investigated using in vitro techniques.[143-145] In cultured human fetal lung, SP-C mRNA levels rise in response to 100 nM dexamethasone, reach maximal levels in 24 hours and are accompanied by a parallel increase (~10- to 12-fold) in the rate of SP-C gene transcription. Compared to that of SP-B, the increase in SP-C mRNA occurs at a slower rate and requires higher hormone concentrations. For SP-C (but not SP-B), the corticosteroid effect also requires ongoing protein synthesis (i.e., it is cyclohexamide inhibitable), which likely reflects involvement of a labile transcription factor, but does not involve a change in mRNA stability.[143-144] In fetal rabbit lung explants, on the other hand, increased SP-C mRNA stability is induced by dexamethasone; this may represent species variability.[145] A palindromic glucocorticoid response element (GRE) exists at -1.9 kb of the human SP-C gene,[83] although confirmation that this is a functional GRE has not been forthcoming.

Preliminary studies suggest that the changes in steady state SP-C message induced by explant culture and corticosteroids are accompanied by changes in SP-C protein expression and processing. By immunocytochemistry, proSP-C immunoreactivity in airway epithelial cells is markedly enhanced by culture of human fetal lung explants in dexamethasone.[135] Immunoprecipitation revealed a 15-fold increase in ^{35}S-proSP-C_{21} in dexamethasone-treated versus control lungs which parallels the reported changes in mRNA content.[131] In contrast to either preculture or culture controls, synthesis of proSP-C_{21} in the steroid treated fetal lung explant is followed by early appearance of a 24-26 kDa form and subsequent appearance of smaller processing intermediates including 6-10 kDa forms. Therefore, dexamethasone treatment of the fetal lung results in coordinated increases in proSP-C protein expression and mRNA content which are mediated by enhanced transcription. Through secondary effects on lung maturation, corticosteroids also appear to increase the ability of the immature lung to process translated protein.

In addition to corticosteroids, several other factors have been shown to regulate expression of SP-C in the fetal lung (Table 5.4).[146,147] However, the effects of thyroid hormone,

Table 5.4. Factors regulating SP-C mRNA expression during development

Agent	SP-C mRNA	Experimental Model	Ref.
Glucocorticoid	↑	HFL	131
Glucocorticoid	↑	RFL; RIV	138-139, 142
Glucocorticoid	↑	Rabbit in vivo	137
cyclic AMP	↔, ↑	HFL	139
Forskolin	↔	HFL	131
Thyroid hormone	↔	HFL	139
Interferon-γ	↔	HFL	147
Hyperglycemia	↓	Streptozotocin treated rat	148-149
Insulin	↔	HFL	150
Androgens	↔	HFL	131
Estrogens	↔	HFL	131
Retinoic acid	↓	RFL	151

Abbreviations used:HFL = Cultured human fetal lung; RFL = Cultured rat fetal lung; RIV = Rat in vivo; MFL = Cultured mouse fetal lung

insulin, cyclic AMP, androgens and hyperglycemia are less clearly defined, appear to be species-dependent and are influenced by experimental conditions (explants vs. in vivo models).[131,137-139,142,148-151] With the exception of corticosteroids and thyroid hormone, the physiological role of other multihormone regimens remains to be defined.

Regulation of SP-C Expression and Metabolism

In the adult lung, expression of SP-C mRNA can be modulated by a variety of factors including in vitro culture, hormones, cytokines and environmental factors (Table 5.5).[111,152-158]

Negative Modulators

There is a time dependent decrease in SP-C mRNA steady-state levels in type II cells cultured on plastic for 24-48 hours or on Engelbreth-Holm-Swarm matrix that is associated with a commensurate loss of proSP-C protein expression.[79] It remains to be established if the downregulation in tissue culture is due to decreased rates of transcription or increased mRNA degradation.

The proinflammatory cytokine TNF-α decreases SP-C mRNA levels in mice within 24 hours of intratracheal administration. Recombinant murine TNF-α also inhibits endogenous SP-C mRNA in vitro in MLE-12 cells in a time- and dose-dependent manner.[152] TGF-β_1, a peptide growth factor often elaborated by many cells during the course of lung injury, decreases the abundance of SP-C mRNA in isolated type II cells; this occurs without altering cellular morphology and is accompanied by an increase in fibronectin mRNA.[153]

Positive Modulators

As in the fetus, corticosteroids increase SP-C mRNA levels in adult lungs. Rats given subcutaneous corticosteroids show upregulation of SP-C mRNA, as well as that of SP-A and SP-B.[154] Adrenalectomy, however, does not alter SP-C mRNA levels, raising doubts as to the physiological role of glucocorticoids in the regulation of SP-C expression in adult lung.

Table 5.5. Factors regulating SP-C mRNA expression in adult (post-natal) lung

Agent	SP-C mRNA	Experimental Model	Ref.
TNF-α	↓	Murine IT in vivo	152
	↓	MLE-12 cells in vitro	152
TGF-β_1	↓	Rat type II cells in vitro	153
Culture	↓	Rat type II cells in vitro	111
Glucocorticoid	↑	Rat in vivo	154
Hyperoxia	↑	Rat in vivo	155
Hyperoxia	↑	Hamster in vivo	156
Hyperoxia	↔	Rabbits in vivo	157
LPS	↑	Rat in vivo	158

Abbreviation: IT = intratracheal administration.; LPS = lipopolysaccharide

Changes in steady state SP-C mRNA levels are also modulated by oxygen. Exposure of rats to 85% oxygen for 5 days causes a 2-fold increase in SP-C mRNA as assayed by Northern blotting.[155] Semiquantitative measurement with a polyspecific antiserum showed a 5-fold increase in the amount of SP-C in lung lavage recovered from oxygen-exposed animals. The precise response to hyperoxia may be dependent on the species and the extent of exposure. Hamsters in a hyperoxic environment (100% oxygen for 8 days) have increases in SP-C mRNA content,[156] but exposure of rabbits to 100% oxygen for 64 hours has no detectable effect on SP-C mRNA levels.[84]

Intratracheal instillation of adult rats with endotoxin (lipopolysaccharide) increases SP-C mRNA levels in alveolar type II cells as determined by Northern blotting and in situ hybridization.[158] As for hyperoxia, it is not certain whether the endotoxin-induced increase in SP-C mRNA is a direct physiological response mediated by induction of gene transcription or a marker of type II cell proliferation which occurs in response to lung injury.

SP-C in Congenital and Acquired Lung Disease

The potential role of SP-C in the pathophysiology of both congenital and acquired lung disease is becoming increasingly recognized. To date, neither a transgenic knockout mouse model of the SP-C gene nor the detection of an SP-C gene abnormality in humans has been reported. Nonetheless, downregulation of SP-C gene expression, disruptions of its normal synthetic processing and altered metabolism have been demonstrated in both clinical and animal models, emphasizing the importance of the protein in normal lung physiology.

SP-B Deficiency

Recently, evidence for the importance of proper intracellular targeting and proteolysis in the biosynthesis and post-translational processing of SP-C is underscored by the phenotypic characterization of congenital SP-B deficiency resulting from mutational disruption of the SP-B gene.[159-161] In both the 121ins2[119] and R236C[159] mutations, the SP-B deficient phenotype is associated with abnormal post-translational processing of SP-C manifested as an abnormal accumulation of a 6-12 kDa proSP-C intermediate with concomitant lack of detectable SP-$C_{3.7}$.[119,120,159] Electron microscopy of SP-B deficient lung tissue has shown absence of well formed lamellar bodies in type II cells and loss of directional secretion.[120]

Similar phenotypic features have been noted in the recently described SP-B knockout mouse.[160] Thus, the severe hypoxia and altered lung mechanics associated with inactivation of the SP-B gene results in a double phenotypic knockout of both the mature SP-B and SP-C proteins.

Pulmonary Alveolar Proteinosis (PAP)

Pulmonary alveolar proteinosis is a clinical syndrome of a diffuse infiltrative lung disease characterized by the excessive accumulation of surfactant phospholipids and proteins within the alveolar space.[161] Although an animal model of the disease has been created with the GM-CSF knockout mouse,[162] the etiology in humans remains unknown. The primary structures of SP-A, SP-B and SP-C have all been reported to be abnormal.[163] While SP-C from normal lung lavage possesses thioester linked palmitate and N-terminal fraying (34-35 amino acids), SP-$C_{3.7}$ from alveolar proteinosis patients is modified by partial removal of palmitate residues and additional N-terminal degradation resulting in some forms with less than 34 amino acids. These results have not been correlated with changes in surfactant biophysics.

Chronic Lung Disease of the Newborn and Adult Respiratory Distress Syndrome

Although chronic lung disease of the newborn and adult respiratory distress syndrome are both associated with abnormalities in surfactant biophysics, SP-C expression and metabolism in these conditions is largely unknown. Based on in vitro and animal data, the presence of TNF-α, TGF-β_1 and other cytokines in bronchoalveolar lavage fluid from patients with these conditions would be expected to alter SP-C mRNA and protein expression. TGF-β1 downregulates SP-C mRNA and proprotein synthesis in fetal lung explant cultures while simultaneously enhancing lung matrix deposition.[164]

Conclusions and Future Directions

Despite the difficulties intrinsic to the unique structure of the smallest surfactant protein, we have learned much since its discovery nearly 20 years ago. Our traditional thinking about surfactant-associated proteins has centered principally around their effects on the biophysical properties of surfactant phospholipid. It is now apparent that the surfactant proteins are a biochemically, biophysically and functionally diverse group of mammalian peptides.

From studies presented in this review, it is apparent that SP-C represents a physiologically important peptide with novel structural properties; extreme hydrophobicity, membrane spanning potential (α-helix) and a unique post-translational modification, i.e., palmitoylation. A full understanding of SP-C remains incomplete, but progress to date includes characterization of its biophysical properties, partial mapping of the SP-C gene including detailed information on the promoter and regulation of transcription, and understanding of proSP-C processing in normal and SP-B deficient conditions.

Some of the future areas which are ripe for investigation include:

1. Development of better methodologies for accurate quantitation of the hydrophobic SP-$C_{3.7}$ in both surfactant and intracellular compartments;
2. Further characterization of SP-C synthetic processing including a detailed understanding of the role of palmitoylation, the determinants of intracellular targeting, the proteases responsible for propeptide cleavage and the potential regulators of proprotein processing;
3. Further progress in understanding the three dimensional structure of SP-C as well as its interactions with phospholipids and the other surfactant proteins;

4. Quantitation of SP-C levels and its biophysical function and metabolism in inflammatory, infectious and congenital lung disease;
5. Additional characterization of the SP-C promoter and development of its use as a tissue specific promoter to augment gene therapy of the lung.

Improved understanding in these and other areas will undoubtedly lead to new applications and permit novel approaches to the treatment of some pulmonary diseases.

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CHAPTER 6

Biosynthesis and Secretion of Surfactant Protein D

Erika C. Crouch

Introduction

Surfactant protein D (SP-D) is a pulmonary glycoprotein that is believed to play important roles in the response of the lung to microorganisms.[1-6] It is structurally and functionally related to surfactant protein A (SP-A), and is a member of the same family of collagenous, carbohydrate-binding host defense proteins, designated collectins. This chapter reviews the structure, biosynthesis and secretion of SP-D. The biologic activities of the protein are only briefly discussed in order to emphasize the importance of certain structural features for SP-D function. For more details relating to the probable host defense activities of SP-D see chapter 9.

Overview of Function

SP-D binds to saccharide ligands expressed on a wide variety of bacterial, fungal and viral organisms with resulting agglutination and enhanced association with alveolar macrophages and neutrophils.[1,7-11] SP-D may also modulate inflammatory and immunologically-mediated events within the airspace through lectin dependent or receptor mediated interactions with macrophages and other host defense cells.[12-14] Underlying these interactions is the capacity of the lectin domain to bind to specific glycoconjugates associated with the microbial cell wall,[7,15] or adsorbed to particles.[16] Although SP-D is a member of the mannose type subgroup of C-type lectins it shows preferential interaction with α-D-glucose-containing sugars or structurally related molecules.[17]

SP-D shows specific binding to specific components of the airspace lining material, primarily phosphatidylinositol and glucosylceramide.[18-20] The binding to both components involves the carboxy-terminal domain and can be inhibited with specific saccharides or EDTA.[21,22] Although there is currently no evidence that SP-D influences the biophysical properties of the surface active layer it is possible that it contributes to the metabolism of airspace surfactant under some conditions in vivo.[23]

Molecular Structure of SP-D

As for other collectins, SP-D is characterized by four discrete structural domains: an amino-terminal crosslinking domain, a triple helical collagen domain, a linking peptide or neck domain and the carboxy-terminal, carbohydrate recognition domain (CRD) (Fig. 6.1).[2,24] The collagen domain contains the single consensus for N-linked glycosylation and contains both hydroxylysine and hydroxylysyl-glycosides. The CRD contains four

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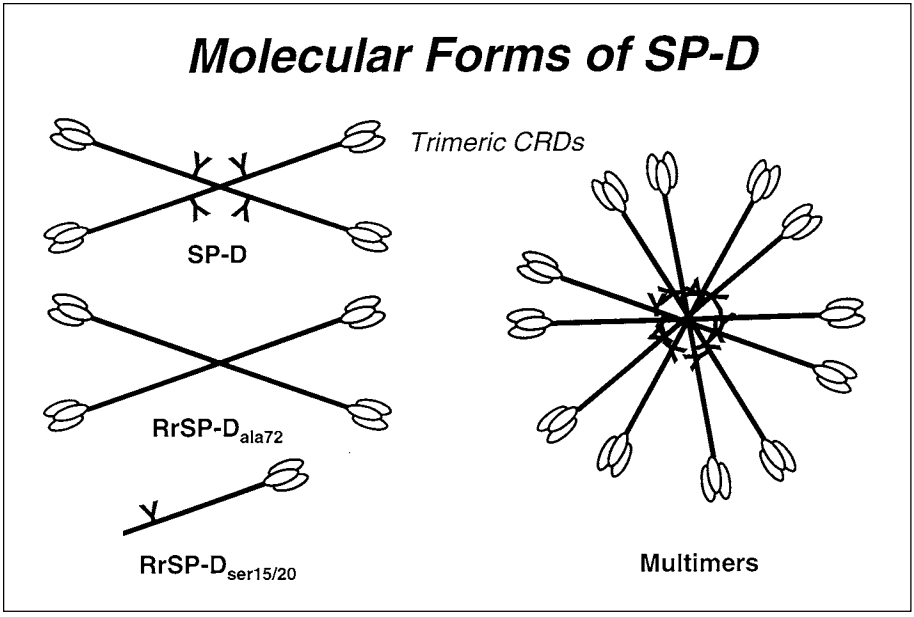

Fig. 6.1. Macromolecular forms of natural and recombinant SP-Ds. The diagram compares natural SP-D (upper left) with multimers of dodecamers (right). Also shown are two recombinant rat (Rr) mutants: The N-linked glycosylation mutant RrSP-Dala$_{72}$ and the single arm mutant RrSP-Dser$_{15/20}$. The former lacks a consensus for asparagine linked glycosylation (asn-gly-ser→asn-gly-ala) while the latter mutant lacks the two conserved cysteine residues within the amino-terminal domain (cys$_{15/20}$→ser$_{15/20}$). The maximum distance between trimeric CRDs is approximately 110 nM. RrSP-Dser$_{15/20}$ bears structural similarity to single arm forms of natural SP-D. SP-D multimers, which can contain as many as eight or more dodecamers, constitute the majority of SP-D extracted from the insoluble fraction of human alveolar proteinosis lavage.

conserved cysteine residues that contribute to the formation of intrachain bonds that stabilize the calcium and ligand binding sites. Each 43 kDa chain participates in the formation of a trimeric subunit.[24] These subunits can further associate at their amino-termini and these amino-terminal interactions are stabilized by interchain disulfide crosslinks. The predominant molecular form of SP-D isolated from normal lung is a dodecamer (4 x 3 = 12 chains). However, individual trimeric subunits (single arms) and higher order multimers with up to 96 or more chains have also been isolated.[25-28]

Structure-Function Correlations

Monomeric CRDs show specific but relatively low affinity binding interactions with ligands in solid phase assays.[2,22] A trimeric CRD, as exists within the trimeric subunits of the natural molecule (3 x 43 kDa), is required for high affinity binding interactions with various saccharide ligands. Trimers can mediate certain effects on leukocytes such as chemotaxis and effects on lymphocyte proliferation. However, multimers of trimers are required for microbial agglutination and the enhancement of microbial binding to host leukocytes.[27,29] Given the above, it should not be too surprising that trimeric subunits can competitively inhibit some agglutination and agglutination-dependent activities. Thus, the regulation of

assembly of SP-D trimers and of higher order multimers is consider to be critical for SP-D function.

Cellular Sites of SP-D Production

Contributions of Lung Epithelial Cells

Alveolar type II and nonciliated bronchiolar epithelial cells are the major sites of SP-D in the lung as assessed by in situ hybridization and immunochemical assays.[30-32] In situ hybridization and immunochemical studies of isolated rat type II and Clara cells suggest that these cell types have a similar capacity for SP-D production (Longmore WJ and Moxley MA, unpublished data). However, given that the lung yields many more type II than Clara cells, it seems likely that type II cells account for the majority of SP-D in the distal airways and alveoli.

There is evidence for heterogeneity in the production or accumulation of SP-D by these cell populations in vivo. For example, double labeling studies have shown that there are differences in the relative production of SP-D and SP-A among populations of rat bronchiolar cells.[33] Many cells show cytoplasmic labeling for both proteins but some cells show labeling for only SP-A or SP-D.[32]

Immunoelectronmicroscopic studies have shown that the majority of cytoplasmic SP-D in rat type II cells is contained within the rough endoplasmic reticulum (RER) and Golgi.[32,34] There is no detectable association with lamellar bodies or extracellular tubular myelin, suggesting the majority of the protein is secreted by a different pathway than SP-A. Nonciliated bronchiolar cells show intense labeling over the periphery of secretory granules with comparatively little staining of other cytoplasmic organelles.[30] Together, these observations are consistent with a constitutive secretion pathway in type II cells and a regulated pathway involving granule exocytosis in Clara cells. Although there appears to be granule to granule variation in SP-D content most apical granules contain some immunoreactive SP-D.[33] Double labeling studies have also shown SP-D and SP-A within the same granule,[32] albeit in different granular sub-compartments, suggesting that there may be coregulation of secretion of these proteins by some nonciliated cells in vivo.

Airspace Accumulation of SP-D

The majority of the secreted SP-D accumulates in the lung as a soluble protein. However, it can also associate with insoluble components of the airspace lining material. Ultrastructural studies have shown that the insoluble immunoreactive material in normal and silicotic rat lavage is associated with granular to amorphous electron-dense material similar to the contents of Clara cell granules.[34] The fraction of soluble protein in bronchoalveolar lavage is species-dependent (e.g., 50% in human to 70-90% in rat),[34-37] but is highly dependent on the conditions of lavage and centrifugation. The insoluble protein can be quantitatively solubilized with specific sugars (e.g., maltose) or EDTA.[17,38]

Nonpulmonary Sites of SP-D Production and Accumulation

Although the lung is the major site of SP-D production and accumulation,[39-41] there is growing evidence that it is not a lung specific protein. SP-D is produced by mucus cells in the stomach and possibly urinary bladder,[42] and SP-D or immunologically crossreactive proteins have been identified in tracheobronchial, lacrimal and salivary glands and at other sites in the bowel of mice and rodents.[41,43] Proteins crossreactive with antibodies to SP-D have been identified in serum from normal human adults (~70 ng/ml), and the protein accumulates in amniotic fluid during late gestation.[31,37,44] Although the lung is almost certainly the site of production of amniotic fluid SP-D,[45] a possible contribution from amnionic

epithelial cells has not been excluded.[44] Significantly, SP-D mRNA has not been detected in liver, the major source of the known serum collectins. Human alveolar macrophages show variable but often strong cytoplasmic staining for SP-D.[1] However, these cells lack SP-D message and the immunoreactive material is entirely contained within phagolysosomal compartments.

Genomic Organization

SP-D Gene Structure

Human SP-D is encoded by a single gene with at least 8 exons spanning >11 kilobases (kb) (Fig. 6.2).[46,47] There are seven exons encoding translated sequence. The first encodes three bases of the 5' untranslated region, the signal peptide, the NH_2-terminal domain and the first seven Gly-X-Y triplets of the collagen domain of SP-D (SNC1). Although this collagenous sequence is homologous to sequences in the first translated exons of SP-A, the remainder of the collagen domain appears distinct from SP-A and highly homologous to the collagen domains of the related bovine serum lectins, conglutinin and CL-43.[26,48] It is encoded by four homologous 117 base pair (bp) exons (C2-C5). The linking peptide and CRD are each encoded by a single exon as described for SP-A and other members of the collectin family.[49] Protein, cDNA and genomic sequencing together suggest the existence of a number of alleles, some of which are characterized by amino acid substitutions in the coding region.

A Collectin Gene Cluster on 10q

The human SP-D gene has been localized to chromosome 10q22.2-23.1 in proximity to the SP-A genes.[46,50-54] Kölble and coworkers have reported the isolation of cosmid clones containing both SP-D and SP-A gene sequences.[50]

Modulation of SP-D Production

Lung Organ Cultures

SP-D production is stimulated by glucocorticoids in rat and human fetal lung explants as well as in fetal rat lung in vivo.[55-57] These effects are in part mediated at the level of gene transcription. Interestingly, expression of SP-D in rat gastric mucosa is increased by administration of glucocorticoids or indomethacin and increased additively in the presence of both.[42] SP-D mRNA is also increased in human fetal lung explants by 8-bromo cyclic AMP.[57] Despite their obvious similarities, SP-D and SP-A show differences in developmental expression and different patterns of regulation by glucocorticoids, cytokines and other factors in human lung explants.[58] Specifically, lipopolysaccharide (LPS) and γ-interferon have no effect on SP-D production in this system.[57]

Primary Epithelial Cell Cultures

The capacity of isolated rat type II cells to produce SP-A and SP-D rapidly declines during the first several hours of culture on plastic concomitant with a marked decrease in the levels of total and translatable mRNA.[41] Although production of SP-A can be maintained when cells are cultured on a suitable matrix and in the presence of various growth factors including keratinocyte growth factor,[59,60] similar studies with SP-D have not yet been published.

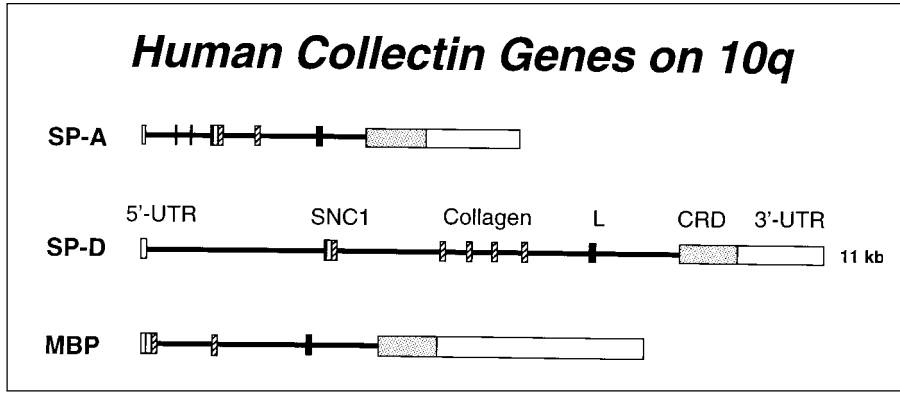

Fig. 6.2. SP-D gene structure compared to SP-A and MBP. The gene for human SP-D is compared to one of the human SP-A genes and the gene for serum MBP. The spatial organization of exons is remarkably similar. In each case, a short segment of the untranslated region (UTR), signal peptide, amino-terminal crosslinking domain and a homologous collagenous sequence are encoded by a single exon (SNC1). The remainder of the collagen helix is encoded by one to four additional exons. Separate exons encode the linking peptide (L) and the entire CRD. A comparison of the bovine conglutinin gene and a bovine SP-D cDNA suggest that there may be an additional untranslated exon within intron 1 of the human gene that is preferentially deleted from mature lung transcripts by alternative splicing.

Lung Tumor Cell Lines

SP-D is synthesized by a few human lung adenocarcinoma cell lines with ultrastructural evidence of type II and/or Clara cell differentiation. The well characterized H441 cell line does not secrete detectable amounts of protein. Unfortunately, the few cell lines which do secrete SP-D are difficult to propagate and systematic studies of SP-D synthesis have not yet been undertaken.

Transcriptional Regulation of SP-D Gene Expression

The SP-D Promoter

Approximately 3 kb of upstream sequence of the human SP-D gene, and smaller regions of the rat gene, have been isolated and sequenced.[46,47] Within the first 1 kb are numerous potential cis-regulatory elements including AP-1 and AP-1 like sequences, CREB-like sequences, E-box motifs and a variety of sequences that may participate in the regulation of the acute phase response (NF-IL-6, PEA3, APF-1). In this regard, most of these putative regulatory elements show high homology and positional conservation with sequences in the bovine acute phase protein, conglutinin.[47,61]

Studies of SP-D promoter activity have thus far primarily utilized H441 cells which express small but detectable amounts of SP-D mRNA. Transient transfection assays in these cells using 5'-deletion mutants in chloramphenicol acetyltransferase (CAT) reporter constructs have demonstrated a complex profile of CAT activity consistent with the existence of important negative and positive regulatory elements in the "distal promoter" between -698 and -804 bp and -285 and -698 bp,[47] and within the "proximal promoter" between -285 and -231 bp and -161 and -231 bp.

Interactions with AP-1 Proteins

A conserved canonical AP-1 consensus (5'-TGAGTCA-3'; at -109 bp) is important for the regulation of basal and dexamethasone-stimulated expression as shown by site-directed mutagenesis of the consensus within XS285-CAT reporter constructs (Crouch EC et al, unpublished data). Gel supershift experiments and cotransfection studies using H441 nuclear extracts or cells, respectively, suggest that the effects are not mediated by c-Jun homodimers or c-Jun/c-Fos heterodimers. The binding site is preferentially occupied by Fos-related antigens and other Jun family members in unstimulated cells, predominantly Fra-1 and JunD. Cotransfections with various sense and antisense Fos and Jun cDNA-containing plasmids suggest that alterations in the composition of the AP-1 complex can yield bidirectional changes in promoter activity. Other experiments suggest that the modulation of gene expression through the conserved AP-1 element involves interactions with upstream sites as well as downstream sequences near the site of transcriptional initiation. Thus environmental modulation of the expression or activity of AP-1 proteins may play an important role in modulating the production of SP-D.

Transcriptional Activation by Glucocorticoids

Transfection studies using CAT reporter constructs in H441 lung adenocarcinoma cells have demonstrated dexamethasone-mediated increases (up to 5-fold) in SP-D promoter activity.[47] However, these sequences lack classical full-site glucocorticoid response elements (GRE), and a deletion construct lacking the single half-site GRE in the SP-D proximal promoter (FS-161) still shows a 2-fold increase in CAT activity in the presence of 50 nM dexamethasone. Under conditions where overexpression of glucocorticoid receptor (GR) stimulates the activity of a GRE-containing control plasmid in H441 cells, SP-D promoter activity is decreased, consistent with transcriptional interference of AP-1 proteins or other SP-D transactivating factors by GR. Thus, at least some of the transcriptional effects of glucocorticoids are indirect and probably involve the binding of transactivating factors other than GR. In this regard, the cells must be exposed to dexamethasone for at least 24 hours to achieve maximal stimulation, suggesting a contribution of new protein synthesis.

Regulation of Cell Type Restricted Expression

Sequences within 161 bp of the start site also appear to confer cell-type restricted expression in pulmonary adenocarcinoma cells compared to hepatoma (HepG2) cells. Although the region immediately upstream of the conserved AP-1 contains a sequence similar to the thyroid transcription factor 1 (TTF-1) consensus, SP-D promoter constructs do not show TTF-1 responsiveness in cotransfection experiments and oligomers containing the sequence do not bind TTF-1 in gel shift and supershift assays (Crouch EC, unpublished data). Preliminary experiments implicating TTF-1 in the regulation of SP-D gene expression by the proximal promoter were apparently spurious, because the pSK-CAT vector used for the original SP-D deletion constructs was itself responsive to TTF-1.

Post-Transcriptional Regulation

Primer extension assays and cDNA cloning have identified a single major transcript in human and rat lung.[39,40,47,62] However, bovine conglutinin, believed to be evolutionarily derived from SP-D, contains 9 exons with an additional untranslated exon (72 bp) between the previously designated first and second exons of the human gene.[61] Comparison of the conglutinin gene sequence with the published 5'-untranslated sequence of a bovine lung SP-D cDNA[51] demonstrates the presence of a sequence virtually identical to exon 2 of conglutinin. Unless this represents a bovine specific sequence, which seems unlikely, this obser-

vation strongly suggests alternative splicing of this exon, with a predominance of short transcripts in the lung.

Pathway of Biosynthesis and Secretion

Model Systems

Until recently, studies of SP-D biosynthesis and secretion have been hampered by the lack of a satisfactory model. As indicated above, isolated type II cells rapidly lose the capacity to produce SP-D within several hours. Clara cells have thus far proven difficult to isolate in sufficient purity and numbers to permit detailed studies. We have therefore used a mammalian expression system in which CHO-K1 cells have been stably transfected with wild-type or mutant SP-D cDNAs.[25,27,63,64]

The validity of this system as a model of assembly and constitutive secretion by type II cells is supported by the apparent structural and functional identity of the natural and recombinant wild-type proteins and by similarities in the kinetics of secretion with freshly isolated type II cells.[63] SP-D is secreted quite slowly from CHO-K1 cells with a half-life of approximately 3 hours and an estimated minimum synthesis/secretion time of 1.2 hours. A synthesis/secretion of approximately 1.4 hours was obtained for partially purified suspensions of freshly isolated rat type II cells.

Pathway of Secretion

Studies using the model system suggest that folding of the CRD, trimerization of monomers, N-linked glycosylation of asn_{70}, prolyl and lysyl hydroxylation of the collagen domain, triple helix formation, the amino-terminal association of trimeric subunits and the formation of intersubunit disulfide crosslinks all occur in the RER (Fig. 6.3).[63] Although monomers can associate to form disulfide-bonded trimers within approximately 30 minutes,[63] the subsequent association and crosslinking of trimeric subunits requires a minimum of 30 additional minutes in the RER. Oligosaccharide maturation and terminal sialylation of the oligosaccharide at asn_{70} are relatively rapid events that occur in the Golgi immediately prior to secretion.

Role of the Neck Region in the Trimerization of 43 kDa Subunits

Recent studies by Reid and coworkers have shown that the linking peptide or neck region of bovine SP-D can spontaneously form stable trimers consistent with a trimeric coiled-coil.[65] The efficient secretion of trimeric species in the absence of a triple helical collagen domain (see below) strongly suggests that trimerization is primarily driven by interactions between the linking peptides in vivo.

Within the context of the full length molecule, the neck domain may serve to register the three polypeptide chains prior to folding of the collagen helix.[65] In addition, interactions between the linking peptide and CRD may precisely determine the spatial distribution of the CRD within a trimeric subunit, thereby contributing to the generation of a single high affinity saccharide binding site.[22]

Oligomerization of Trimeric Subunits and Inter-Subunit Crosslinking

Studies of recombinant rat SP-D (RrSP-D) biosynthesis indicate that the amino-terminal interchain disulfide bonds also form very rapidly, covalently stabilizing the association of trimeric intermediates.[63] The assembly of multimers of trimeric subunits, each with a high affinity saccharide binding site, confers multivalency and the capacity to participate in bridging interactions between spatially segregated ligands.

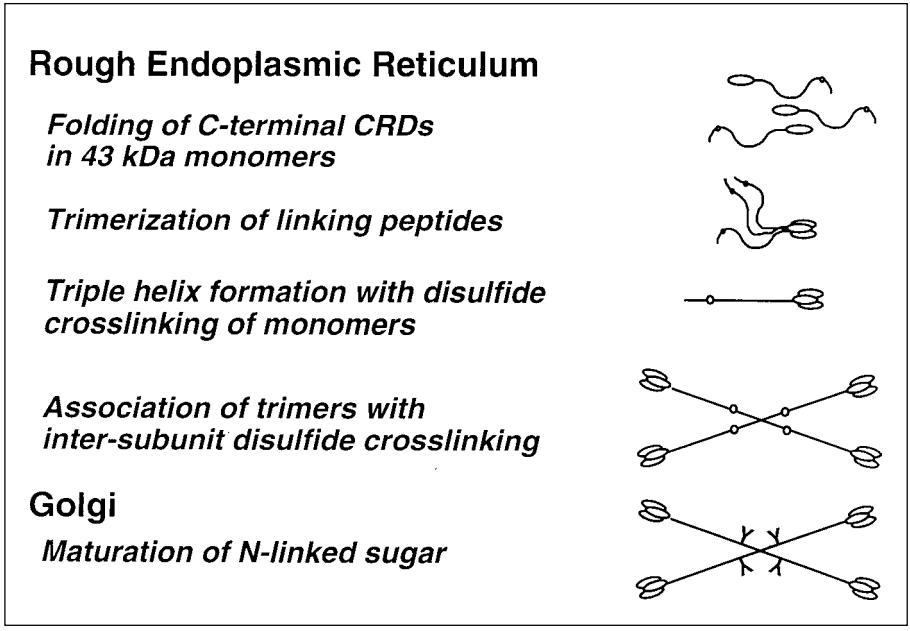

Fig. 6.3. Model pathway of SP-D assembly and secretion. Pulse-chase studies suggest that newly synthesized monomers rapidly associate to form disulfide-bonded trimers.[63] The subunits then associate to form crosslinked oligomers which gradually assemble to form mature dodecamers. Oligomers appear to be specifically retained within the RER until the processes of subunit association and disulfide crosslinking are complete. Transport of the assembled dodecamer to the Golgi with final trimming and sialylation is accomplished shortly prior to secretion.

The oligomerization of trimeric subunits with the formation of specific disulfide crosslinks between the subunits appears to be rate limiting for dodecamer assembly and secretion.[63] The important role of disulfide bonding in the secretion of the full-length molecule is supported by several observations:

1. 2-2'dipyridyl (DP), an inhibitor of prolyl hydroxylation, interferes with the formation of interchain disulfide bonds and completely blocks the secretion of rat SP-D by freshly isolated type II cells or transfected CHO-K1 cells;
2. low concentrations of dithiothreitol reversibly inhibit disulfide crosslinking and secretion;
3. rat SP-D mutants with a single substitution of serine for cysteine 15 or cysteine 20 are not secreted. These effects probably result from a mispairing of cysteine residues (or the presence of free cysteines) rather than from the absence of disulfide crosslinks, because a mutant protein with serine substitutions at both positions ($RrSP\text{-}Dser_{15/20}$) is efficiently secreted as trimeric subunits.[64]

Biosynthetic studies have also shown that the major trimeric cellular intermediate, which elutes from gel filtration columns in the position of SP-D trimers (i.e., 3 x 43 kDa) under nondenaturing conditions, migrates as disulfide-bonded trimers by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the absence of reducing agents.[63] This strongly suggests that newly synthesized trimeric subunits are initially stabilized by intrasubunit interchain disulfide bonds. These subunits then associate to form oligomers which

gradually assemble to form mature dodecamers. Thus, the formation of mature crosslinks involves the apposition of previously unpaired cysteines and a process of rearrangement that converts a specific subset of intra-subunit disulfide bonds to inter-subunit bonds. The mechanism driving chain association prior to stabilization by interchain disulfide bonds is not known. However, the high predicted hydrophobicity of sequences surrounding the amino-terminal cysteines suggests that the initial interactions are hydrophobic.

Role of the Collagen Domain in Assembly and Secretion

The inhibition of SP-D secretion by DP was initially believed to result from inhibition of triple helix formation and specific retention of molecules with unfolded collagen domains.[66] However, RrSP-Dser$_{15/20}$, which lacks amino-terminal cysteines, is efficiently secreted as a trimer both in the absence and presence of DP even though the trimers secreted in the presence of the inhibitor have a pepsin-sensitive (nontriple helical) collagen domain. Thus, inhibition of secretion by DP more likely results as a consequence of interfering with the normal crosslinking of amino-terminal cysteines.[63]

Nevertheless, there is evidence that the formation of dodecamers and higher order multimers depends on conformational constraints imposed by the triple helical collagen domain.[67] RrSP-D mutants lacking the collagen domain are secreted as trimers stabilized by interchain crosslinks but fail to assemble as dodecamers. In the latter case, trimerization of the coiled coil domain (neck region) is presumably sufficient to allow correct folding and crosslinking of the amino-terminal domain without an intervening collagen helix.

In summary, the folding of the collagen helix is required for the correct pairing of amino-terminal cysteine residues in the full length protein. Efficient secretion in turn requires an appropriate pairing of cysteine residues, either within or between trimeric subunits, and the elimination of free cysteines. If the amino-terminal peptide is deleted or if both cysteines are mutated there is no potential for abnormal crosslinking or folding and the mutant protein is efficiently secreted. The assembly and secretion of stable dodecamers involves the formation of appropriate intersubunit disulfide bonds as well as the collagen domain.

Acquisition of Lectin Activity

The formation of intrachain disulfide bonds within the CRD occurs very rapidly, probably on the nascent chains.[63] The subsequent trimerization of 43 kDa SP-D monomers appears to provide all the additional structural information needed for extracted intermediates to bind to saccharide-affinity columns. For example, intermediates accumulating in the presence of DP, which prevents collagen helix formation and normal interchain crosslinking, bind efficiently to maltosyl-agarose. This is also consistent with the ability of bacterially expressed trimeric carboxy-terminal domains to bind to specific saccharide ligands in solid phase assays.[68]

Given that many glycoproteins are transiently glucosylated in the RER, it seems likely that the SP-D intermediates are maintained in an inactive form or are somehow sequestered from other glycoproteins within the cell. It is possible that local calcium concentrations are important in this regard. Inhibition of SP-D activity by EDTA or EGTA is readily reversible by the addition of excess calcium.[17] Thus, exposure of isolated intermediates to calcium may allow rapid acquisition of an appropriate conformation for ligand binding even though they are inactive prior to secretion in vivo.

Glycosylation of Asparagine 70

All SP-Ds thus far characterized show utilization of the single consensus for N-linked glycosylation at asn$_{70}$ within exon C2. Although some (but not all) metabolic inhibitor studies

of SP-A biosynthesis suggest that N-linked glycosylation is required for secretion,[69,70] tunicamycin did not inhibit the secretion of rat SP-D by freshly isolated type II cells and RrSP-D by CHO-K1 cells.[63] Consistent with this observation, elimination of the conserved N-linked oligosaccharide at asn_{70} in SP-D by site directed mutagenesis did not significantly alter the kinetics of secretion of dodecamers.[63]

Interestingly, the secretion of a single arm mutant of SP-D ($RrSP\text{-}Dser_{15/20}$), which cannot form stable dodecamers, is inhibited by tunicamycin. Thus, the intracellular pathway of SP-D secretion may depend on the conformation, oligomerization or crosslinking of the amino-terminal peptide domain. It is possible that the oligosaccharide determines the interaction of SP-D with specific chaperones under some circumstances in vivo, or participates in regulating the secretion of single arm forms of the protein. A subtle secretory role is consistent with the observation that the deglycosylated mutant appears indistinguishable from the wild type protein with respect to biologic activity and resistance to proteolytic degradation in vitro.

There is evidence for heterogeneity in the structure of the oligosaccharide at asn_{70}. SP-D migrates as a fairly broad band on SDS-PAGE. When SP-D dodecamers are resolved by DEAE chromatography under nondenaturing conditions the larger or more slowly migrating species elute earlier in the gradient than more rapidly migrating species (Crouch EC and Persson A, unpublished data). This observation strongly suggests that the larger components are more acidic than the smaller species and indicates that similarly modified chains preferentially associate within a single SP-D dodecamer. Interestingly, mutations that interfere with the multimerization of trimeric subunits are associated with alterations in oligosaccharide structure and increased sialylation, leading to a slower mobility on SDS-PAGE.[64] Thus, differences in the level of sialylation probably account for at least part of the charge heterogeneity and polydispersity on SDS-PAGE. The significance of these observations is uncertain.

Modulation of SP-D Production In Vivo

The "Acute-Phase" Response

Immunohistochemical and in situ hybridization studies strongly suggest that the production of SP-D is usually increased in association with epithelial activation as a consequence of acute and chronic lung injury. Because the mRNAs for the lung collectins are increased within several hours to a few days following injury it has been suggested that they are pulmonary acute phase proteins, similar to liver-derived mannose binding protein (MBP) and conglutinin, which are generally recognized as systemic acute phase reactants.[71] Although SP-D gene expression is not increased by LPS in human fetal lung organ culture,[57] intratracheal instillation of LPS in adult rats results in a relatively rapid increase in SP-D mRNA and protein.[71] It seems likely that secondary inflammatory mediators or more general effects on epithelial function are involved. In any case, such findings suggest that it is prudent to be cautious in extrapolating from fetal lung explants to the situation in mature lung in vivo.

Experimental Lung Injury

SP-D is also increased in other forms of experimental lung injury and human disease. Intratracheal instillation of crystalline silica in rats results in an increase in SP-D mRNA and protein associated with the development of alveolar proteinosis.[34,72] Reactive pneumocytes and Clara cells show intense staining and increased labeling by in situ hybridization at 2-3 weeks following instillation (Crouch EC, unpublished data). Increases in

immunostaining of reactive pneumocytes have also been observed within several days following bleomycin instillation in rats (Crouch EC, unpublished data) and in association with hyperoxic injury.[73]

Alterations in SP-D Accumulation Associated with Human Disease

Immunohistochemical studies have demonstrated increased cytoplasmic staining of reactive type II cells in biopsies from patients with pulmonary fibrosis.[1] Idiopathic pulmonary fibrosis and pulmonary alveolar proteinosis are also associated with marked increases in the serum levels of proteins immunologically crossreactive with well characterized antibodies to SP-D.[45] Cigarette smoking is associated with a decreased recovery of SP-D from lung lavage[74] but the mechanism remains to be elucidated.

Questions and Directions for Future Research

What are the extra-pulmonary sites of SP-D production? How is the tissue-restricted gene expression of SP-D regulated? Are there tissue specific differences in post-transcriptional regulation and post-translational modification? How are differences in the extent of sialylation regulated? How is SP-D targeted to secretory granules in Clara cells, and how is secretion regulated? Is there coordinate regulation of SP-A and SP-D secretion by these cells in vivo? How is the acquisition of lectin activity regulated within the cell? How is the degree of subunit oligomerization regulated? What is the site and mechanism of the multimerization of dodecamers? How are the trimeric subunits oriented in the molecule? Are they in parallel as for SP-A? What is the specific pattern of interchain disulfide bonding within the dodecamer? What are the major cellular mechanisms contributing to the increased production and accumulation of SP-D in various forms of lung injury? Is it part of a generalized, activated or postinjury phenotype involving keratinocyte growth factor or other mediators?

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Chapter 7

Regulation of Surfactant Secretion

Seamus A. Rooney

The goal of this chapter is to review current knowledge of the mechanisms regulating surfactant secretion. Surfactant homeostasis is maintained by a balance between synthesis, secretion and removal. Upon synthesis, surfactant components can be either secreted immediately into the alveolar lumen or stored and subsequently secreted in response to an appropriate signal. After participating in lowering surface tension or the additional functions described in other chapters, surfactant components are ultimately removed from the alveolar space either by catabolism or by re-uptake into the type II cells and recycling into new surfactant. Although secretion is but one of many steps in the overall context of surfactant metabolism, there is every indication that it is a vital one and critical in maintaining adequate levels of extracellular surfactant.

Lamellar Bodies and Surfactant Secretion

Secretion from mammalian cells can be either constitutive or regulated. Constitutive secretion is a continuous process that is limited only by the amount of material available for secretion. In the regulated mechanism, the material to be secreted is stored in secretory organelles and only released in response to an appropriate stimulus. There is evidence that surfactant components are secreted by both mechanisms.

The phospholipid components of surfactant are secreted largely by a regulated mechanism. As discussed subsequently, surfactant phospholipids are secreted in response to a variety of pharmacological and physiological stimuli. The secretory organelle is the lamellar inclusion body, a characteristic morphological component of the type II pneumocyte (Fig. 7.1). Consistent with being intracellular storage sites, isolated lamellar bodies are enriched in phospholipid,[1,2] the composition of which is very similar to that of extracellular surfactant obtained from lung lavage.[3] Phospholipid secretion occurs by exocytosis, a process in which the lamellar body membrane fuses with the plasma membrane of the type II cell and the lamellar body contents are released into the alveolar lumen. Lamellar bodies in the process of exocytosis have been captured by electron microscopy.[4,5] An example of such an event is illustrated in Figure 7.2. Although surfactant phospholipids are largely secreted by regulated exocytosis, the possibility of some constitutive secretion cannot be excluded. The basal secretion of phospholipids that is observed in isolated type II cells cultured without secretagogues may well be a constitutive process.

Secretion of the protein components of surfactant has been much less studied than that of the phospholipids. There is considerable evidence that surfactant protein A (SP-A) is secreted independently of the lipid components of surfactant. SP-A secretion in cultured type II cells is not stimulated by agents that increase phospholipid secretion[6,7] and secretion of SP-A and phosphatidylcholine (PC) exhibit different time courses.[7] Radiolabeled SP-A

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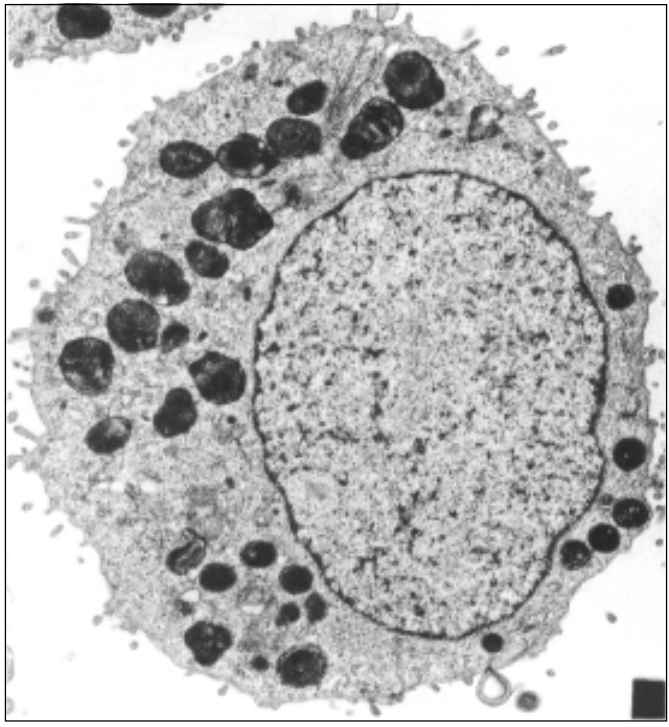

Fig. 7.1. A type II pneumocyte isolated from human lung. Reproduced with permission from Dobbs LG. Isolation and culture of alveolar type II cells. Am J Physiol 1990; 258:L134-L147.

appears considerably earlier in alveolar lavage than in lamellar bodies from the lungs of animals injected in vivo with ^{35}S-methionine suggesting that secretion of SP-A is independent of lamellar bodies.[8,9] Although lamellar bodies were reported to be enriched in SP-A is some studies,[10-12] they were reported to contain relatively little in others.[2,13,14] Clearly, if only a fraction of SP-A is associated with lamellar bodies, its secretion must be independent of those organelles. Taken together, these data suggest that the bulk of SP-A is secreted independently of lamellar bodies and by a constitutive rather than a regulated mechanism.

Secretion of surfactant protein D (SP-D) also appears to be constitutive (chapter 6). Lamellar bodies have been reported to be devoid of SP-D;[15,16] therefore, it cannot be secreted by the same regulated mechanism as the phospholipids. However, whether secretion of SP-D is stimulated by surfactant secretagogues has not been examined. As discussed in chapters 4 and 5, there is evidence that surfactant protein B (SP-B) and surfactant protein C (SP-C) are processed through lamellar bodies. These hydrophobic proteins were first described in lamellar bodies[17] and isolated lamellar bodies were reported to be enriched in SP-B and SP-C.[2] Therefore, SP-B and SP-C are most likely secreted from lamellar bodies by regulated exocytosis along with the phospholipids. Indeed, in vivo experiments with radiolabeled precursors showed that the labeling kinetics of PC and SP-B in alveolar lavage were similar and that both products appeared in lamellar bodies earlier than in the lavage.[18] There is currently no information on whether secretion of SP-B and SP-C is stimulated by surfac-

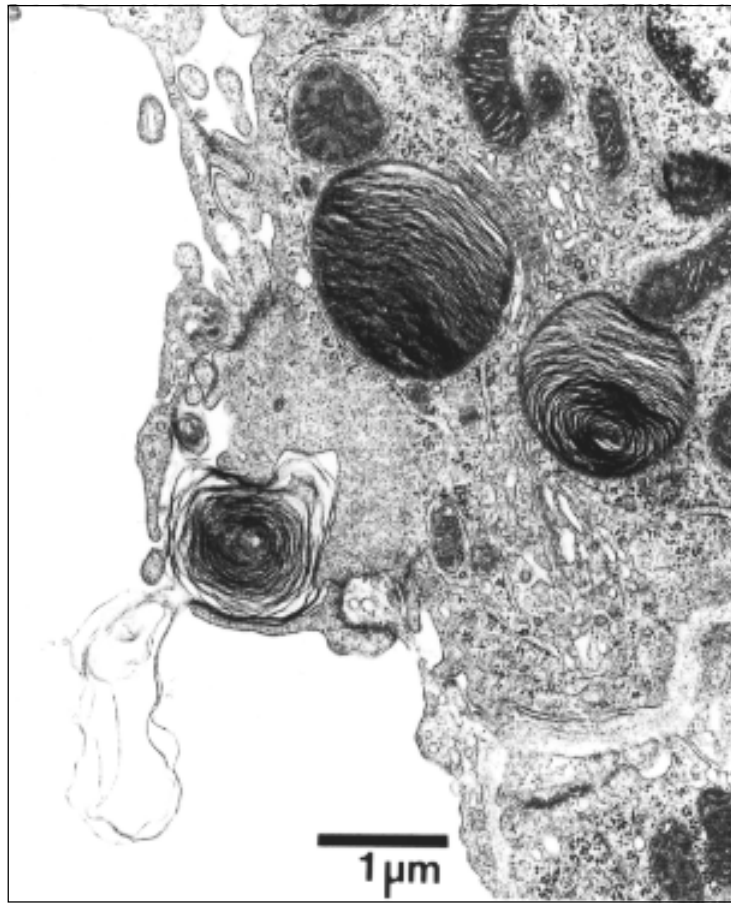

Fig. 7.2. A lamellar body in the process of exocytosis from a type II cell. Reproduced with permission from Rooney SA, Young SL, Mendelson CR. Molecular and cellular processing of lung surfactant. FASEB J 1994; 8:957-967.

tant phospholipid secretagogues. Indeed, because of the difficulty of accurate quantitation (chapter 5) such experiments would be difficult to carry out in the case of SP-C.

In summary, secretion of surfactant phospholipids, as well as that of SP-B and SP-C, occurs largely by regulated exocytosis of lamellar bodies, whereas secretion of SP-A and SP-D appears to be constitutive.

Experimental Models

Surfactant secretion has been investigated in several experimental models. Early studies employed intact animals in vivo, isolated perfused lungs and lung slices.[3,19,20] The intact animal is undoubtedly the best model in which to study the physiological regulation of surfactant secretion, but it suffers from the disadvantage that direct effects on the lung cannot be distinguished from those mediated by other organs. As the end-point is usually the amount of surfactant recovered in lung lavage, another disadvantage of the intact animal model, and one that is shared by the isolated perfused lung, is that effects on surfactant

synthesis, as well as those on its removal or degradation, are not easily distinguished from effects on secretion.

The isolated type II cell in primary culture is now the model of choice for most studies on regulation of surfactant secretion. The majority of such studies have been carried out on cells isolated from adult rats, but surfactant secretion has also been examined in type II cells isolated from rabbits[21-23] and humans.[7,24] Isolated type II cells in primary culture secrete surfactant as defined by its lipid composition,[3,25] surface activity,[25] presence of SP-A[6,25] and morphological appearance.[25] Although secretion of SP-A has also been examined,[6,7,25] most studies on surfactant secretion from isolated type II cells have focused on its lipid components, particularly PC or disaturated PC. However, it appears that all surfactant phospholipids are secreted together, as the phospholipid composition of the secreted material is not altered by secretagogues.[24,25]

In most secretion studies, the intracellular PC pool is labeled by incubating the cells overnight with ^{3}H- or ^{14}C-choline, after which the rate of appearance of radiolabeled-PC in the medium is measured. In some studies PC was separated from other lipids, but that step is not necessary when the cells are pulsed with radiolabeled choline, as PC accounts for over 95% of the total lipid radioactivity in both the cells and culture medium.[26-29] PC secretion is expressed as the amount of radiolabeled PC in the medium as a fraction of the total in the cells and medium combined. The basal rate of PC secretion is about 0.5% to 1% per hour under such conditions.[27,28,30-32] That is undoubtedly an underestimate of the true rate of surfactant secretion because the denominator includes total cellular PC and not just that in lamellar bodies. Although surfactant secretagogues do not increase the rate of lactate dehydrogenase release, the basal rate of surfactant phospholipid secretion is similar to or even less than the rate of lactate dehydrogenase release.[26,33,34] That raises the question of whether basal secretion is a reflection of cell leakiness rather than true secretion. However, the composition of the material released is similar to that of surfactant isolated from lung lavage[3] and different from that of the cells,[25] indicating that it is indeed true secretion.

Although the type II cell in primary culture has been extensively used for studies on the regulation of surfactant phospholipid secretion, it is not an ideal model. Culture of cells on plastic or extracellular matrix is obviously an unphysiological system and the possibility of tissue culture artifacts must always be considered. Type II cells rapidly loose their characteristic phenotype, particularly their distinct phospholipid profile and the presence of lamellar bodies, after not much more than a day in culture.[35-38] The response to secretagogues also decreases with time in culture.[39] However, that is not a serious problem, as phospholipid secretion can easily be measured within 24 hours of isolating the cells. Some phenotypic characteristics of the type II cells can be preserved for an extended period when culture is carried out in defined media and on special substrata.[40,41] However, it is not clear if the ability to secrete surfactant and to respond to secretagogues is retained under such conditions.

A practical disadvantage in using primary cultures is that fresh cells have to be isolated for each experiment. A continuous cell line would be more convenient and less expensive and would also be useful for molecular approaches that are impossible in primary cultures. However, although a number of immortalized type II cell lines have been developed,[42-45] none are suitable for studies on surfactant PC secretion either because they lack lamellar bodies, do not have the typical phospholipid composition of type II cells or do not respond to surfactant secretagogues. Two older cell lines were considered as type II cell models before methods for isolation of authentic type II cells were established: A549 cells, derived from a human lung carcinoma,[46] and L-2 cells, clonally derived from rat lung.[47] Although both were initially reported to contain lamellar bodies,[46,47] the cells currently available do not.[48] Furthermore, neither A549[49,50] nor L-2[50] cells have the characteristic phospholipid composition of authentic type II cells. In summary, at present there is no type II cell line

available that is suitable for studies on surfactant PC secretion, so that the isolated type II cell in primary culture remains the model of choice.

Surfactant Secretagogues

A large number of physiological and pharmacological agents can stimulate surfactant secretion. In early studies, β-adrenergic and cholinergic agonists were reported to stimulate surfactant secretion in vivo.[3,19] It was subsequently established that β-agonists act directly on the type II cell. Beta-receptors exist on the type II cell[51,52] and β-agonists stimulate surfactant secretion in isolated type II cells in primary culture.[3,33,53] On the other hand, cholinergic agonists do not stimulate surfactant secretion in the isolated perfused lung,[54] in lung slices[55] or in isolated type II cells.[33,53] The stimulatory effect of cholinergic agonists in vivo is an indirect effect on the lung that is probably mediated by catecholamines released by the adrenal medulla.[56] Beta-receptors are coupled to adenylate cyclase (AC) and their activation is characteristically associated with increased formation of cyclic AMP (cAMP).[57] Not surprisingly, other agents that stimulate cAMP formation also stimulate surfactant secretion in type II cells. They include cholera toxin,[58] forskolin[59] and agonists that activate an adenosine receptor that is also coupled to AC activation.[60,61]

Other well established surfactant secretagogues in type II cells include ionophores, ionomycin and A23187, which promote Ca^{2+} influx into the cell, and the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), and cell-permeable diacylglycerols (DAGs), 1-oleoyl-2-acetylglycerol and dioctanoylglycerol, which activate protein kinase C (PKC).[3]

Although the extent of stimulation can vary considerably, in general surfactant secretion is increased 2- to 3-fold by agonists that activate AC and by Ca^{2+} ionophores, and 5-fold or more by TPA and other PKC activators.[3] Combinations of agonists acting via different signaling mechanisms have been reported to stimulate surfactant secretion as much as 12-fold.[27,62] Mastoparan, an agent that activates several signaling mechanisms, has been reported to increase surfactant secretion 8- to 10-fold over the rate in control type II cells.[63] The P2 purinoceptor agonist adenosine 5'-triphosphate (ATP), which also acts via a number of signaling mechanisms, has been reported to stimulate surfactant secretion as much as 5- to 6-fold,[30,34,64-66] although less stimulation has also been reported.[26,61,67]

Other agonists that have been reported to stimulate surfactant secretion in type II cells include arachidonic acid,[68,69] other unsaturated fatty acids,[69] prostaglandins,[70] leukotrienes,[71] vasopressin,[72] histamine,[73] antihistamines,[74] serum lipoproteins,[75] gastrin-releasing peptide[76] and endothelin-1.[29]

Agents that Inhibit Surfactant Secretion

There are a number of agents that inhibit surfactant phospholipid secretion in type II cells. SP-A can completely abolish the stimulatory effects of secretagogues and it also inhibits its basal secretion.[77-79] The plant lectins concanavalin A[78,80,81] and wheat germ[80] and *Maclura pomifera* agglutinins,[78,80] proteins that have some structural characteristics in common with SP-A, also inhibit surfactant phospholipid secretion. Other inhibitors of surfactant secretion include substance P,[82] adenosine A_1 receptor agonists[26] and compound 48/80, an agent that promotes histamine release from mast cells.[83]

Signal Transduction Mechanisms

Transduction of an extracellular signal into a physiological response can be a complex process involving a number of intermediate proteins and signaling molecules.[84] The existence of multiple forms of transducer proteins among which positive and negative interactions can occur has led to the realization that signal transduction can be finely regulated.[85] A schematic representation of the signal transduction mechanisms that mediate surfactant

secretion is presented in Figure 7.3. Although an oversimplification, it summarizes most of the currently elucidated signaling steps in the type II cell.

Surfactant secretagogues include agents that activate receptors on the surface of the type II cell as well as those that penetrate the cell and activate downstream signaling steps. At least three cell surface receptors can regulate surfactant secretion. Those are β-adrenergic receptors that are activated by secretagogues such as epinephrine,[33] terbutaline[33,74] and isoproterenol,[33,53,74] adenosine receptors that are activated by adenosine and adenosine analogs such as 5'(N-ethylcarboxyamido)adenosine (NECA)[60,61,86] and P2 purinoceptors that are activated by ATP,[30,61,64] uridine 5'-triphosphate (UTP)[67,87] and ATP analogues.[88-90] Surfactant secretagogues that bypass cell surface receptors include cholera toxin and forskolin, which activate AC; TPA and cell-permeable DAGs that activate PKC; and ionophores that promote Ca^{2+} influx.[3]

The signaling mechanisms mediating surfactant secretion consist of three distinct pathways, although there are overlap and interactions among them:[5,62]

1. Activation of AC, generation of cAMP and activation of cAMP-dependent protein kinase (protein kinase A, PKA);
2. Activation of PKC, either directly or indirectly as a consequence of receptor activation;
3. Increased levels of intracellular Ca^{2+}, either as uptake from outside the cell promoted by ionophores or as release from intracellular storage sites in response to receptor activation.

Beta-Adrenoceptors and Adenosine Receptors Coupled to Adenylate Cyclase Activation

Three β-adrenoceptors, β_1, β_2 and β_3, are known to exist.[57] The β_3 receptor does not appear to be expressed in the lung[57] but the β_1 and β_2 genes are both expressed in the type II cell.[91] However, pharmacological data[51,52] suggest that surfactant secretion is regulated by the β_2 receptor. There are four subtypes of adenosine receptors (P1 purinoceptors), A_1, A_{2A}, A_{2B} and A_3,[92] and all four are expressed in the type II cell (Xu ZX and Rooney SA, unpublished). However, whereas the A_2 subtypes are coupled to activation of AC, activation of the A_1 and A_3 receptors leads to decreased cAMP formation.[92] As adenosine and the adenosine analogs that stimulate surfactant secretion increase cAMP formation,[60,61] it is evident that surfactant secretion is regulated by one of the A_2 receptors. The adenosine receptor on the type II cell could not be characterized with radioligands because of the presence of large amounts of a NECA-binding protein.[86] However, the potency order of adenosine receptor agonists and antagonists in the type II cell[60,86] is consistent with that reported for the A_{2B} receptor in other systems[92] and suggests that it is the adenosine A_{2B} receptor which regulates surfactant secretion.

The β_2-adrenergic[57] and A_{2B} adenosine[93] receptors are coupled to AC via the heterotrimeric guanosine 5'-triphosphate-binding protein (G-protein) G_s in other systems, and are presumably similarly coupled in the type II cell (Fig. 7.3). Activation of AC results in formation of cAMP, and that second messenger in turn activates PKA. Two surfactant secretagogues bypass a receptor to activate AC. Forskolin directly activates AC, while cholera toxin irreversibly activates G_s. Cholera toxin[58] and forskolin,[59] as well as surfactant secretagogues that activate β-adrenergic[33,53,60] and adenosine A_{2B}[60,61] receptors, have all been shown to stimulate cAMP formation in the type II cell. The increase in cAMP in response to terbutaline and forskolin in type II cells was reported to be associated with increased PKA activation.[59]

At least nine mammalian AC isoforms[94] and multiple PKA isoforms[95] are known to exist. AC II and AC IV, but not AC I, are expressed in the type II cell.[96] However, there is no information on which PKA isoform(s) exist in the type II cell or which AC and PKA

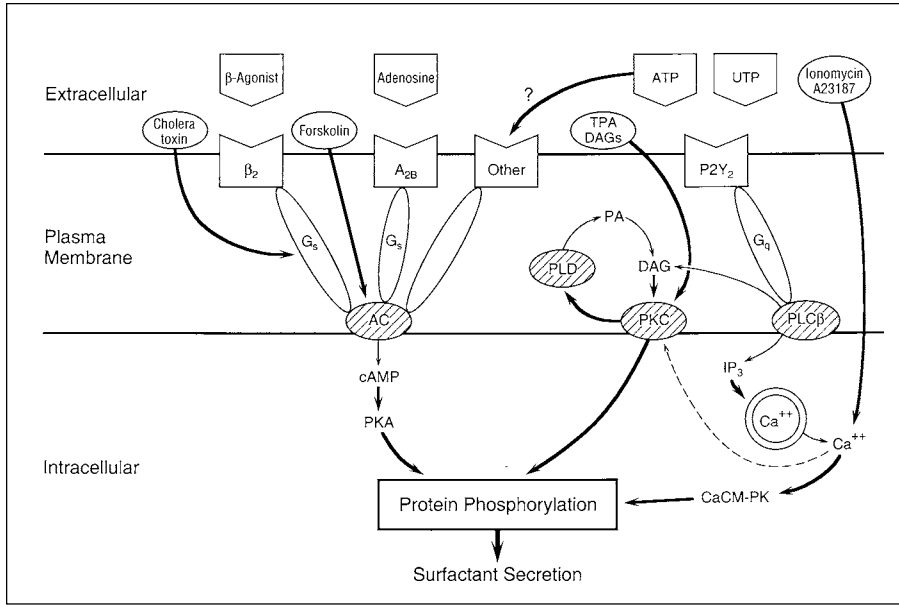

Fig. 7.3. Schematic representation of signal transduction mechanisms mediating surfactant phospholipid secretion in type II cells. Beta-adrenergic agonists bind to β_2-receptors that are coupled to adenylate cyclase (AC) via the heterotrimeric GTP-binding protein (G-protein) G_s. Adenosine and its analogs bind to adenosine A_{2B} receptors that are similarly coupled to AC. Forskolin directly activates AC, while cholera toxin permanently activates G_s. Activation of AC results in the generation of cAMP, which in turn activates cAMP-dependent protein kinase (PKA). ATP and UTP bind to $P2Y_2$ purinoceptors that are coupled to phospholipase Cβ (PLCβ) via G_q. Activation of PLCβ results in the hydrolysis of phosphatidylinositol bisphosphate (not shown) and generation of diacylglycerol (DAG) and inositol trisphosphate (IP_3). DAG activates protein kinase C (PKC) which in turn activates phospholipase D (PLD). PLD hydrolysis of phosphatidylcholine leads to the formation of choline (not shown) and phosphatidic acid (PA). Phosphatidate phosphatase (not shown) converts PA to DAG which then further activates PKC. 12-O-tetradecanoylphorbol-13-acetate (TPA) and cell permeable DAGs, such as 1-oleoyl-2-acetylglycerol and dioctanoylglycerol, directly activate PKC. ATP also binds to an AC-coupled methylxanthine-sensitive receptor that is distinct from the adenosine A_{2B} receptor. IP_3 promotes mobilization of calcium from intracellular stores, while the ionophores ionomycin and A23187 promote calcium influx into the cell. Calcium activates a Ca^{2+}-calmodulin-dependent protein kinase (CaCM-PK) and may also act synergistically with DAG to activate PKC. Protein phosphorylation by PKA, PKC or CaCM-PK ultimately leads to surfactant secretion. See text for further details and supporting references. Adapted from Rooney SA, Young SL, Mendelson CR. Molecular and cellular processing of lung surfactant. FASEB J 1994; 8:957-967.

isoform(s) are activated by surfactant secretagogues. The finding that the stimulatory effect of terbutaline on surfactant secretion is not additive to that of adenosine or NECA[60] suggests that β-receptors and adenosine A_{2B} receptors are coupled to activation of the same AC and PKA isoforms. On the other hand, the effects of terbutaline and forskolin on surfactant secretion are additive,[59] suggesting that forskolin may activate different isoform(s) from those coupled to receptor activation.

P2 Purinoceptors and Activators of Protein Kinase C

P2 receptors are divided into two major groups: P2X and P2Y.[97,98] P2X receptors are ion channels, whereas P2Y receptors are coupled to G-proteins.[97,98] At present, seven P2X subtypes and seven P2Y subtypes have been cloned.[97] There is good evidence that the P2 purinoceptor responsible for stimulation of surfactant is $P2Y_2$. (The $P2Y_2$ receptor was termed P_{2u} in an older nomenclature system).[92] ATP and UTP are equally potent at $P2Y_2$ receptors[92,98] and both nucleotides stimulate surfactant secretion in type II cells, with similar concentrations eliciting half maximal effects (EC_{50} values).[87] Additional agonist potency order data are also consistent with the $P2Y_2$ receptor.[87-89] The $P2Y_2$ receptor is expressed in the type II cell.[87,99] Indeed the rat $P2Y_2$ receptor was first cloned using a type II cell cDNA library.[99]

Signal transduction data are also consistent with $P2Y_2$ receptor regulation of surfactant secretion. The $P2Y_2$ receptor is coupled to phosphoinositide-specific phospholipase C (PPI-PLC),[98] an enzyme that acts on phosphatidylinositol bisphosphate to generate the two second messengers inositol trisphosphate (IP_3) and DAG (Fig. 7.3).[100] DAG activates PKC[100] and IP_3 promotes the mobilization of Ca^{2+} from intracellular stores.[101] ATP has been shown to promote formation of IP_3 and DAG,[30,32,61,102,103] to decrease phosphoinositide levels,[32,102] to increase intracellular Ca^{2+} levels[30,65] and to activate PKC[30,66] in type II cells. The $P2Y_2$ receptor is generally coupled to the pertussis toxin resistant G-protein G_q.[104] Effects of ATP in the type II cell are only partly inhibited by pertussis toxin,[65,102,105] so it is likely that the $P2Y_2$ receptor on the type II cell is similarly coupled. However, the fact that there is some pertussis toxin inhibition suggests that the subunits of G_i may also have a role in coupling the $P2Y_2$ receptor to PPI-PLC.[85] In that context, it is of interest that G_i was recently reported to mediate lipoprotein induced surfactant secretion in type II cells.[106]

Eleven mammalian PKC isoforms are known to exist[100] and the α, βI, βII, δ, η, θ, ζ and μ isoforms have been identified in the type II cell by immunoblotting.[107-109] However, little is currently known concerning the identity of the PKC isoform(s) activated by $P2Y_2$ agonists in the type II cell. PKCα, PKCβ (βI and βII were not distinguished),[107] PKCδ and PKCη were reported to be activated by TPA[107] in the type II cell and, in a preliminary report, PKCα and PKCβII were reported to be activated by ATP.[108] It remains to be determined if the same PKCs are activated by $P2Y_2$ agonists and by the cell-permeable DAGs that stimulate surfactant secretion. There are three groups of PPI-PLC enzymes, PLC-β, PLC-δ and PLC-γ,[110,111] but only PLC-β is activated by G-proteins.[85] As the $P2Y_2$ receptor is coupled to a G-protein, it follows that PLC-β is involved in surfactant secretion. Four isoforms of PLC-β exist.[111] The β_1 and β_3 isoforms are widely distributed whereas β_2 and β_4 are largely confined to hematopoietic cells and the retina, respectively.[111] Immunoblotting revealed the presence of PLC-β_3 in type II cells whereas the β_1, β_2 and β_4 isoforms were not detected.[109] It remains to be determined if PLC-PLC-β_3 is activated by surfactant secretagogues that activate PKC in the type II cell.

Activation of Phospholipase D

There is biphasic formation of DAG in response to ATP in the type II cell.[32,102] There is an initial transient increase within seconds after addition of ATP, followed by a larger and more sustained increase 5-10 min later. There is also a sustained increase in phosphatidic acid formation which corresponds to the second DAG peak.[102] The same increases in DAG and phosphatidic acid formation occur in response to UTP.[87] The initial increase in DAG is accompanied by IP_3 formation and is, therefore, consistent with activation of PPI-PLC. The second DAG peak is not accompanied by an increase in IP_3[102] and cannot, therefore, be due to activation of PPI-PLC.

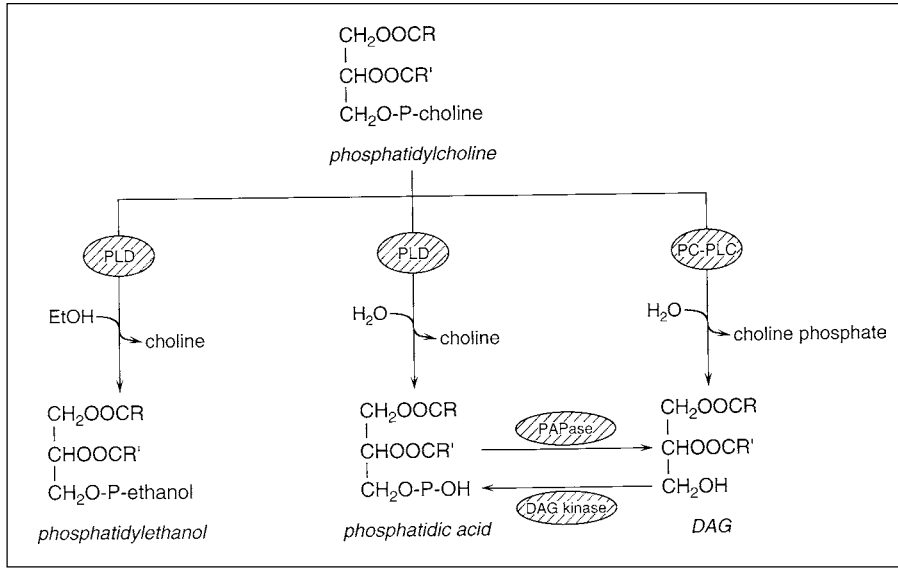

Fig. 7.4. The actions of phospholipase D (PLD) and phosphatidylcholine-specific phospholipase C (PC-PLC) on phosphatidylcholine. PLD normally catalyzes a hydrolytic reaction in which the choline moiety of phosphatidylcholine is replaced by water and phosphatidic acid is the lipid product. However, in the presence of an alcohol, PLD catalyzes a transphosphatidylation reaction in which the lipid product is a phosphatidylalcohol rather than phosphatidic acid. When the alcohol is ethanol, the product of the PLD reaction is phosphatidylethanol. The action of PC-PLC leads to the formation of a diacylglycerol (DAG) and choline phosphate. Phosphatidic acid can be hydrolyzed to DAG by the action of phosphatidate phosphatase (PAPase), and DAG can be converted to phosphatidic acid by the action of DAG kinase.

Phospholipases other than PPI-PLC are known to be involved in signaling mechanisms.[100] Activation of phospholipase D (PLD) occurs in many systems[112] and there is also evidence of activation of a phospholipase C (PLC), distinct from PPI-PLC, that acts on phospholipids other than phosphoinositides, e.g., PC.[113,114] (That PLC is designated PC-PLC, although it can act on a number of glycerophosphatides in addition to PC).[115] The reactions catalyzed by PLD and PC-PLC are illustrated in Figure 7.4. Phosphatidic acid is the immediate product of the action of PLD, whereas DAG is the immediate product of PC-PLC. Phosphatidic acid and DAG are metabolically interconvertible. Phosphatidic acid can be converted to DAG in a dephosphorylation reaction catalyzed by phosphatidate phosphatase (PAPase) and can be formed from DAG in a phosphorylation reaction catalyzed by DAG kinase (Fig. 7.4). Thus, the increase in DAG and phosphatidic acid formation in response to ATP and UTP in the type II cell could be due to activation of either PC-PLC or PLD.

It is possible to distinguish between PC-PLC and PLD by taking advantage of the fact that PLD catalyzes a transphosphatidylation reaction[112,116] whereby in the presence of ethanol it forms phosphatidylethanol rather than phosphatidic acid (Fig. 7.4). Addition of ethanol and either ATP or UTP to type II cells results in the rapid formation of phosphatidylethanol.[87,105] Such data suggest that the second peak of DAG formation is due to activation of PLD.

PLD is also activated by TPA and dioctanoylglycerol,[105] surfactant secretagogues that activate PKC. However, it is not activated by secretagogues that do not activate PKC.[105] In addition, phosphatidylethanol formation in response to ATP, TPA and dioctanoylglycerol is antagonized by PKC inhibitors.[105] Those data suggest that activation of PLD in the type II cell, as in many other systems,[112] is mediated by PKC. Both ATP and TPA stimulate formation of choline in type II cells, suggesting that PC is the substrate for PLD.[87]

Similar to the other signaling proteins discussed above, several isozymes of mammalian PLD are believed to exist and one has recently been cloned.[112] Currently, there is no information on whether more than one PLD isoform exist in the type II cell.

As shown in Figure 7.3, activation of PLD provides a mechanism by which $P2Y_2$ receptor agonists can sustain secretion of surfactant. Activation of PPI-PLCβ results in formation of DAG, which in turn activates PKC. Protein phosphorylation then initiates PC secretion. As phosphatidylinositol bisphosphate is a minor lipid in most cells, it is unlikely that that mechanism could maintain prolonged stimulation of surfactant secretion. However, PKC also activates PLD and that enzyme, acting on the much more abundant PC,[87] generates larger quantities of DAG. Such DAGs in turn further activate PKC and perpetuate the cycle. Sustained PKC activation then maintains surfactant secretion for a prolonged period.

Intracellular Ca^{2+} Elevation

In addition to the PKC pathway, activation of the $P2Y_2$ receptor results in the formation of IP_3 (Fig. 7.3) and that second messenger promotes release of Ca^{2+} from intracellular stores.[101] ATP increases type II cell intracellular Ca^{2+} levels[30] and they are also increased by ionophores that stimulate surfactant secretion.[20] Ca^{2+} is known to activate a number of the PKC isozymes.[100] Hence, there is overlap between the PKC and Ca^{2+} signaling pathways (Fig. 7.3). Interestingly, effects of both ATP and ionomycin on surfactant secretion are decreased by calmodulin antagonists,[62,65] suggesting that a calmodulin dependent step is involved in the regulation of surfactant secretion. Calmodulin has been reported to be associated with lamellar bodies.[117] Whether it is Ca^{2+}/calmodulin-dependent protein kinase (CaCM-PK) that regulates surfactant secretion, as shown in Figure 7.3, or another calmodulin dependent step, remains to be determined.

Adenylate Cyclase Coupled ATP Receptor

ATP stimulates cAMP formation in the type II cell.[61,87,89] That suggests that, in addition to the PKC and calcium-activated pathways, ATP also activates the AC pathway of surfactant secretion. UTP does not promote cAMP formation,[87] so that ATP activation of AC is not mediated by the $P2Y_2$ receptor. Indeed, there is no evidence that the $P2Y_2$ receptor is coupled to AC in any system.[118] The fact that ATP activates the AC signaling mechanism in addition to $P2Y_2$ receptor-coupled mechanisms may explain the fact that ATP stimulates surfactant secretion to a greater extent than UTP,[67,87] even though the two agonists have the same EC_{50}s.[87] In contrast, both nucleotides stimulate formation of DAG and phosphatidic acid as well as PLD activation to similar extents,[87,105] showing that they are equally effective as $P2Y_2$ agonists.

At least three mechanisms could account for ATP stimulation of cAMP formation. Firstly, ATP could be degraded by ectonucleotidases to adenosine, which could then act directly at the adenosine A_{2B} receptor on the type II cell.[118,119] ATP could also be degraded to adenosine 5'-monophosphate (AMP), another potential adenosine receptor agonist.[92] Secondly, ATP itself could act directly at the adenosine A_{2B} receptor on the type II cell. Thirdly, ATP could activate an unrelated AC-coupled receptor.

There are synergistic interactions between the AC and PLC signal transduction pathways of surfactant secretion.[89] The stimulatory effects of surfactant secretagogues acting via

the AC pathway are synergistic with those that activate PKC[88,120] or increase Ca^{2+} levels.[120,121] Thus, the effect of NECA was reported to be synergistic with those of TPA, dioctanoylglycerol and ionomycin,[120] as were the effects of cAMP and forskolin with that of ionomycin[121] and the effect of adenosine with that of TPA.[88] It is therefore difficult to assess the ATP-activated AC and PLC signaling pathways when both are active. Ideally, the AC pathway should be investigated in the absence of the $P2Y_2$ pathways. That situation is difficult to achieve in adult type II cells, as there are no selective $P2Y_2$ antagonists available at present. However, type II cells from newborn animals provide a model in which the $P2Y_2$ receptor is not functional. UTP does not stimulate surfactant secretion, and neither ATP or UTP stimulate DAG formation or PLD activation in type II cells isolated from 1-2 day old rats.[67] On the other, ATP does stimulate surfactant secretion, albeit to a lesser extent than in adult type II cells, and also promotes cAMP formation.[67] Therefore, the AC-coupled signaling mechanism is functional in the newborn cells, whereas the $P2Y_2$ receptor-coupled mechanisms are not. Consequently, type II cells from 1-2 day old rats provide a natural model in which the AC-coupled ATP signaling mechanism can be investigated in the absence of interactions with the $P2Y_2$ receptor pathway.

Newborn type II cells were, therefore, employed to examine the AC-coupled ATP signaling mechanism of surfactant secretion.[67] Inclusion of sufficient adenosine deaminase in the culture medium to completely abolish the stimulatory effect of adenosine on surfactant secretion has no effect on that of the same concentration of ATP. Similarly, a combination of adenosine deaminase and 5'-nucleotidase completely blocks the stimulatory effect of AMP on secretion but does not significantly decrease that of ATP. The stimulatory effect of ATP on surfactant secretion in adult rat type II cells is similarly unaffected by adenosine deaminase[88] or AMP deaminase.[89] Such data establish that the effect of ATP on cAMP formation is not mediated by its degradation to metabolites that directly activate the adenosine A_{2B} receptor.

Subsequent experiments suggested that ATP and adenosine A_{2B} receptor agonists activate the same receptor on type II cells from newborn rats.[67] First, the stimulatory effects of ATP and NECA on surfactant secretion and cAMP formation are not additive. Second, several adenosine A_{2B} receptor antagonists decrease the stimulatory effects of both ATP and NECA on surfactant secretion. Methylxanthine adenosine receptor antagonists also decrease the stimulatory effects of ATP on PC secretion and cAMP formation in adult rat type II cells.[61,64,89] Third, desensitization of the A_{2B} receptor results in a marked decrease in the stimulatory effects of NECA, adenosine and ATP. In those experiments, type II cells from 1-2 day old newborn rats were cultured overnight with NECA, washed with fresh medium and then incubated with NECA, adenosine, ATP or terbutaline. cAMP formation in response to NECA, adenosine and ATP is drastically reduced in the cells incubated overnight with NECA compared to cells similarly incubated without it. In contrast, the stimulatory effect of terbutaline on cAMP formation is not affected by the prolonged exposure to NECA. Similarly, prolonged exposure to ATP virtually abolishes the subsequent stimulatory effects of NECA, adenosine and ATP on cAMP formation.

As there is substantial evidence that it is the adenosine A_{2B} receptor that mediates the stimulatory effects of adenosine, NECA and other adenosine analogs on surfactant secretion,[60,67,86] the above data lead to the conclusion that ATP also activates the A_{2B} receptor. Rat A_{2B} receptors have been cloned and expressed in Chinese hamster ovary (CHO) cells that normally do not contain an A_{2B} receptor.[122,123] The ability of ATP to stimulate cAMP formation in the transfected CHO cells was therefore examined. Although adenosine and NECA produce a more than 100-fold increase in cAMP formation in the transfected cells compared to control CHO cells, ATP produces only a small increase (2-fold at best), and that is significantly decreased by adenosine deaminase (Xu ZX and Rooney SA, unpublished). It is,

therefore, clear that ATP does not activate the adenosine A_{2B} receptor. This suggests that ATP activates a separate AC-coupled receptor (Fig. 7.3).

AC-coupled ATP receptors have also been described in other systems. An AC-coupled receptor activated by NECA, adenosine and ATP was reported in *Xenopus laevis* follicular oocytes and considered to be a novel P1 purinoceptor subtype.[124] It differs from the AC-coupled ATP receptor on the type II cell, however, as it is activated by CGS-21680, an adenosine A_{2A} receptor agonist[92] that has little effect on surfactant secretion.[86] A receptor in a neuroblastoma-glioma hybrid cell line is similar to the AC-coupled ATP receptor on the type II cell in that it is activated by ATP and adenosine but not by UTP, effects of ATP and adenosine are not additive, adenosine receptor antagonists block the effect of ATP, and prolonged exposure to NECA markedly decreases the subsequent response to NECA, adenosine and ATP.[125] AC-coupled ATP receptors have also been reported in a number of other systems.[126-132] Although it has often been assumed that increased cAMP formation in response to ATP is mediated by adenosine resulting from ATP degradation,[118,119] that possibility was clearly eliminated in most of the above studies,[124-131] as well as in type II cells.[67] As in type II cells[67] and brain cells,[125] cAMP formation in response to ATP in aortic endothelial cells[126] and thyroid cells[128] is antagonized by methylxanthines. On the other hand, the AC-coupled ATP receptor in kidney epithelial cells is activated by UTP but not by adenosine,[129] whereas adenosine and ATP activate different AC-coupled receptors in myoblasts[127] and vascular smooth muscle cells.[130] ATP stimulation of cAMP formation in myoblasts is antagonized by suramin,[127] a P2 antagonist[92] that has little effect on ATP stimulated surfactant secretion in type II cells.[133] Therefore, it is possible that there are a number of different AC-coupled ATP receptors. In several studies the effect of ATP on cAMP formation was postulated to be mediated by a novel P2 receptor.[125-127,130-132,134]

In summary, it is clear that ATP stimulation of surfactant secretion is mediated by an AC-coupled receptor in addition to the $P2Y_2$ receptor (Fig. 7.3). Molecular cloning will be necessary to elucidate the precise identity of the AC-coupled ATP receptor on the type II cell and to determine if it is the same as those described in other systems.

Other Mechanisms

The above data show that surfactant secretion in type II cells is mediated by at least three signal transduction pathways (Fig. 7.3). However, the possibility of additional signaling mechanisms cannot be excluded. In addition to PLCβ and PLD, other phospholipases may also be involved in signaling mechanisms that regulate surfactant secretion. TPA was reported to generate formation of lyso-PC in type II cells, suggesting activation of phospholipase A_2.[103] P2 receptors have also been reported to activate phospholipase A_2 in other systems,[119] including airway epithelium.[135]

Based on the observation that ATP stimulated formation of choline phosphate in type II cells, it was concluded that activation of PC-PLC was quantitatively more important than that of PLD in the generation of DAGs.[32] In other studies on type II cells, however, ATP was reported to stimulate release of choline but not of choline phosphate.[87,103]

The stimulatory effects of terbutaline, NECA, ATP, TPA, dioctanoylglycerol and ionomycin on surfactant secretion in type II cells are drastically reduced by Ro-318220,[136] a purported specific inhibitor of PKC.[137,138] Furthermore, the concentrations of Ro-318220 that decrease the effects of terbutaline, NECA, TPA and ionomycin by 50% (IC_{50} values) are similar,[136] suggesting that Ro-318220 antagonizes a common step in the AC, PKC and calcium signaling pathways of surfactant secretion. Such a step is likely to be distal to the reactions depicted in Figure 7.3. Ro-318220 inhibits expression of mitogen activated protein (MAP) kinase phosphatase-1[139] and the activity of MAP kinase activated protein kinase;[140] in a recent preliminary communication, components of the MAP kinase signaling pathway

were reported to be activated by TPA and ATP in type II cells.[108] The MAP kinase signaling cascade is also activated by ATP and UTP in rat kidney cells[141] and in a human endothelial cell line,[142] and in the latter system MAP kinase activation is inhibited by Ro-318220. It remains to be established if components of the MAP kinase signaling pathway or other kinases are inhibited by Ro-318220 in type II cells and if inhibition of such a step accounts for the Ro-318220 inhibition of agonist-stimulated surfactant secretion.[136]

Distal Steps in Surfactant Secretion

Downstream steps in surfactant secretion have not been extensively investigated. As shown in Figure 7.3, all of the signal transduction pathways result in activation of specific protein kinases and phosphorylation of proteins. However, the steps distal to protein kinase activation that ultimately lead to fusion of the lamellar body membrane with the plasma membrane of the type II cell and exocytosis of surfactant have not been elucidated.

Although protein kinases and protein phosphorylation occupy key positions in cell signaling mechanisms,[143] little is known about the identity of the proteins that are phosphorylated by the protein kinases activated by surfactant secretagogues. It is not known if the same or different proteins are phosphorylated by PKA, PKC and CaCM-PK in type II cells. Many of the synergistic effects observed to occur between surfactant secretagogues[62,120] could be due to interactions at the level of protein phosphorylation. It is possible, for instance, that phosphorylation of a protein by one agonist facilitates subsequent phosphorylation by a second.

Activation of PKA in response to cAMP[144,145] and terbutaline[146] promotes phosphorylation of a number of proteins in the type II cell. In one study, actin (43 kDa) was identified as the major PKA substrate in type II cell cytosol,[145] although cAMP promoted the phosphorylation of three additional proteins, 260 kDa, 240 kDa and 22 kDa.[144] In another study, terbutaline was reported to promote the phosphorylation of 47 kDa and 112 kDa proteins, but not of actin.[146] Inclusion of TPA in the culture medium was reported to promote phosphorylation of three proteins, 50 kDa pI 5.8, 50 kDa pI 5.7 and 25 kDa, in the type II cell, and addition of the protein phosphatase inhibitor okadaic acid together with TPA resulted in phosphorylation of additional proteins.[147] With the exception of actin,[145] the type II cell proteins that are phosphorylated in response to surfactant secretagogues have not been identified.

Limited proteolysis of PKA in response to calpain, Ca^{2+}-activated neutral protease, was suggested to prolong protein phosphorylation and thus sustain surfactant secretion in response to agonists acting via the PKA signaling mechanism.[146] A role for calpain in surfactant secretion is further suggested by the findings that A23187, TPA and terbutaline activate endogenous calpain in type II cells,[148] and that a calpain inhibitor antagonizes the effects of those secretagogues.[149]

The steps beyond protein phosphorylation that ultimately result in exocytosis of lamellar bodies are also largely unknown. Involvement of cytoskeletal elements is suggested by the findings that colchicine and vinblastine, agents that disrupt microtubules, inhibit surfactant secretion in intact lungs,[150] lung slices[55,151] and isolated type II cells.[152-154] Actin is generally involved in secretion[155] and, in agreement with the finding that actin phosphorylation is promoted by cAMP,[145] a number of studies suggest a role for actin filaments in surfactant secretion.[55,154,156-159] In an elegant morphological study, lamellar bodies in the process of exocytosis at the apical surface of the type II cell were reported to be surrounded by actin filaments, whereas actin was less frequently associated with lamellar bodies at the basal surface of the cell.[156] In the same study, the amount of actin in the type II cell was increased by isoproterenol, a β-agonist that also promotes lamellar body exocytosis.[157] Cytochalasin D, an agent known to disrupt actin filaments, decreased lamellar body exocytosis

as measured morphologically[156] and abolished the stimulatory effect of isoproterenol.[157] Cytochalasin B was also reported to inhibit surfactant secretion when measured biochemically in lung slices.[55] Such data suggest that intact microfilaments are necessary for surfactant secretion. In other studies, however, cytochalasins stimulated surfactant secretion,[154,158] suggesting that disruption of actin filaments promotes secretion. Some of these discrepancies may be due to differences in cytochalasin concentrations. A delicate balance between actin polymerization and depolymerization is known to be essential for secretion, and disruption of actin filaments also has both stimulatory and inhibitory effects on secretion in other systems.[155] Two surfactant secretagogues, terbutaline and TPA, were recently reported to promote actin depolymerization in type II cells,[159] a finding consistent with the notion that disruption of microfilaments is associated with secretion.[155]

Fusion of the limiting membrane of the lamellar body with the plasma membrane of the type II cell is obviously a prerequisite for surfactant exocytosis. Studies have been designed to identify proteins involved in such membrane fusion. Annexins are a group of proteins that bind phospholipids in a Ca^{2+}-dependent manner and that are implicated in membrane fusion and exocytosis in a number of systems.[160,161] Several annexins are expressed in type II cells[162-164] and there is evidence that annexins may have a role in surfactant secretion.

Synexin (annexin VII) promotes fusion of lamellar bodies and plasma membranes in vitro.[165,166] A physiological role for synexin in surfactant secretion is suggested by the findings that stilbene disulfonates antagonize the increase in surfactant secretion in response to alkalosis, ATP and terbutaline in type II cells[167] and also inhibit synexin-dependent membrane aggregation and fusion.[168] Another annexin, annexin II, also promotes fusion of lamellar bodies with liposomes at physiological concentrations of calcium, an effect similarly antagonized by a stilbene disulfonate.[169] In addition, annexin II enhances surfactant secretion in response to Ca^{2+} in permeabilized type II cells that are depleted of the normal complement of annexins.[163] Finally, although there is no evidence that it has a role in surfactant secretion, annexin IV binds to both lamellar bodies and SP-A in type II cells.[162]

A combination of genetic studies in yeast and biochemical studies on mammalian neurosecretory cells has led to a generalized paradigm of the final steps in exocytosis.[155,161,170] In that model the cytoplasmic proteins, N-ethylmaleimide sensitive fusion protein (NSF) and soluble NSF attachment proteins (SNAPs), form a complex with SNAP receptors (SNAREs). The SNARE hypothesis states that in the process of vesicle docking, proteins on the vesicle (v-SNAREs) bind to their counterparts on the target membrane (t-SNAREs). The NSF-SNAP-SNARE fusion complex disassembles after ATP hydrolysis, mediated by the ATPase NSF, in a process that results in membrane fusion and release of vesicle contents. Numerous proteins are known to be involved in the SNARE mechanism of exocytosis[161,171] and several were recently reported to be expressed in the type II cell.[172] In a preliminary communication, expression of NSF, SNAP and SNAREs was reported in intact type II cells while the t-SNARE syntaxin and the v-SNARE synaptobrevin were identified in plasma membrane and lamellar body fractions, respectively.[172] These data are consistent with the involvement of the SNARE mechanism in surfactant secretion. Whether annexin-mediated secretion is part of the SNARE mechanism in type II cells or a separate secretory pathway remains to be established. In other systems, however, annexins have been integrated into the SNARE exocytosis model, although their precise role has not been established.[161,171]

An increase in intracellular Ca^{2+} is often the trigger of exocytosis and has been postulated to be involved both in the SNARE model[155,170] and in annexin-mediated effects.[160] Whether an increase in Ca^{2+} is obligatory for the initiation of exocytosis, however, is less clear. A number of surfactant secretagogues clearly promote an increase in Ca^{2+} levels in the type II cell. They include ionophores[20,121,173] and ATP.[30,65] Some surfactant secretagogues,

however, do not appear to promote an increase in Ca^{2+}. There are data that suggest that a minimum level of Ca^{2+} may be necessary for surfactant secretion,[173] but the evidence that β-agonists increase Ca^{2+} in the type II cell is not impressive and there is no evidence that TPA or diacylglycerols increase Ca^{2+} levels.[121,173] Clearly much remains to be learned regarding distal steps in surfactant secretion and particularly how annexins and the SNARE paradigm can be integrated into a generalized model of surfactant exocytosis.

Physiological Regulation of Surfactant Secretion

The fact that surfactant secretion can be stimulated by many different agonists suggests that its physiological regulation may be a highly redundant process. It is likely that secretion is a critical factor in overall surfactant homeostasis and that such redundancy assures that surfactant secretion is not disrupted. There is no known pathological condition attributable to a defect in surfactant secretion. The insufficiency in surfactant that occurs in premature infants and that can lead to the respiratory distress syndrome (RDS) appears to be largely due to lung immaturity and insufficient surfactant synthesis. There is no evidence that a defect in surfactant secretion contributes to RDS.

How is surfactant secretion regulated in vivo? A number of agonists stimulate surfactant secretion in vivo. They include β-agonists,[56,174] cholinergic agonists[56,175,176] and adenosine.[177] However, the mere fact that an agent has an effect in vivo does not establish that it has a physiological role. Blockade of a physiological response, on the other hand, can yield information on the pharmacological mechanism involved. At least two physiological factors, ventilation and labor, are know to stimulate surfactant secretion in vivo.

Ventilation stimulates surfactant secretion in both adult[174,175] and newborn[176,178] animals as determined by the amount of surfactant phospholipid recovered in lung lavage. A morphological study confirmed that the effect of ventilation is on lamellar body exocytosis.[179] Ventilation also stimulates surfactant secretion in the isolated perfused lung.[54] At least part of the effect of ventilation may be due to a direct mechanical effect on the type II cell, as stretch increases surfactant secretion in cells cultured on a stretchable membrane.[180] Labor is another physiological factor that stimulates surfactant secretion. Significantly greater amounts of surfactant phospholipid are recovered in lung lavage from newborn rabbits delivered after labor than from those delivered by cesarean section before the onset of labor at the same gestational age.[181] The stimulatory effect of labor on surfactant secretion is also apparent in lung slices and in that model it was established that the effect was primarily on secretion rather than on synthesis.[182] Labor also increases lamellar body secretion as measured morphologically in the newborn rat.[183]

The stimulatory effects of ventilation[174] and labor[182] are antagonized by β-antagonists. As discussed earlier, β-receptors exist on the type II cell[51,52] and β-agonists stimulate surfactant secretion in primary cultures of type II cells,[3,33,53] isolated perfused lungs[54] and in vivo.[174] Taken together, such data strongly suggest that the β-adrenergic system has a role in the physiological regulation of surfactant secretion.

The effect of ventilation in vivo is also blocked by atropine,[175,176,178] suggesting that a cholinergic mechanism has a role in the physiological regulation of surfactant secretion. However, although cholinergic agonists stimulate surfactant secretion in vivo their effects are blocked by both β-antagonists and adrenalectomy.[56,184] Such data, together with the findings that cholinergic agonists do not stimulate surfactant secretion in isolated perfused lungs,[54] lung slices[55] or isolated type II cells[33,53] led to the conclusion that cholinergic regulation of surfactant secretion is an indirect effect mediated by the adrenal medulla.[56,184] Nevertheless, the cholinergic system may be very important in the physiological regulation of surfactant secretion.

A physiological role for prostaglandins in the regulation of surfactant secretion is suggested by the findings that inhibitors of prostaglandin synthesis antagonize the stimulatory effects of labor[182] and ventilation[174,176] in the rabbit. Such inhibitors also antagonize the normal developmental increase in surfactant secretion in fetal lambs.[185]

Some of the other agents reported to stimulate surfactant secretion in type II cells may also have a physiological role. Indeed, the finding that the ventilation-induced increase in surfactant secretion in the isolated perfused lung is not antagonized by propranolol, atropine or indomethacin[54] suggests that agents other than β-agonists, cholinergic agonists and prostaglandins mediate surfactant secretion. That the ventilation-induced increase in surfactant in lung lavage in the newborn rabbit is decreased by leukotriene antagonists and by a lipoxygenase inhibitor[176] suggests a physiological role for leukotrienes. The possibility that a methylxanthine-sensitive receptor may play a physiological role in surfactant secretion is suggested by the finding that the injection of newborn rabbits with 8-phenyltheophylline significantly attenuates the ventilation-induced increase in surfactant secretion that occurs immediately after birth.[176] Such a receptor could be the adenosine A_{2B} receptor or the AC-coupled ATP receptor. That it may be the A_{2B} receptor is suggested by the finding that infusion of pregnant rabbits with adenosine increased surfactant secretion in the fetuses.[177] On the other hand, injection of newborn rabbits with NECA does not increase the amount of surfactant in lung lavage.[176] It is possible, however, that endogenous levels of adenosine or ATP in the newborn lungs are already increased in response to ventilation, so that the exogenous agonist has no further effect.

There is no information on whether the $P2Y_2$ receptor on the type II cell has a physiological role in the regulation of surfactant secretion. There is good evidence that ATP may be a physiological agonist in other systems, but a physiological role for UTP is less well established.[92] ATP levels were measured in adult rat bronchoalveolar lavage fluid and, based on tissue culture data,[90] were sufficient to stimulate surfactant secretion.[186] However, there was no correlation between ATP concentration and surfactant content as ATP levels were decreased in animals exposed to oxygen, whereas surfactant levels were increased.[186] It is, therefore, not clear if ATP has a physiological role in regulation of surfactant secretion. Because there are no specific $P2Y_2$ antagonists, it is difficult to establish such a role for ATP or UTP by blocking the increase in surfactant secretion induced by ventilation or labor. The stretch-induced increase in surfactant secretion in type II cells was accompanied by an increase in cellular Ca^{2+} levels.[180] A mechanical stimulus was reported to promote ATP release[187] and IP_3 formation[188] in airway epithelial cells. These data raise the possibility that the stimulatory effect of stretch on surfactant secretion is mediated by the type II cell $P2Y_2$ receptor.

Apart from ATP and UTP, the only well established surfactant secretagogues that activate the PKC signaling pathway are nonphysiological agonists such as TPA and cell permeable DAGs, while those that activate the CaCM-PK pathway are the equally unphysiological ionophores ionomycin and A23187. It is highly likely that some physiological agonist activates those signaling mechanisms and it is tempting to speculate that ATP or UTP may be such an agonist. However, at present there is no direct evidence to support a physiological role for $P2Y_2$ receptors in the regulation of surfactant secretion.

The finding that SP-A inhibits surfactant phospholipid secretion[77-79] and promotes its uptake[189] by type II cells in vitro led to the notion that SP-A might have a physiological role in the regulation of extracellular surfactant phospholipid levels. However, the findings that surfactant secretion and uptake appear to be normal in SP-A knockout mice[190] make a physiological role for SP-A in regulation of surfactant metabolism unlikely.

Future Directions

There is considerable information available on agents that stimulate surfactant phospholipid secretion in isolated type II cells and on the signal transduction mechanisms by which their effects are mediated. However, information on the ACs, PKCs and PLCs involved in the signaling process is only beginning to become available and little is known about the identity of other signaling molecules. Much also remains to be learned about distal steps in surfactant secretion, in particular the steps beyond activation of protein kinases. Virtually nothing is known about how the lamellar body membrane fuses with the plasma membrane of the type II cell to ultimately expel surfactant components into the alveolar lumen. The elucidation of such steps and, in particular, the identification of individual proteins involved in the process will be a major goal of future research.

Other aspects of surfactant secretion that need to be elucidated include the mechanisms by which the surfactant proteins are secreted. Is secretion of SP-A entirely constitutive? How is secretion of SP-D effected? Is secretion of the hydrophobic proteins SP-B and SP-C subject to the same regulatory mechanisms as that of surfactant phospholipids?

Finally, although several groups of agonists and at least three signal transduction mechanisms can regulate surfactant secretion in isolated type II cells, relatively little is known about how surfactant secretion is physiologically regulated. There is good evidence that the β-adrenergic system has a physiological role and it is also likely that cholinergic regulation is of physiological importance. However, virtually nothing is known about other physiological mediators. Is the $P2Y_2$ receptor involved in the regulation of surfactant secretion? Does ATP or UTP have a physiological role? What other physiological agonists might activate the PKC and calcium-mediated signaling mechanisms? Are there negative modulators of surfactant secretion? Although the recent data on the SP-A deficient mouse suggests that SP-A is probably not involved in the physiological regulation of surfactant metabolism, other similar proteins such as SP-D and C1q may have such a role.[190] What are the physiological consequences of deleting expression of specific genes involved in surfactant secretion signaling? Is there such redundancy that deletion of one pathway is compensated by activation of others? What are the consequences of deleting genes in more than one signaling pathway? Is survival possible in situations where surfactant secretion is seriously compromised?

It is likely that the elucidation of these and related questions will occupy those interested in surfactant secretion for the foreseeable future.

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CHAPTER 8

Lung Surfactant Clearance and Cellular Processing

Aron B. Fisher

Initial studies following the discovery of lung surfactant focused on its chemical composition and pathways for synthesis and secretion. More recently, there has been increasing exploration of the mechanisms and pathways for removal of surfactant from the alveolar space. Since either a deficit or an excess of alveolar surfactant can be detrimental, the process and mechanisms for surfactant clearance are important for understanding lung function. Furthermore, secretion and removal of surfactant from the alveolar space require coordination in order to prevent significant alterations in the extracellular pool size. As described in chapter 1, surfactant represents a complex mixture of lipids and proteins rather than a uniform product, and extracellular surfactant may exist in multiple forms, including structured tubular myelin, multilamellar or unilamellar liposomes, micelles, molecular aggregates and monomers. It is possible that the various surfactant components and its physical forms are cleared by different mechanisms. While much has been learned in recent years, the precise pathways for clearance, the role of physical form and mechanisms for coordination of secretion and clearance are still not fully understood. This chapter will emphasize the mechanisms for clearance of phosphatidylcholine (PC) and surfactant protein A (SP-A), the major lipid and protein components of surfactant, by the normal adult lung with reference to the neonatal lung where appropriate. Currently, there is little reliable data available on clearance of other components of surfactant or on abnormalities of clearance associated with disease.

Various terms have been used to describe the processes involved in surfactant removal from alveoli. This chapter will use the following definitions: Surfactant clearance represents the disappearance of surfactant (or a surfactant component) from the alveolar space; surfactant reuptake represents removal of surfactant from the alveolar space by the lung epithelium; surfactant recycling indicates resecretion of surfactant lipid or protein components (without degradation) following their reuptake; surfactant reutilization indicates the use of degraded surfactant components for surfactant resynthesis. Recent reviews have emphasized one or more of these processes.[1-6]

Surfactant Turnover

Biological Half Time of Alveolar Lipids

What is the life time of surfactant in the alveolar space following secretion? Many studies to evaluate the turnover of alveolar surfactant lipids have utilized a common experimental design: intravenous (or intraperitoneal) injection of a radiolabeled precursor of PC

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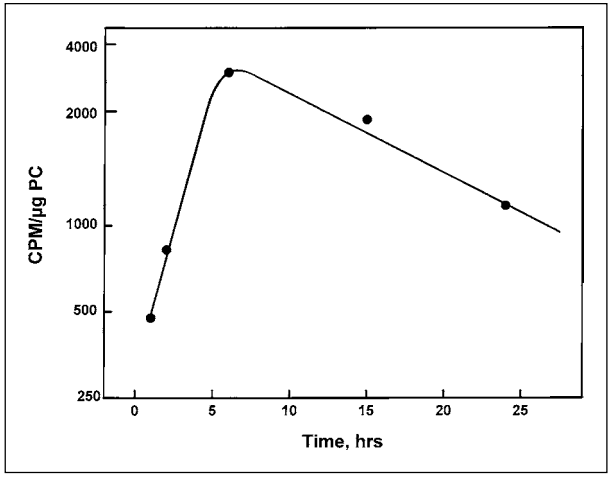

Fig. 8.1. Turnover of lung surfactant as evaluated by PC specific activity in lung lavage. Rats were injected intravenously with radiolabeled palmitate and sacrificed at intervals from 1-24 hours. PC specific activity (cpm per µg PC) is shown on a log scale and plotted vs. time of sacrifice. Increasing specific activity indicates secretion of PC into the alveolar space followed by clearance with a half time of 12 hours. Derived from original data in ref. 8.

followed by sacrifice of animals at varying time points for alveolar lavage to evaluate PC specific activity. The radiolabeled substrates utilized as tracers for PC synthesis are palmitate for the fatty acyl moiety, choline to label the head group, glycerol or glucose for the glyceride backbone and phosphate to label the lipid phosphorus. PC specific activity in the extracellular fraction obtained from the alveolar space reflects the balance between surfactant PC secretion and removal. With this protocol, there is a rapid increase in alveolar PC specific activity that peaks at about 6 hours, followed by a semilogarithmic decline (Fig. 8.1). The biological half time is calculated from the slope of the decreasing specific activity, representing the time required for PC specific activity in the alveolar wash to decrease by 50%. Results obtained by various laboratories and with several species (rats, rabbits, mice) have shown remarkable similarity.[7-15]

A striking observation is that the value for biological half time varies with the PC precursor label (Table 8.1). Based on palmitate and glyceryl labels, the biological half time for surfactant removal is approximately 16 hours. In contrast, the biological half times are consistently prolonged for the choline and phosphate labels in PC. A proper understanding of these differences is clouded by the uncertain kinetics for labeling of the individual precursor pools. The actual reason(s) for the differences is not known and could be trivial. A potentially important explanation for the discrepancy in half times between palmitate/glyceryl and choline/phosphate labels is that the latter are regenerated by PC degradation and are reutilized by the lung cells for resynthesis and resecretion of PC. In contrast to choline (and choline phosphate) which are utilized in the lung primarily for PC synthesis, regenerated palmitate and glycerol would enter the general metabolic pool where they could be metabolized to CO_2 or utilized for synthesis of other complex lipids. Additional evidence has now confirmed the metabolic reutilization of components of PC.

If surfactant components are extensively reutilized, results for biological half time are difficult to interpret in terms of actual rates of surfactant clearance. This issue has been approached through calculations of turnover time using precursor-product analysis, with the assumption that lung lamellar bodies are the precursor of alveolar surfactant (the product). The analysis, based on the relationships proposed by Zilversmit et al,[16] requires measurement of PC specific activity in lamellar bodies and in the lung lavage fraction. Assuming bi-directional flux between the lung tissue and alveolar space, the calculated surfactant PC turnover time in adult rats and rabbits is approximately 4-11 hours.[10,14,17-19] The bio-

Table 8.1. Biological half time in hours of lung surfactant PC in the alveolar space

Species	Radiolabeled PC Precursor			
	Palmitate	Glycerol/Glucose	Choline	Phosphate
Rat[7]	14	14		
Rat[8]	12			
Rat[9]	15		42	45
Rat[10]	16	16	47	
Rabbit[11,12]	17	16	35	61
Rabbit[13]	18			
Rabbit[14]	20		45	90
Mouse[15]		16		
Mean	*16*	*16*	*42*	*65*

Values were calculated from a semilogarithmic plot of PC specific activity in material obtained by lung lavage vs. time after intravenous injection of radiolabeled precursor.

logical half time in the absence of recycling will equal turnover time multiplied by 0.69 (i.e., ln 0.5); thus, the turnover time required to give a biological half time of 16 hours for palmitate would be approximately 22 hours. This is more than twice the turnover time estimated with the Zilversmit equations. This discrepancy is compatible with extensive recycling and/or reutilization of palmitate-labeled PC. However, precise quantitation of turnover time is complicated by difficulties in isolating pure fractions (surfactant; lamellar bodies) and their compositional heterogeneity.[20] In any event, the primary data for surfactant PC clearance indicate rapid turnover with replacement of approximately 5-10% of alveolar PC per hour.

Models for the Study of Surfactant Clearance

The primary experimental protocol used to evaluate of the clearance of lung surfactant in the intact lung has been to measure the disappearance of label from alveoli and/or its appearance in lung tissue at intervals following endotracheal instillation of radiolabeled lipid or protein. Anesthetized animals and the isolated perfused lung preparation have both proven useful as models. Each has its advantages, and the two approaches have resulted in complementary data. Distribution of lipid following its instillation is an important issue for the interpretation of results. Fortunately (both for study and therapy), surfactant lipids instilled into the trachea rapidly distribute throughout all lung regions although there is heterogeneity at both macro- and microscopic levels.[21-25] The precise effects on clearance of this sort of heterogeneity have not been determined. Another unexplored factor is the extent and type of association between instilled and endogenous alveolar lipids and the consequent effects on clearance.

Cell culture systems also have been utilized to study surfactant clearance. Availability of isolated cells presents an opportunity to evaluate mechanisms and pathways, although they obviously cannot reproduce the kinetics of the intact lung. Unfortunately, no cultured cell line appears to represent a satisfactory model for these investigations so that primary cell cultures have been necessary. Cuboidal alveolar epithelial cells (type II cells) isolated from rat or rabbit lungs by enzymatic digestion and maintained in primary culture retain the capacity to accumulate phospholipids from the incubation medium. However, phenotypic expression in these primary cell cultures is relatively unstable, and rigorous consideration of culture conditions is required for interpretation of uptake data. The cell culture

matrix appears to be important in maintaining the differentiated phenotype; as an example, cells cultured on microporous membranes show significantly greater rates of dipalmitoyl PC (DPPC) and SP-A internalization and SP-A receptor density as compared to cells cultured on plastic supports.[26,27] Different coatings for plastic supports, including collagen, polylysine and Engelbreth-Holm-Swarm tumor basement membrane, have significant effects on intracellular trafficking patterns by type II cells,[28-30] indicating the need for utmost caution in the interpretation of results. Sadly, there seems to be no ideal system at present for adequately maintaining the differentiated type II cell phenotype in long term culture.

Another important issue for surfactant clearance studies is the choice of substrate. Radiolabeled natural surfactant can be prepared in a donor animal by addition of an appropriate precursor to the perfusion medium of an isolated lung[31-33] or by intravenous injection[20,34,35] or endotracheal instillation[36] in an intact animal. While clearly the most physiological substrate, the labor intensity involved in the preparation of labeled natural surfactant represents a major drawback. Furthermore, important sub-forms may be lost during the surfactant isolation procedure and alteration of the natural product may occur with storage and reconstitution. Radiolabeled natural surfactant also has been generated by its coincubation with radiolabeled liposomes.[37] The surfactant and added lipids associate and the product generally appears to behave similarly to endogenously labeled material,[34] although subtle differences could exist and would be difficult to corroborate experimentally.

Synthetic lipids represent an alternative and readily available substrate for study of surfactant clearance. Because of their hydrophobicity, these cannot be presented as a simple solution, but rather are presented as liposomes prepared from mixtures of synthetic or natural lipids. Unilamellar liposomes of relatively uniform size and defined composition can be generated reproducibly, although this approach does not reproduce the precise physical forms present in the alveolar space. Since the phase transition temperature for DPPC is above physiologic temperature, it must be mixed with other lipids in order to generate a fluid liposome at 37°C. The composition of lipids affects the fluidity of the mixture and undoubtedly will influence interactions with cell membranes. Composition and method of preparation can also have a variety of other effects. For example, multilamellar liposomes appear to be poorly internalized by cells, possibly because of their bulk.[38] As another example, the presence of phosphatidylglycerol (PG) in the liposome increases cellular uptake, presumably related to its negative charge.[21,39] To standardize conditions, our laboratory has utilized negatively charged unilamellar liposomes of uniform size (approximately 100 nm in diameter) prepared from a lipid mixture that approximates the composition of lung surfactant: 50% DPPC, 25% mixed PC, 10% PG and 15% cholesterol.

Clearance of Alveolar Lipids

Clearance of DPPC from Lung Alveoli

Clearance of surfactant lipids from the alveoli has been studied following endotracheal instillation of radiolabeled natural surfactant or liposomes. Administration of exogenous surfactant augments the alveolar pool so that subsequent clearance represents a combination of normal turnover plus any regulatory mechanisms designed to restore the normal alveolar surfactant pool size. For example, endotracheal instillation of 0.5 mg DPPC in a 250 g rat will approximately double the alveolar DPPC content.

Lung lavage of animals sacrificed at intervals has demonstrated a rapid decrease in lipid label in the alveolar space with a corresponding increase in lung tissue-associated label that occurs within minutes following endotracheal instillation (Fig. 8.2). The recovery of labeled lipid in the lung tissue at this initial time point is not decreased by adding mild

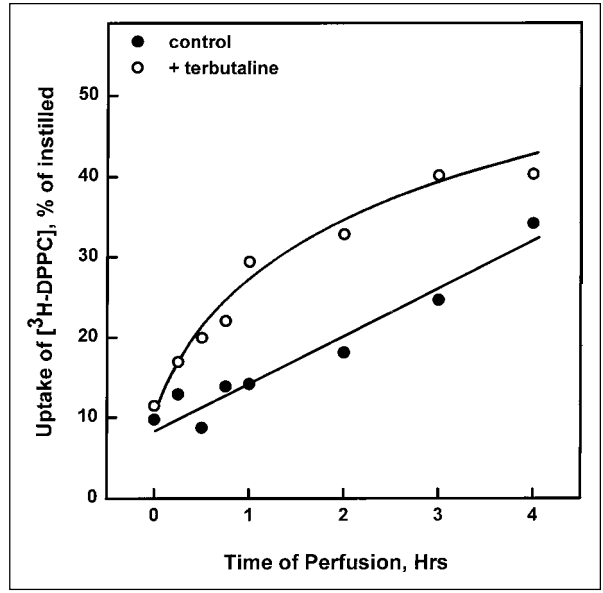

Fig. 8.2. Uptake of ^{3}H-DPPC in mixed unilamellar liposomes from the alveolar space by the isolated perfused rat lung under control conditions and with addition of 10^{-4} M terbutaline to the perfusate. Zero time represents lung uptake 5 minutes after endotracheal instillation of liposomes in the anesthetized rat. Compiled from original data in refs. 23 and 41.

detergents or proteolytic enzymes to the lavage fluid, indicating that the instilled lipid has become cell-associated.[23] Radioautography of rat lungs fixed at one minute following aerosolization of ^{3}H-DPPC has confirmed its rapid cell association.[40] This very rapid uptake may represent, in large part, the tight binding or fusion of lipids with epithelial cell membranes. Following endotracheal administration of liposomes (1 μmol PC), approximately 10% of label is incorporated into the tissue within five minutes after instillation; with labeled natural surfactant, the rapid association of label with tissue is approximately three times greater, suggesting an important effect of lipid physical form, the presence of surfactant-associated proteins or other undetermined factors.[23]

Following the initial rapid phase, there is a progressive decrease in label recovered by lung lavage and a corresponding linear accumulation of radiolabel in the lung. Studies with the isolated perfused rat lung show clearance at about 5% per hour for PC in liposomes (Fig. 8.2) with slightly greater values for clearance of natural surfactant.[23] The addition of terbutaline to the perfusate significantly stimulates uptake,[41] as shown in Figure 8.2; the effect of this and other secretagogues is discussed in greater detail below. Values for clearance measured in vivo for various species are similar: Uptake in rabbits is 4-8% of instilled dpm per hour[13,21] and in mice about 3-5%.[15,42] Thus, these experiments with endotracheally instilled natural surfactant or lipids confirm the rapid turnover of alveolar surface active material.

Using increasing concentrations of instilled liposomes under conditions of uptake stimulated by the presence of terbutaline, the rate of clearance of DPPC instilled into the alveolar space is saturable in the perfused rat lung model.[41] In this system, the stimulated maximal uptake rate of about 0.35 μmol PC per hour is reached at about 1 μmol of instilled PC. Delayed clearance of very large amounts (3 times the endogenous pool) of instilled surfactant from alveoli of neonatal rabbits is compatible with a saturable process.[24] Saturation suggests the presence of specific mechanisms for the uptake of surfactant lipids.

Pathways for Surfactant Lipid Clearance

Theoretically, there are five possible pathways for clearance of surfactant from the alveolar space. These are:

1. transport up the mucociliary escalator;
2. pericellular transport (or transcytosis) into the pulmonary interstitium for removal by lymphatics or capillary blood;
3. degradation in situ by extracellular enzymes;
4. uptake and degradation by alveolar macrophages; and
5. reuptake by the alveolar epithelium.

The possible role of tracheobronchial clearance mechanisms (i.e., transport up the mucociliary escalator) was studied by collecting gastric contents in rabbits following endotracheal instillation of labeled surfactant or following intravenous injection of labeled surfactant precursors.[43] Only 3-7% of total labeled surfactant is recovered in the gastric contents after 24 hours, indicating that this pathway plays a minor role in lung surfactant clearance. Possible clearance of surfactant from the alveoli by pericellular mechanisms (i.e., transcytosis into the lung interstitium) for removal by lymphatic drainage or pulmonary blood flow was evaluated by collecting lung lymph in a chronic sheep preparation following intravenous injection of labeled palmitate.[44] Lipid label recovered in lung lymph during 100 hours is only 4% of the activity in lung tissue and does not follow the pattern of disaturated PC in alveolar surfactant. Thus, lymphatic clearance does not play a role in clearance of alveolar surfactant PC. A study of lymph and blood clearance following endotracheal instillation of labeled DPPC reached a similar conclusion.[45] With respect to possible in situ degradation, several studies have shown the presence of phospholipase activity in lung lavage fluid. However, activity of these enzymes is low even when assayed in vitro under optimal conditions.[46-49] Degradation products are not detected in the lung lavage fluid after endotracheal instillation of lipid.[47,50] DPPC analogs (D-isomer of DPPC and L-α-DPPC ether) that are resistant to degradation by the major phospholipase (phospholipase A_2, PLA_2) are cleared to the same extent as DPPC.[21,51] Finally, clearance in the perfused rat lung is unchanged by the presence of a phospholipase A_2 inhibitor.[52] These results indicate that DPPC clearance does not depend on its intra-alveolar degradation.

The ability of alveolar macrophages to ingest and degrade surfactant phospholipids has been shown clearly by electron microscopic examination of lung tissue[53] and by in vitro studies with alveolar macrophages obtained by lung lavage.[54-57] However, the distribution of alveolar macrophages in the lung implies that a major role in the normal clearance of surfactant is unlikely. For example, the normal rat lung contains approximately 28×10^6 alveoli and 23×10^6 alveolar macrophages.[58] Since macrophages are not evenly distributed throughout the gas exchange region, it appears that they are not reasonably located in order to maintain a relatively constant surfactant concentration in individual alveoli and to promote rapid surfactant turnover. For reference, the normal rat lung has approximately 126×10^6 type II epithelial cells (granular pneumocytes), which are relatively evenly distributed throughout the alveolar region.[58] In vivo studies with lipids instilled into the lung have confirmed that the fraction of alveolar surfactant PC removed by alveolar macrophages is relatively small. At 2 hours after treatment of rats with nebulized labeled DPPC, autoradiography showed that three times more type II cells than alveolar macrophages contain significant radiolabel.[40] At 8 hours after instillation of radiolabeled surfactant into rabbit lungs, only 8% is recovered in alveolar macrophages, compared to 52% present in the postlavage lung tissue.[31] As a confounding variable, alveolar macrophages could rapidly catabolize ingested surfactant and release the radiolabeled products into the extracellular space, which would result in an underestimate of their role. With isolated alveolar macrophages, degradation of internalized DPPC is about 50% during a 2 hour incubation,[57]

suggesting that the total macrophage contribution to clearance is somewhat greater than predicted from analysis of the cellular contents. Using a nonmetabolizable DPPC analog, the clearance of label by alveolar macrophages from the alveolar space of rabbits is about 20% of the instilled lipid.[59] This would appear to be the approximate upper limit for normal clearance of lipid by alveolar macrophages.

Thus, tracheobronchial transport, transcytosis with lymphatic (or blood) removal, in situ degradation or uptake by alveolar macrophages appear to contribute relatively little to surfactant clearance from the alveoli. It is now clear that surfactant clearance is primarily by reuptake into the alveolar epithelium.

Uptake of DPPC by Alveolar Epithelium

Considerable evidence has now accumulated to indicate that type II cells are mainly responsible for clearance of surfactant from the alveolar space. The endocytic potential of these cells was originally suggested by Niden,[60] who presented electron microscopic evidence of carbon ingestion by mouse type II cells in situ and proposed their role in surfactant clearance. However, this idea was not readily accepted because the notion that a cell would secrete and remove the same material was considered unlikely. It has since been demonstrated that some cells re-accumulate their secretory products by receptor-mediated endocytosis, and the idea that type II cells may do the same is no longer counterintuitive. An example of secretion followed by receptor-mediated endocytosis in response to a physiological stimulus is the processing of thyroglobulin by the thyroid follicular cell. This macromolecule is secreted into the thyroid acinus, where it forms a storage pool for thyroid hormone. Subsequent receptor-mediated endocytosis of the protein by the thyroid follicular cell results in internalization of the complex for generation of free thyroid hormone. Endocytosis by type II cells has been confirmed by electron microscopic studies which show uptake of cationic ferritin from the alveolar space into an endosomal compartment and subsequent transfer to lamellar bodies.[61] Of note, type II cells seem incapable of phagocytosis (the ingestion of material of large dimension), and the original studies showing uptake of colloidal carbon[60] could not be confirmed.[61,62] This would suggest a preference for smaller lipid forms for uptake by the cells.

Several studies have provided direct experimental evidence for uptake of surfactant by type II cells. Surfactant lipids incubated with rabbit lung slices or instilled into the airway appear in lamellar bodies, the unique type II cell organelle.[13,63] Isolated type II cells in primary culture show time dependent uptake of labeled liposomal PC from the incubation medium.[39,64] Type II cells isolated from rat lungs instilled with labeled DPPC and then perfused in vitro for varying times show the presence of the label.[33] Electron microscopic autoradiography of rat lungs following nebulization or instillation of radiolabeled DPPC show accumulation primarily within type II cells.[25,40] Localization of a lipid PC analog by confocal fluorescence microscopy gives similar results,[65] as shown in Figure 8.3. While both radiolabeled as well as fluorescent lipid can associate with membranous alveolar epithelial (type I) cells at early time points, subsequent accumulation of label in these cells has not been demonstrated. Careful cell fractionation studies during the 24 hours following endotracheal instillation of labeled DPPC in rabbits shows that more than 65% of recovered label is associated with type II cells.[59]

Mechanisms for Cellular Uptake of Lipids

Potential mechanisms for lipid uptake by cells include lipid exchange between cell membranes and extracellular lipids, fusion of liposomes with the cell membrane and endocytosis. The initial rapid association of lipid vesicles with type II cells is sensitive to treatment with trypsin, but the percentage released by trypsin treatment decreases progressively

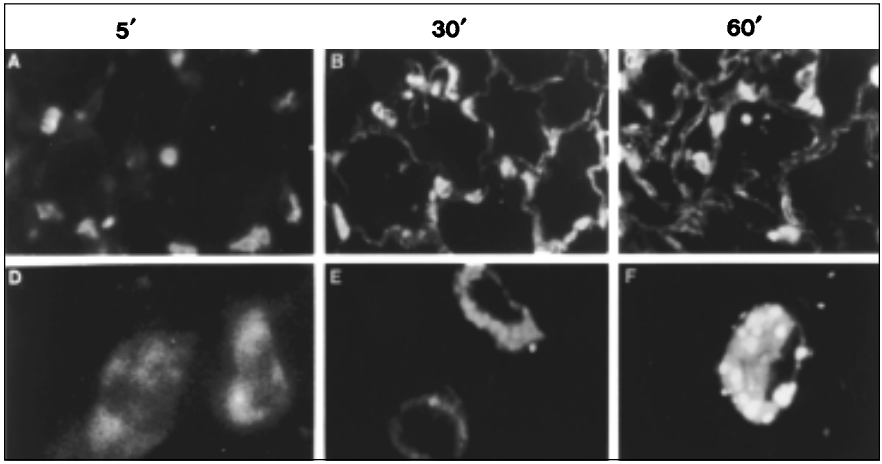

Fig. 8.3 Fluorescence microscopy of rat lungs fixed at intervals (5, 30 and 60 minutes) following endotracheal instillation of mixed unilamellar liposomes labeled with the fluorescent PC analog NBD-PC. Lungs were examined at low (upper panels; A, B, C) and high (lower panels; D, E, F) magnifications. A low level of diffuse fluorescence at the early (5 minute) time point leads to subsequent greater lipid accumulation with primarily discrete fluorescence in type II cells by 60 minutes. Reproduced with permission from Chinoy MR, Fisher AB, Shuman H. Confocal imaging of time-dependent internalization and localization of NBD-PC in intact rat lungs. Am J Physiol 1994; 266:L713-L721.

with time, compatible with their internalization.[39] In addition to the lipid "envelope," cells internalize the enclosed aqueous contents of liposomes.[39] Uptake is inhibited by incubation at 4°C and by metabolic inhibitors that decrease ATP availability.[39,64,66,67] Thus, these results exclude lipid exchange as a significant mechanism and are compatible with liposome binding to the cell membrane, with subsequent endocytosis as the major mechanism for lipid uptake by the cells.

Effect of Surfactant Proteins on Lipid Uptake

Several observations suggest a role for surfactant-associated proteins in lipid endocytosis:

1. with instillation of labeled natural surfactant into rabbit lungs, there is significantly greater uptake of surfactant subfractions containing surfactant proteins;[20]
2. In isolated rat type II cells, labeled lipid in natural surfactant is accumulated at a more rapid rate than synthetic liposomes and metabolic inhibitors have a greater effect on its uptake;[64]
3. Uptake of lipid in natural surfactant by isolated cells is decreased following treatment with a protein reducing agent or with an antibody to SP-A;[64]
4. SP-A added to liposomes significantly stimulates the uptake of liposomal DPPC by isolated type II cells.[27,49,66,68-70]

The degree of stimulation appears to vary greatly depending primarily on the rate of lipid uptake in the absence of SP-A. Increased internalization of lipid by the cells is indicated by insensitivity of cell-associated label to treatment with trypsin, its increased appearance in lamellar bodies and an increased intracellular generation of degradation products (described below).[27,49,70] Stimulation of DPPC uptake has been demonstrated with freshly

isolated type II cells[68,70] and with cells in culture on microporous membranes,[27] but may be more difficult to demonstrate with cells cultured on plastic.[66] This effect correlates with decreased binding of SP-A to type II cells that have been cultured on plastic vs. microporous membrane.[27] The effect of SP-A on lipid uptake is saturable and is not observed with unrelated proteins or with other cell types (fibroblasts), indicating specificity of the effect.[27,68] Specific site directed mutations in SP-A ($glu_{195} \rightarrow$ gln and $arg_{197} \rightarrow$ asp) reverses the effect of SP-A on DPPC uptake by type II cells.[71]

In the isolated rat lung, uptake of natural surfactant radiolabeled in lipid (PC) and protein (primarily SP-A) components after 2 hours of perfusion is similar for the two labels, suggesting that they are internalized as a unit.[72] Since DPPC is known to preferentially bind to SP-A,[49,73] a reasonable conclusion is that the SP-A:DPPC complex is taken up more efficiently by type II cells than is DPPC itself. Supporting evidence is the observation that SP-A is much less effective in promoting uptake of lipids that it binds less well.[70]

Is the SP-A mediated stimulation of DPPC uptake as demonstrated with isolated type II cells physiologically relevant? While SP-A can promote surfactant lipid uptake, it inhibits secretion, at least in isolated cell systems.[71,74] Thus, SP-A is possibly the regulator to fine tune the extracellular pool of surfactant through coordination of secretion and clearance. Although an attractive possibility, knockout mice totally deficient in SP-A have relatively little alteration in the extracellular pool size of surfactant lipids.[42] This could imply upregulation of non-SP-A mediated processes for DPPC uptake or could indicate that the stimulation of uptake noted in isolated cells is an in vitro phenomenon. Thus, the possible role of SP-A in the regulation of the extracellular DPPC pool size remains to be established.

The hydrophobic surfactant proteins, surfactant protein B (SP-B) and surfactant protein C (SP-C), may also modify lipid uptake by type II cells, although evidence for a physiological role is less compelling. Liposome uptake by isolated cells was stimulated by a hydrophobic protein[75] (later determined to be SP-C) but subsequently it was demonstrated that lipids coisolating with the protein could account for the effect.[76] Cellular uptake of lipid is stimulated by synthetic SP-B and SP-C peptides, but these effects are not saturable and are not type II cell specific.[69] These effects of hydrophobic peptides may be due to their interaction with lipids, with a resultant change in physical form as the mechanism for increased uptake by isolated cells. For example, a shift to smaller forms could account for the increased uptake of multilamellar liposomes in the presence of SP-B or SP-C.[38] SP-C appears to be more effective, as predicted from its greater hydrophobicity.

Mechanism for DPPC Association with Type II Cells

Although type II cells make up only about 5% of the cells lining the alveolar surface, they are responsible for the majority of DPPC uptake. Thus, specific mechanisms must exist to promote the role of type II cells in the clearance of DPPC. A reasonable hypothesis is that the type II cell membrane possesses specific recognition sites for the lipid or its ligands. By contrast, the type I epithelial cell, which constitutes the bulk of the alveolar surface, appears to be unable to endocytose DPPC. Although association of DPPC with type I epithelium has been demonstrated in situ, this is limited to the initial time observed and most likely represents lipid exchange or fusion with the cell membrane rather than lipid endocytosis.[25,40]

Since DPPC can specifically bind to SP-A, specificity for lipid uptake by type II cells could be mediated through cellular recognition of the lipid:protein complex. While there is still debate concerning its precise identification, it is now clear that type II cell membranes possess a protein(s) with SP-A receptor-like activity. In the presence of SP-A, binding of liposomes to plasma membranes isolated from type II cells increases more than 4-fold.[77] Specificity, at least in part, is evident, since the association of SP-A with type II cell plasma

membranes is significantly greater than that observed with plasma membranes from liver. Currently, three candidate receptor proteins have been described with molecular masses of about 30 kDa,[78] 55 kDa[79] and 210 kDa[80] based on gel electrophoresis under reducing conditions. An anti-idiotypic antibody to the 55 kDa "receptor" protein inhibits both binding of SP-A and SP-A mediated lipid uptake by isolated type II cells.[81] Site specific SP-A mutations result in loss of both the SP-A effect on DPPC uptake by type II cells and the ability to compete with labeled SP-A for binding to the cell membrane.[71] Thus, the cell membrane SP-A receptor potentially confers specificity for DPPC uptake by type II cells through cellular recognition of the SP-A:lipid complex.

Receptor-mediated endocytosis is a well-recognized mechanism for internalization of ligands and occurs predominantly through receptors clustered on the cell membrane at sites of clathrin-coated pits.[82] These receptors, with or without the ligand, continuously recycle between the membrane-associated coated pit and internal coated vesicles. Electron microscopy using immunogold techniques has demonstrated that SP-A binding is clustered in coated pits of type II cells.[28] These observations suggest a role for clathrin-mediated uptake of SP-A and, by inference, the SP-A mediated uptake of DPPC.

While SP-A stimulates uptake of DPPC by type II cells, these cells also endocytose lipid vesicles in the absence of added SP-A.[26,39,64,66,67] This may be important physiologically, since the molar ratio of DPPC to SP-A in the alveolar space is approximately 10,000 (the stoichiometry of DPPC binding to SP-A is not known). Uptake of DPPC by isolated cells is inhibited in part by cytochalasin D,[66,67] suggesting that internalization of lipid is associated with actin-dependent cell membrane retrieval. Thus, the type II cell membrane may have a specialized affinity or high affinity binding sites for DPPC unrelated to SP-A receptors. One possibility is that these specialized binding sites represent patches of lamellar body membrane inserted into the type II cell plasma membrane (Shuman H, Zen K, personal communication), but the precise mechanism is not yet known.

Coordination of Secretion and Clearance

Is clearance of surfactant from the alveolar space regulated, i.e., under physiological control, or is it cleared at some constitutive rate which could vary with the alveolar pool size? Intuitively, it would seem likely that the extracellular pool size of a substance as crucial as lung surfactant would be tightly controlled. If the pool size is regulated, then secretion and clearance should be physiologically linked. Several lines of evidence have provided evidence for physiological regulation of surfactant clearance and its linkage to secretion, although the precise mechanisms are not yet known.

Increased lung ventilation is a potent stimulus for surfactant secretion (see chapter 7) and also enhances the rate of surfactant clearance. Alveolar clearance of lipid instilled in the trachea of anesthetized rabbits increases by 70% (to 13.3% of instilled PC cleared per hour) when ventilation doubles consequent to added external dead space.[21] Vigorous exercise in rats (swimming) results in an increase in alveolar surfactant pool size, possibly related to hyperventilation, that returns to control level within 3 hours following cessation of the exercise,[83] indicating balance between secretion and clearance pathways. On the other hand, instillation of large amounts of surfactant into lungs has no apparent gross effect on the rate of surfactant lipid secretion.[24,84] Thus, endogenous signals related to secretion may be important for its coordination with uptake.

Agonists for β-adrenergic, protein kinase C (PKC) and purinergic systems are potent secretagogues for surfactant (see chapter 7) and have been shown to also increase the rate of alveolar lipid clearance. In the isolated perfused rat lung, addition to the perfusate of terbutaline, 8-bromo cyclic AMP (cAMP), 12-O-tetradecanoylphorbol-13-acetate (TPA) or ATP increases the rate of alveolar DPPC clearance by the lung.[41] Uptake is approximately

5% of instilled label per hour under control conditions and approximately doubles in the presence of any one of the secretagogues. A similar effect is observed by addition of a β-adrenergic agonist (metaproterenol) to radiolabeled surfactant instilled endotracheally in intact rabbits.[85] Secretagogues also stimulate uptake of lipid in isolated type II cells in primary culture on microporous membranes, although not in cells cultured on plastic.[26,66] Since the secretory response to secretagogues is maintained under both culture conditions, the linkage between secretion and reuptake of DPPC would appear to be an indirect rather than a direct coupling.

A possible mechanism for the secretagogue-mediated increase in lipid clearance is suggested by studies of the SP-A receptor. Exposure of type II cells to agonists (terbutaline, cAMP analog, TPA and ATP) results in increased SP-A binding to the cell membrane compatible with increased receptor expression.[86] Increased SP-A receptor expression on the cell surface could promote increased reuptake of DPPC through its binding to SP-A, as described above. Stimulation of uptake by secretagogues also occurs in the absence of SP-A,[26] so that an effect of these agonists on the SP-A receptor-independent pathway of endocytosis is likely. This effect could be mediated through stimulation of the rate of plasma membrane internalization.

Clearance of Other Lipids

Compared to DPPC, only a smattering of studies related to clearance of other lipids have been reported. Lung uptake of mixed liposomes containing labeled PG, phosphatidylethanolamine, cholesterol or lyso-PC has been described.[85,87,88] Since uptake of liposomal DPPC is by endocytosis, it follows that any lipid presented in mixed liposomes with DPPC should be internalized by type II cells. Furthermore, liposomes instilled into the alveolar space will mix with endogenous surfactant so that independent uptake of the lipid of interest cannot be assumed. Under these experimental conditions, mechanisms for recognition of the DPPC component of the mixed liposome could dominate the uptake process and determine the kinetics. With plasma membranes from isolated type II cells, SP-A mediated binding of 1-palmitoyl-2-linolenyl PC in mixed liposomes is decreased by 45% compared with DPPC,[77] compatible with the experimentally-determined binding affinity of lipids for SP-A.[73] This suggests that lung clearance of non-DPPC lipids through SP-A mediated uptake could be relatively impaired. However, the preponderance of DPPC as a lipid component in the alveolar space makes it unlikely that its total segregation from other lipids is unlikely to occur physiologically. More studies are required to define clearance of these other lipids, although the imperative for physiological understanding is not great.

Intracellular Lipid Trafficking

Fate of Internalized Lipid

Potential fates for lipid internalized by type II cells are:

1. degradation;
2. transport to lamellar bodies for resecretion; or
3. transfer to the interstitium (transcytosis) for clearance by lymphatics or blood.

As described above, clearance of intact lipid via the lymphatics or blood does not occur. Both degradation and resecretion of DPPC have been described, and the relative contribution of the two pathways may vary with physiological conditions. The partition between these pathways represents a potential additional control point for regulation of surfactant turnover.

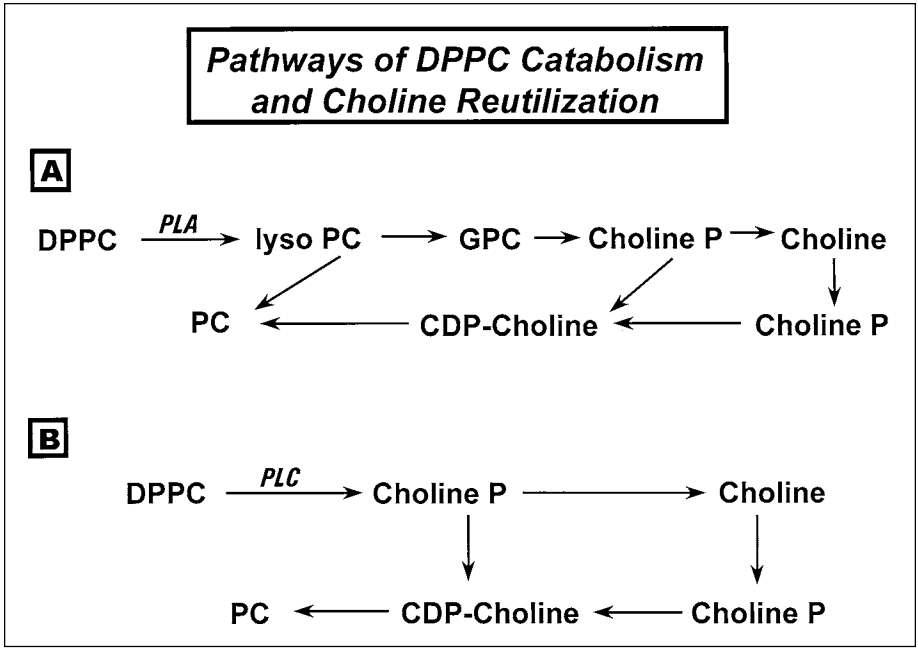

Fig. 8.4. Pathways for catabolism and reutilization of choline-labeled DPPC via phospholipase A (PLA_1 or PLA_2) (panel A) or phospholipase C (panel B). Only choline-containing products are indicated. Catabolism via phospholipase D (not shown) would generate labeled choline directly, but would otherwise follow the reactions in panel B. GPC, glycerophosphorylcholine.

Degradation of Internalized Lung DPPC

In the isolated perfused rat lung, there is significant degradation of the labeled lipid in the 2 hours following endotracheal instillation of choline-labeled liposomes or natural surfactant.[32,52,72] Choline label in these experiments is recovered in both the aqueous fraction (containing glycerophosphorylcholine, choline phosphate and free choline) and organic fraction of the lung homogenate, as expected from known degradative pathways (Fig. 8.4). The aqueous fraction also shows radiolabel in CDPcholine, indicating reutilization of choline for generation of the rate-limiting substrate for PC synthesis via the de novo pathway (see chapter 2). In the organic fraction, label is found in DPPC (the original substrate), in lyso-PC (a degradation product) and in PC containing unsaturated fatty acids. The latter also indicates reutilization of the choline label for resynthesis of PC, either through reacylation of lyso-PC (with an unsaturated fatty acid) or through the de novo pathway. Degradation of lipid and reutilization of the resulting components are seen in isolated type II cells incubated with liposomes or natural surfactant.[64,89,90]

Determination of the fractional degradation of internalized lipid is difficult in the intact lung because the metabolic pool cannot be pulse-labeled, and recovery of metabolic products is cumbersome. Nonetheless, estimations of rates of metabolism suggest that about two-thirds of lipid internalized by the perfused lung is degraded,[50,91] as indicated in Figure 8.5. Since reutilization of the choline label would be expected to regenerate DPPC as a major product, the extent of DPPC degradation in the perfused lung experiments may be underestimated. Less extensive studies evaluating degradation of radiolabeled natural surfactant yield similar results.[50]

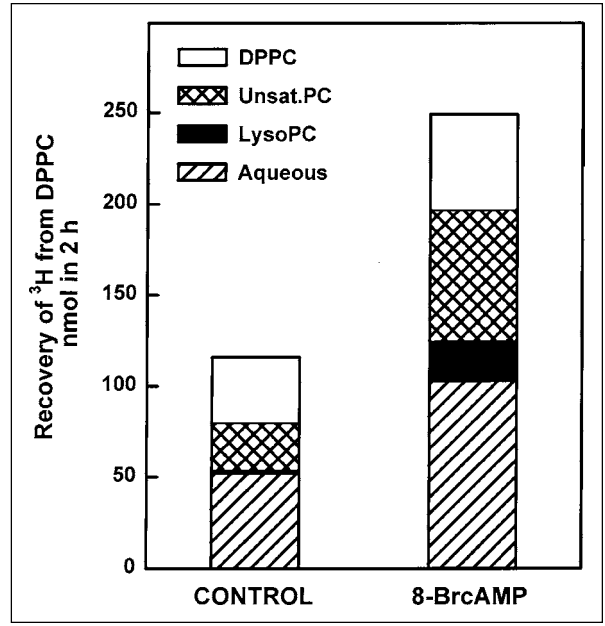

Fig. 8.5. Uptake and degradation of ^{3}H-choline labeled DPPC by the isolated rat lung during a 2 hour perfusion in the presence or absence (control) of 10^{-4} M 8-bromo cAMP (8-BrcAMP) in the perfusate. ^{3}H-DPPC in mixed unilamellar liposomes was instilled into the trachea. Uptake and degradation were calculated assuming linear rates between 5 minutes and 2 hours of perfusion. The height of the bar indicates total DPPC uptake by the lung. The total height minus the DPPC fraction represents degradation. The recovery of ^{3}H in radiolabeled components is indicated by the different shadings. The lyso-PC and aqueous fractions represent degradation products. The unsaturated PC fraction represents degradation followed by metabolic reutilization for PC synthesis. DPPC represents either the original material or that resynthesized from labeled degradation products. The presence of the cAMP analog resulted in a significant stimulation of both DPPC uptake and degradation. Compiled from original data in Fisher et al[32] as modified in Fisher and Dodia.[50]

The foregoing analysis indicates reutilization of choline-label from DPPC for resynthesis and resecretion. Reutilization of fatty acid (palmitate) from monoenoic PC or from PG for synthesis of phospholipids has been observed in rabbit lungs and isolated rat type II cells.[39,88,89] Reutilization of a particular component will depend on its metabolic pool. For example, choline is actively transported into the type II cell against a concentration gradient with an affinity of the transporter for choline in the micromolar range.[92,93] Therefore, it is expected that the choline liberated intracellularly following lipolytic activity would be avidly retained. Further, choline is utilized almost exclusively for PC synthesis, so that its rate of reutilization in the type II cell would be reflected in an analysis of surfactant. Fatty acids, on the other hand, enter a pool that is utilized for various metabolic reactions including mitochondrial oxidation to CO_2 and synthesis of glycerides and other complex lipids. Consequently, its rate of reutilization for PC synthesis would be expected to be lower, reflecting its multiple potential fates. These differences in reutilization could account for the apparent differences in biological half times for PC as determined by various substrates (Table 8.1). The similar half times for choline and phosphate suggest that choline may be reutilized primarily as choline phosphate rather than as the free base.

The rate of intracellular surfactant degradation is normally not rate limiting for surfactant clearance. As evidence, rates of clearance for DPPC and its nonmetabolizable analogs (the D-isomer or the ether analog) are similar.[21,51,94] Uptake is also not altered in the presence of an inhibitor of DPPC catabolism.[52] Clearly, long term alterations of surfactant degradation could have consequences for surfactant clearance, but this potential effect has not yet been evaluated.

Phospholipases and DPPC Degradation

Liposomal lipids are degraded during incubation with lung homogenate,[21,52] indicating the action of one or more phospholipases. The pattern of recovery of metabolic products in the isolated perfused rat lung following endotracheal instillation of labeled DPPC indicates that PLA_2 is the predominant degradative enzyme. In perfused lung experiments with label in the *sn*-2 palmitate of DPPC, the ratio of label recovery in free fatty acids vs. diacylglycerol is 34:1.[52] The action of PLA_2 generates lyso-PC and the free fatty acid. Lyso-PC in turn can be reacylated to PC or degraded by lysophospholipases and various esterases to simpler water soluble constituents as described above. Activities of other phospholipases (phospholipase A_1, phospholipase C and phospholipase D), which are characterized by their site of hydrolytic cleavage (Fig. 8.4), have been identified in lung tissue and type II cells (see Fisher and Dodia[90] for a more extensive discussion). A significant role for them in the turnover of internalized surfactant DPPC is not likely, although one or more may be crucial in terms of intracellular signaling pathways (see chapter 7).

PLA_2 represents a diverse family of enzymes including both Ca^{2+}-dependent and Ca^{2+}-independent forms. Examples of the former include pancreatic PLA_2 (type I secreted), PLA_2 associated with inflammatory exudates (type II secreted) and the cytosolic PLA_2 that appears to be coupled to eicosanoid biosynthesis.[95] Secreted PLA_2 forms have a molecular mass of about 15 kDa and the cytosolic PLA_2 of about 85 kDa; all have pH optima in the neutral to alkaline range. Several of these Ca^{2+}-dependent PLA_2 forms have been demonstrated in alveolar type II cells.[90,96] Ca^{2+}-independent PLA_2s represent another large group that have been categorized as brush border (membrane), cytosolic and lysosomal enzymes.[97] Ca^{2+}-independent PLA_2 activity has been identified in lungs, type II cells and lamellar bodies.[98-100] Of particular interest are in vitro assays showing considerable activity with a pH 4 optimum, indicating a lysosomal type enzyme. Studies with a transition-state phospholipid-analog inhibitor indicate that this Ca^{2+}-independent lysosomal type PLA_2 is responsible for the major fraction of surfactant DPPC degradation in type II cells.[52,90] This enzyme (designated $aiPLA_2$) has now been identified with a cDNA clone and in vitro expression of a 26 kDa protein.[101] It has been localized to lung lamellar body and lysosomal fractions,[90] both acidic organelles.[102] Lysosomatrophic agents inhibit degradation of DPPC in type II cells, providing evidence for involvement of an acidic compartment as required for $aiPLA_2$. activity.[90] Colocalization of surfactant phospholipid with a lysosomal marker enzyme has been demonstrated in freshly isolated type II cells, compatible with its intracellular transfer to that organelle.[29] Accumulation of a nonmetabolizable DPPC analog in a lysosome-enriched fraction isolated from rabbit lungs provides additional evidence for lysosomes as the primary site for DPPC degradation.[103]

Degradation of DPPC by isolated type II cells is decreased when SP-A is added to liposomes prior to incubation with the cells.[49,68,70] One possibility for this effect is that SP-A mediates direct transfer of DPPC to lamellar bodies, i.e., recycling as described in the next section. Another possibility is that SP-A directly inhibits the degradative enzyme. Inhibition of $aiPLA_2$ activity in vitro by the presence of SP-A has been demonstrated.[49] Inhibition requires the native SP-A form and can be reversed by reduction/alkylation of the protein. Activity of ai PLA_2 in isolated rat lung lamellar bodies is increased significantly by addition of reducing/alkylating agents presumably through reversal of in situ SP-A inhibition ($aiPLA_2$ itself is insensitive to these reagents).[49] Thus, the presence of SP-A in lamellar bodies may protect DPPC against the action of phospholipase, although the "reason" for the presence of $aiPLA_2$ in these organelles is not yet apparent. These data are compatible with a role for SP-A in modulation of intracellular DPPC degradation through its regulation of $aiPLA_2$ activity.

Recycling of DPPC

Is the DPPC molecule recycled, i.e., internalized and resecreted, without prior degradation? Intact DPPC could follow a pathway similar to ferritin, which is transported directly from the alveolar space to lamellar bodies.[61] However, evidence for recycling of intact DPPC has been difficult to obtain. Jacobs et al[19] used a two-compartment model of surfactant flux to calculate a reutilization rate for DPPC in rabbits, assuming bidirectional flux between lamellar bodies and alveolar space, with provision for loss of label from the alveolar space due to other processes. Using precursor-product relationships, they estimated PC reutilization at 23% for the adult rabbit. A subsequent study reported a reutilization rate in adult rabbits of 40-45%, although specific activity in lamellar bodies was not measured directly.[94] These results must be interpreted cautiously since the scatter in the calculations is great. As discussed above, the inability to pulse label the relevant pools and difficulty in isolating pure fractions can result in gross inaccuracies in calculation of flux based on the assumed precursor-product model. Thus, the extent of direct surfactant recycling in adult animals remains unresolved, but in any event does not appear to be the major pathway. Surfactant lipid recycling may be more prominent in neonatal animals since the calculated recycling rate in newborn rabbits is greater than 90%.[34]

It is possible that SP-A is important for the direct recycling of lipid and that DPPC bound to SP-A is preferentially targeted to lamellar bodies for resecretion. SP-A promotes the incorporation of radiolabeled DPPC into lamellar bodies in rabbit lungs and isolated rat type II cells.[68,70] At 2 hours of isolated rat lung perfusion, both labeled DPPC and SP-A are found in isolated lamellar bodies, although the DPPC:SP-A ratio is about twice that in the original surfactant.[72,104] The label in the isolated lamellar bodies could represent lipid that is transferred directly in association with SP-A. However, lipid that has been resynthesized utilizing the labeled products of DPPC degradation could also contribute. As described above, degradation of DPPC is decreased in the presence of SP-A,[49,68,70] possibly due to SP-A inhibition of aiPLA$_2$.[49] Thus, SP-A could mediate the direct transfer of DPPC to lamellar bodies or could inhibit DPPC degradation. These mechanisms are not incompatible but their relative contributions are not known.

In rat lungs, internalized lipid plus protein labels are found in a lamellar body fraction that is relatively heavy on sucrose density centrifugation (dense lamellar bodies), while a less dense fraction (light lamellar bodies) is enriched in labeled lipid.[104] These results can be interpreted as reflecting two populations of lamellar bodies, a lighter population (possible containing newly synthesized lipid) and a denser population (possibly containing recycled lipid and protein),[105,106] but it is difficult to distinguish this concept from possible loss of protein components during lamellar body isolation.

Clearance of Surfactant-Associated Proteins

SP-A Clearance

The role of SP-A in the clearance of lipids from the alveolar space is described above. The proposed mechanism is endocytosis of the SP-A:DPPC complex following its binding to a type II cell membrane receptor for SP-A. This process, of course, would also result in SP-A internalization. Both DPPC and SP-A can be taken up individually by isolated type II cells but, based on estimated binding affinity and their respective concentrations, it is unlikely that free SP-A (i.e., without associated DPPC) exists in the alveolar space.

Clearance of SP-A and DPPC has been compared in several types of experiments. The ratio of SP-A to DPPC clearance by isolated perfused rat lungs is similar to that in the original surfactant, suggesting that these components are internalized as a unit.[72] In rat and

mouse lungs, clearance in vivo of reconstituted liposomes plus iodinated SP-A are similar,[104,107] but in rabbits the rate of SP-A clearance from the air space is approximately twice that of DPPC (the half time for DPPC clearance is significantly longer in rabbits compared to mice).[108] SP-A is also cleared more rapidly than DPPC from dog lungs following in vivo labeling with radioactive precursors[109] and from lungs of preterm lambs and newborn rabbits after endotracheal instillation.[110-112] On the other hand, type II cells in culture showed an approximately 2-fold greater rate of uptake of lipid compared with labeled protein in natural surfactant,[26] but this difference is likely a manifestation of decreased expression of cell membrane receptors for SP-A in cultured cells[27] and may not reflect the physiological condition.

A greater rate of SP-A clearance from the alveolar space after endotracheal instillation of labeled SP-A and DPPC can be explained by similar rates of uptake of the two components with greater resecretion of the lipid label. Indeed, the ratio of labeled lipid to SP-A in the secretory organelle (lamellar body) after endotracheal administration of surfactant is approximately double that of the original instillate.[72,104] However, SP-A may be resecreted by other pathways as described below. Since the epithelial surfactant uptake system is saturable, the apparent removal rates for each component will depend on dilution of the respective SP-A and DPPC pools by the exogenous labeled material. If the SP-A pool is diluted to a lesser degree by the instilled labeled SP-A (compared to DPPC dilution of the DPPC pool), its apparent clearance rate might be greater. The kinetics are obviously complex and will require additional study for full understanding of relative clearance rates for these major surfactant components.

Mechanism of SP-A Uptake

SP-A uptake by the type II cell appears to occur through receptor mediated endocytosis and, therefore, the nature of the receptor and its affinity for SP-A are of interest. As described above, recent studies provide evidence for at least one and possibly two or three binding proteins for SP-A on type II cell membranes;[78-80] continuing investigations will likely precisely define the molecular characteristics and relationships among these candidate "receptors" in the near future. Using immunogold techniques, SP-A binding was localized to coated pits on the surface of type II cells[28] suggesting clustering of SP-A receptors in those sites. Thus, uptake of SP-A by the cells is apparently mediated through the classical pathway of binding to a receptor and endocytosis via clathrin-coated pits.[82] Receptor binding by SP-A is mediated through its noncollagenous, carbohydrate recognition domain at the C terminus.[71,113]

SP-A binding to the receptor on isolated type II cells is saturable at an SP-A concentration of about 1-5 μg/ml.[86,113] The number of binding sites is sensitive to culture conditions and is increased approximately 5-fold in cells cultured on microporous membranes as compared with plastic dishes.[86] SP-A binding is greater in the presence of phospholipids, possibly related to conformational changes in either SP-A or in the membrane receptor.[27] The number of binding sites approximately doubled when cells were exposed briefly (about 20 minutes) to a β-adrenergic agonist or PKC activator, suggesting recruitment of an intracellular pool of "receptors" to the cell surface.[86] This could be the mechanism for the secretagogue-mediated increase in SP-A uptake by perfused rat lung and cultured rat type II cells.[26,72]

Intracellular Processing of Internalized SP-A

The pathway for intracellular trafficking of SP-A following internalization by type II cells appears to follow the expected route for receptor mediated endocytosis,[82] although none of the studies with lung have been definitive. Using colloidal gold or ^{125}I radioautographic techniques to follow SP-A, the label associated with clathrin coated pits on the cell

membrane appears to move rapidly (within 2-5 minutes) to clathrin coated and then uncoated vesicles, within 15 minutes to multi-vesicular bodies (MVB) and finally to lamellar bodies.[25,28,30,114] There is significant transfer of labeled SP-A to lamellar bodies in rat lungs within 2 hours following its endotracheal instillation,[25] whereas the process is slower in isolated type II cell cultures. The latter most likely represents alteration of normal intracellular trafficking by cell culture conditions. For example, with type II cells cultured on plastic, significant labeling of lamellar bodies with SP-A-bound colloidal gold label is not observed,[28] while 20% of lamellar bodies in cells cultured on a polylysine coated surface are labeled during the first hour.[30] Immunofluorescence studies show colocalization of SP-A and a lysosomal protein in cells cultured on plastic but a different pattern for cells grown on a more physiologic matrix.[29] Forms representing apparent fusion between MVB and lamellar bodies are prominent on ultrastructural analysis of type II cells. However, the precise role of MVB has not been defined since autoradiography of intact rat lung failed to demonstrate time-dependent appearance of alveolar ^{125}I-SP-A in these organelles.[25] Further, the relationship between electron lucent MVB as possible transfer organelles and electron dense MVB as possible degradative organelles has not been resolved.[25]

At 2 hours of isolated lung perfusion following endotracheal instillation of biosynthesized radiolabeled surfactant, subcellular fractionation showed a ratio of protein to lipid accumulation in the lamellar bodies that was about 50% of the ratio of material bound to the plasma membrane; the ratio of material bound to plasma membranes was similar to the original surfactant.[72] A similar disparity between endocytosed DPPC and SP-A in lamellar bodies is observed in rats in vivo using liposomes reconstituted with iodinated SP-A obtained from alveolar proteinosis patients[104] and, in rabbits, using iodinated rabbit SP-A.[108] These results indicate at least partial dissociation of the lipid:protein complex after internalization with subsequent differential targeting of the two surfactant components. While the "free" lipid may be preferentially targeted to a degradative organelle (e.g., lysosomes) as suggested by the high rate of DPPC degradation, the pathway for "free" SP-A has not been determined. The low ratio of labeled protein to lipid in lamellar bodies is evidence against targeting of excess SP-A to this organelle. Current evidence suggests that SP-A is not measurably degraded by type II cells.[115] Preferential targeting of iodinated SP-A (possibly degraded) to the Golgi has been suggested, although the total flux to this compartment is small.[25] These results suggest an endosomal storage pool for internalized SP-A which has yet to be identified. The putative storage process is likely a prelude to resecretion, perhaps through a constitutive secretory pathway. This process, i.e., recycling from the milieu to endosomes and back, has been termed retroendocytosis and has been described for other carrier proteins[116,117] but not as yet for SP-A.

SP-A Metabolism and Alveolar Macrophages

While uptake and recycling represent the primary fate of SP-A, it is also degraded in the lung, but at a significantly slower rate than DPPC. SP-A fragments have been identified in rat and rabbit lungs 1-4 hours after endotracheal instillation of iodinated SP-A.[104,118] In the isolated perfused rat lung, only 2% of internalized surfactant protein is degraded to trichloroacetic acid-soluble components compared with 10% degradation of phospholipid during a 2 hour study period.[72] In vitro, alveolar macrophages are capable of SP-A degradation,[56,115] a process that can be upregulated significantly by simple overnight culture on plastic dishes.[57] These cells show Ca^{2+}-dependent binding of SP-A through a receptor (210 kDa) that may be identical to a type II cell membrane SP-A receptor.[80,119] The binding protein on alveolar macrophage is not present on macrophages isolated from other sites.[120] Freshly isolated rat alveolar macrophages degrade approximately 50% of internalized iodinated SP-A during a 3 hour continuous incubation, while isolated type II cells have markedly

lower rates of degradation which could be due entirely to macrophage contamination of the preparation.[115] Therefore, macrophages appear to be the primary cell for SP-A degradation in the lung. It is possible that macrophages primarily degrade SP-A that has been modified from its native state, as would occur with iodination of protein. Thus, protein modified in vivo, by oxidation or other alterations related to repeated cycling in and out of the cell, could be a target for removal by macrophages. These alterations would have to be subtle, since biosynthesized and iodinated SP-A are handled by the lung in a grossly similar fashion.[108] Of note, macrophages lavaged from the lungs of rabbits following endotracheal instillation of iodinated SP-A contain little of the label.[108] Thus, the role of alveolar macrophages in the normal turnover of SP-A remains undefined.

Clearance of Hydrophobic Surfactant Proteins

There is relatively limited information concerning the clearance of the hydrophobic surfactant proteins. Iodinated SP-B instilled into the lungs of rabbits is cleared with a half time of 7 hours, similar to that for SP-A but more rapid than that for DPPC (11 hours).[121] However, the half time for clearance of ^{125}I-SP-B in mice is 14 hours vs. 8-9 hours for DPPC and 6 hours for SP-A.[42,107] The caveats described above for the role of pool sizes in analysis of relative rates of SP-A vs. DPPC clearance apply here as well. As an example, clearance of SP-B (as well as SP-A and SP-C) in neonatal rabbits is similar to that for DPPC between 2 and 4 hours after endotracheal instillation of surfactant, although the uptake during the initial 2 hours is greater for each of the proteins.[112] At best, it can be concluded that SP-B turnover is rapid and comparable to the other major surfactant components.

Electron microscopic autoradiography demonstrated binding of labeled SP-B to the plasma membrane of isolated type II cells which is independent of coated pits.[122] Thus, unlike SP-A, uptake of SP-B may not involve clathrin. Protein endocytosis through nonclathrin pathways has been demonstrated in nonlung cultured cell lines and appears to result in delivery to the same endosomes as for endocytosis through coated pits.[123] Binding of iodinated SP-B to type II cells is saturable, suggesting the presence of a cell membrane receptor for the protein,[124] although this putative "receptor" has not yet been identified. Internalization of labeled SP-B by type II cells through endosomes to lamellar bodies was demonstrated.[122] SP-B accumulates in lamellar bodies proportionately to DPPC,[121] in contrast to SP-A where, as described above, the ratio of accumulation is about half that of DPPC. Also in contrast to SP-A, approximately 20% of the iodinated SP-B internalized during a 4 hour incubation is degraded by type II cells, as well as by alveolar macrophages.[125] How the clearance kinetics for SP-A and SP-B relate to the observed differences in uptake and metabolism of these proteins in vitro remains to be determined.

Study of recombinant iodinated SP-C has demonstrated clearance from the alveoli and transfer to lamellar bodies with a time course similar to that for DPPC in adult rats,[126,127] while biosynthesized SP-C is cleared from alveoli at a slightly greater rate than that observed for DPPC in neonatal rabbits.[128] The difficulties related to SP-C assay and lack of satisfactory antibodies for the mature peptide (see chapter 5) have hampered studies of SP-C turnover.

Summary and Future Directions

The cellular turnover and processing of lung surfactant components is certainly complex. Based on current evidence, the major clearance pathways for SP-A and DPPC would appear to occur through the following steps (Fig. 8.6):

1. Binding of an SP-A:DPPC complex to a membrane SP-A receptor localized to the clathrin-coated pit of type II cells. This is the receptor-mediated endocytosis pathway.

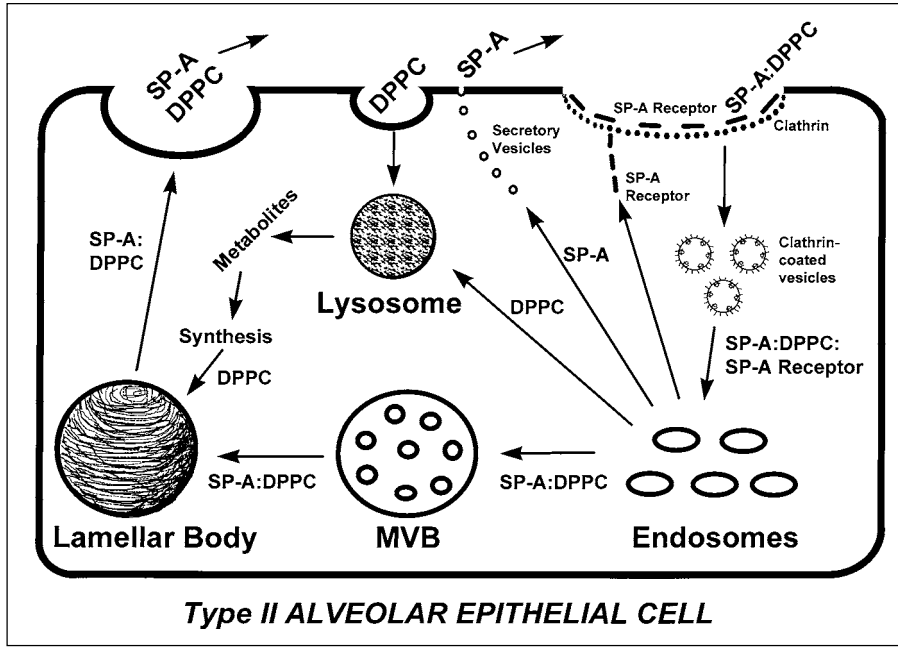

Fig. 8.6. Schematic representation of pathways for uptake of SP-A and DPPC by type II cells. These pathways are described in the text.

2. Internalization of the SP-A:DPPC complex and the SP-A receptor into a coated vesicle and then to an endosomal compartment where the receptor is dissociated for return to the cell surface.
3. Processing of endosomal SP-A:DPPC is a branch-point in the clearance pathways. The SP-A:DPPC complex can be processed in two ways: (a) Dissociation of the complex with transfer of SP-A to vesicles for resecretion (retroendocytosis) and transfer of DPPC to lysosomes; (b) Transfer of the complex to lamellar bodies for resecretion (recycling).
4. DPPC in the lysosomes is degraded by a Ca^{2+}-independent 26 kDa PLA_2 that is regulated by SP-A.
5. A second pathway also functions in DPPC uptake. DPPC binds to the type II cell membrane (at sites of inserted lamellar body membrane?) with internalization by actin-dependent cell-membrane retrieval pathways. Internalized DPPC is directed to lysosomes for degradation (or possibly to lamellar bodies for resecretion).
6. Components of degraded DPPC are "reutilized" by the cellular synthetic processes, including the resynthesis of DPPC, and its transfer to lamellar bodies.
7. "Damaged" SP-A is cleared by alveolar macrophages via a cell membrane receptor and degraded (not shown in Fig. 8.6).

Is the above scheme fact or fancy? Probably some of both. It should be apparent from the foregoing that the evidence in many important areas is fragmentary or inconclusive so that the synthesis presented in Figure 8.6 represents largely "one man's opinion." Key questions remain, notably regarding the mechanisms for intracellular sorting of endocytosed material and the mechanisms for coordinating secretion and removal pathways. Further-

more, this chapter has not addressed a very basic question: Why is surfactant turnover so rapid? A logical possibility is to remove "damaged" and restore "normal" components. Another possibility is that rapid turnover is the most efficient means to maintain the normal extracellular concentration of surfactant. These questions remain for future experimentation. However, the indisputable fact is that the lung alveolar type II epithelial cell plays in the major leagues of cell biology.

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CHAPTER 9

Host Defense Functions of Surfactant

Jo Rae Wright

In addition to reducing surface tension at the air-liquid interface (see chapter 1), surfactant also plays an important role in regulating host defense and inflammation in the lung. Although investigations of the host defense functions of surfactant began over twenty years ago, the field was not of major interest until two relatively recent developments. First, two of the surfactant proteins, surfactant protein A (SP-A) and surfactant protein D (SP-D), were found to be collectins—a family of proteins located in both the lung and the blood that serve as important host defense molecules (see below). Second, the introduction of surfactant replacement therapy for treatment of premature infants suffering from respiratory distress syndrome (RDS) has resulted in a growing awareness that such therapy may have multiple actions in the lung.

The goal of this chapter is to review the current literature on the host defense functions of surfactant. A rationale will be presented for these potential functions, important issues related to studies on host defense will be discussed and a summary of the effects of surfactant on a variety of immune cell functions will follow. The changes in the levels of surfactant components that have been documented in various disease states will be summarized, and a general model for a role of surfactant in host defense will be presented in conclusion.

A Rationale for Host Defense Functions of Surfactant

What is the rationale for proposing or investigating the possibility that surfactant may regulate immune cell function? The literature indicates that many of the early studies from the 1970s investigating the effects of surfactant on alveolar macrophage function were motivated by the fact that surfactant and macrophages exist in close physical proximity in the alveolar hypophase, which is the thin layer of liquid that covers the alveolar epithelium. It has been proposed that the existence of a local defense system in the lung would be highly beneficial since an enormous number of particles and pathogens are likely to be inhaled during normal breathing[1] and deposited onto the thin, delicate lung epithelium. Damage to this epithelium, which can occur if the inflammatory cascade spirals out of control, often leads to flooding of the alveolar space with serum components and a resultant impairment of gas exchange. Thus, a fast, local, well controlled host defense response in the lung seems beneficial.

Further rationale for the involvement of surfactant in the immune response has been supported by the observation that SP-A is structurally homologous to C1q,[2] the first component of the complement cascade, and the recent categorization of surfactant proteins SP-A and SP-D as collectins.[3] Collectins are identified by an N-terminal collagen-like (col-) domain and a C-terminal lectin-like domain (-lectin) (reviewed in refs. 4, 5). In addition to SP-A and SP-D, this family includes the serum mannose binding protein (MBP) and two

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proteins that have thus far been identified only in the sera of cows, collectin-43 and conglutinin (see Fig. 9.1).

The collectins and the complement proteins are all involved in innate[6,7] (nonantibody mediated) host defense. Innate immunity differs from adaptive (antibody-mediated) immunity in several ways. First, innate immunity is not improved by repeated infection; in adaptive immunity antibody levels are elevated by repeated infection. Second, the effector cells of the adaptive immune system are T lymphocytes; the effector cells of the innate immune system are phagocytic cells including macrophages and monocytes.[8] Finally, the innate immune system is generally regarded as a rapid, first line of defense, in which pathogens are opsonized or coated by a protein opsonin and targeted for uptake and killing by the phagocytic cell.

The finding that low levels of serum MBP correlate with an increased and unexplained incidence of infection in children provided compelling evidence that this family of proteins may be involved in host defense (reviewed in ref. 9). The molecular basis for the opsonic defect has been traced to three point mutations in the MBP gene, all occurring in its coding region (reviewed in refs. 10-12). Although an equivalent disease of SP-A deficiency in humans has not been identified, recent studies with mice in which the SP-A gene has been ablated have shown that the SP-A deficient mice have an increased susceptibility to infection[13] and provide strong support for an in vivo role of SP-A in host defense (discussed in detail below).

In vitro studies have shown that MBP (and surfactant proteins, see below) bind to a variety of pathogens, including bacteria and viruses, and stimulate the respiratory burst of immune cells (reviewed in refs. 4, 9). Although MBP, SP-A, and C1q are all structurally homologous, only MBP can substitute for C1q in activation of the complement pathway.[14-16] It is interesting that the genes for human SP-A,[17] MBP[18] and SP-D[19] are all localized on chromosome 10. That raises the possibility that these proteins may be subject to similar regulatory mechanisms.

The collectins are all highly oligomerized and bind to specific carbohydrates. For example, SP-A binds to many monosaccharides including mannose, glucose, fucose and galactose, but does not bind to amino sugars.[20] SP-D binds to glucose containing sugars and phosphatidylinositol (PI).[21] The functional significance of the carbohydrate binding specificity is not clearly understood, although it has been proposed, and some data support the possibility, that the interaction of SP-A and SP-D with various pathogens and immune cells may involve their lectin-like domains.

Issues Related to the Study of Host Defense Functions of Surfactant

"Whole surfactant" is a very complex mixture of lipids, surfactant proteins, and other (e.g., serum-derived) proteins and is usually isolated by lung lavage and a series of differential or density gradient centrifugations. Many of the early studies on host defense investigated the regulatory effects of whole surfactant that was not fractionated into its separate lipid or protein components, and many of these studies generated conflicting results. Although the reasons for all of the conflicts are impossible to discern, many of them are likely related to the variable composition of the isolated surfactant used in the studies.

The studies of King and Clements in the 1970s[22-24] rigorously defined an isolation procedure based on functional and biochemical data; however, this technique, which employs multiple differential centrifugation steps, is used relatively infrequently. Other commonly used procedures include differential centrifugation and step gradients. Depending on the technique used for lavage and isolation, the composition of surfactant, in particular the relative abundance of surfactant proteins,[25] can vary dramatically. Because different lipids and proteins have different effects on immune cell function, it seems likely that some of the

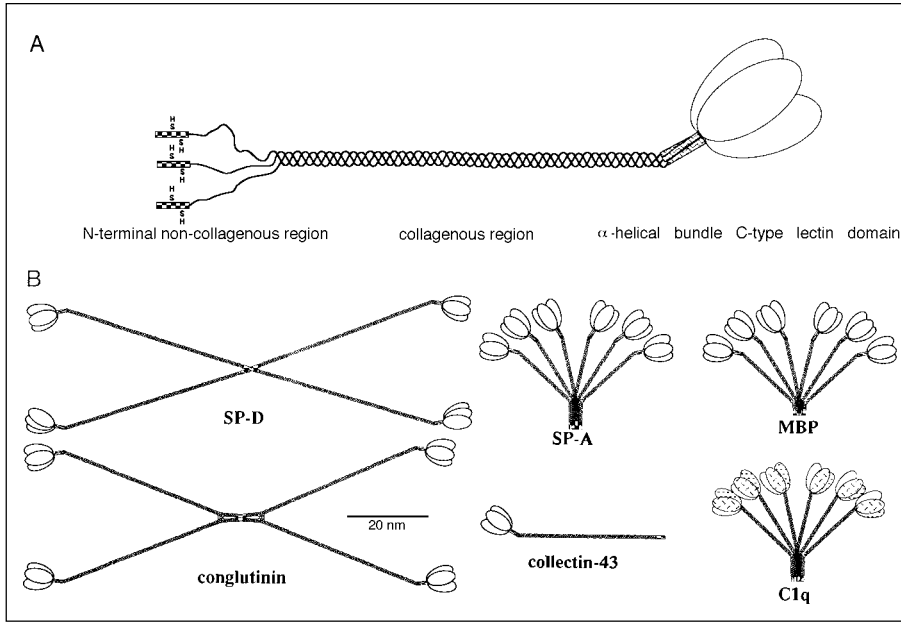


Fig. 9.1. Structure of the collectins and the structurally related complement component, C1q. (A) Collectins have three chains, each containing 4 distinct domains. The first domain is a short, N-terminal region involved in cysteine-mediated disulfide bond formation. The second domain is a collagen-like triple helix. The third domain is an α-helical bundle that is the 'neck' between the collagen-like domain and the fourth domain, which is the carbohydrate recognition domain (CRD). (B) Schematics of the oligomerized forms of SP-D, SP-A, MBP, conglutinin, collectin-43 and C1q. Figure courtesy of Drs. Hoppe and Reid, University of Oxford. Reprinted with permission of the authors and Cambridge University Press from Hoppe HJ, Reid KB. Collectins—soluble proteins containing collagenous regions and lectin domains—and their roles in innate immunity. Protein Sci 1994; 3:1143-1158.

variable results obtained with different preparations of whole surfactant may be a result of the variable composition of the surfactant preparations.

Similar issues relate to the studies using purified surfactant proteins. A variety of methods of purification are utilized by different laboratories (e.g., refs. 26-28), and it has been demonstrated that the method of purification can affect the ability of the protein to regulate immune cell function.[26] For example, van Iwaarden et al[26] compared different methods of purification of SP-A both from the therapeutic lavage of patients with alveolar proteinosis and from lavage of normal rats and dogs. The SP-A isolated from the lavage of both rats and dogs by butanol extraction, a commonly used method of purification, has impaired ability to regulate macrophage function. In contrast, SP-A isolated from alveolar proteinosis lavage is resistant to inactivation by butanol extraction and retains its ability to stimulate macrophages. SP-A purified by high pressure liquid chromatography and detergent solubilization from all three sources effectively stimulates macrophage function. These studies suggest that SP-A isolated from the lavage of normal lungs may be susceptible to inactivation by butanol and raise the concern that various methods of isolation may affect the protein's functionality. Furthermore, SP-A from alveolar proteinosis patient lavage is highly aggre-

gated,[29] and it has been shown that the state of aggregation of SP-A[30,31] and SP-D[32] affects their ability to stimulate immune cells.

Purified surfactant lipid and proteins may often be contaminated with endotoxin,[33,34] the bioactive component of bacterial cell walls that causes fever and inflammation and that is a potent stimuli of immune cells.[35] This is perhaps not surprising since both SP-A and SP-D bind to bacteria and bacterial cell wall components (see below). Although SP-A and SP-D may be associated with endotoxin in vivo, caution must be used in interpretation of data obtained with surfactant preparations containing endotoxin. In many cases, it may be difficult to ascribe a measured response specifically to the surfactant protein or to the endotoxin, since many immunoregulatory cells are exquisitely sensitive to endotoxin. Furthermore, Kalina et al[36] have reported that association of SP-A with bacterial cell wall components alters the ability of SP-A to affect cell functions.

Effects of Surfactant on Phagocytosis and Clearance of Pathogens

Studies by LaForce et al[37] almost 25 years ago showed that surfactant increases the phagocytosis of *Staphylococcus aureus* by alveolar macrophages. Although LaForce et al were unable to show that bacterial killing is enhanced, subsequent studies by Juers et al[38] did demonstrate that surfactant enhances killing of *S. aureus.* Interestingly, the effect of the surfactant varies depending upon the species from which it is isolated. For example, human surfactant either enhances phagocytosis but not killing,[39] or it enhances neither phagocytosis nor killing,[40] whereas surfactant from rat lung lavage enhances both.[39] Although some of these variable effects may depend upon the species from which the surfactant was derived, many of these preparations were obtained by different techniques and purification methods and may therefore have had different compositions.

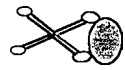
Recent studies have provided more consistent support for a role of surfactant in enhancing phagocytosis. The uptake of *S. aureus* was increased by whole surfactant and lavage fluid,[41,42] and the enhancement in both studies was at least partially attributed to SP-A. Thus, these recent studies suggest that at least part of the ability of whole surfactant to stimulate phagocytosis is due to the SP-A component of surfactant.

In fact, many of the collectins, including SP-A and SP-D, act as opsonins by binding to the surface of particles. SP-A and SP-D have been shown to bind to a variety of pathogens (see Table 9.1), although to date only SP-A has been shown to stimulate phagocytosis. van Iwaarden and coworkers demonstrated that SP-A can act as an opsonin by binding to the surface of a bacterium and facilitating phagocytosis.[41] These investigators demonstrated that whole surfactant also stimulates phagocytosis and that the effect is inhibited by an anti-SP-A antibody. These studies are significant, since it appears that the bulk of SP-A isolated by lung lavage is associated with lipid.[43]

SP-A also increases the binding of numerous other organisms to phagocytic cells, including *Haemophilus influenza* type a (but not type b),[44] *Pseudomonas aeruginosa*,[45] *S. pneumoniae*,[42] *S. auereus*[41] and Group A streptococcus,[42] herpes simplex virus,[46] influenza virus,[47] *Klebsiella pneumoniae*,[48] *Mycobacterium tuberculosis*[30,49] and *Pneumocystis carinii.*[50,51] Some studies have clearly shown that SP-A enhances phagocytosis of specific organisms (e.g., refs. 30, 42, 44, 52), but in many cases the effects of SP-A on binding or phagocytosis were not distinguished.

Additionally, there are some inconsistencies in the literature. For example, McNeely and Coonrod[53] reported that SP-A bound to but did not enhance the uptake of *S. auereus,* whereas Tino and Wright[42] reported that SP-A enhanced uptake of this organism. There have been reports that SP-A both enhanced[45] and had no effect[42] on phagocytosis of *P. aeruginosa.* One possible explanation for these apparently inconsistent findings may be found in the study of Meinz-Keinke et al[45] who reported that the effects of SP-A varied with

Table 9.1. Binding of SP-*A and SP-D to pathogens*

SP-A		**Ref.**
	Candida albicans	54
	Cryptococcus neoformans	61
	Group A streptococcus	42
	Haemophilus influenzae (type a)	44
	Haemophilus influenza	42
	Herpes simplex virus type I	46
	Klebsiella pneumonia	48
	Influenza A virus	59
	Mycobacterium tuberculosis	30
	Pneumocystis carinii	50
	Salmonella minnesota	58
	Staphylococcus aureus	53, 67
	Streptococcus pneumonia	42
SP-D		**Ref.**
	Cryptococcus neoformans	61
	Escherichia coli	56, 57
	Influenza virus A	47
	Klebsiella pneumonia (LPS)	57
	Pneumocystis carinii	55
	Pseudomonas aeruginosa	57

SP-A and SP-D interact with different pathogens via different mechanisms. For example, SP-A has been shown to bind to some strains of *E. coli* by interacting with lipid A, and the oligosaccharide chain of SP-A is not required for binding. In contrast, the oligosaccharide chain of SP-D is required for interaction with influenza virus.

the bacterial growth phase (e.g., log or stationary). In addition, different laboratory or clinical strains may have different affinities for SP-A. A recent study by Rousseau et al[54] showed that SP-A actually interferes with the binding of *Candida albicans* to alveolar macrophages, and under certain conditions SP-A inhibits its uptake.

Although SP-D has also been shown to bind to a variety of pathogens, it has not yet been reported that SP-D is capable of stimulating phagocytosis of these pathogens. In fact, Pikaar et al[52] directly compared the ability of SP-A and SP-D to act as opsonins and found that both SP-A and SP-D bind and induce aggregation of a rough *Escherichia coli* strain, J5, but neither induce aggregation of a smooth strain, 0111. SP-A, but not SP-D, was shown to enhance phagocytosis in a very convincing electron microscopy study. SP-D also binds to *P. carinii*, but does not enhance its phagocytosis.[55] SP-D binds to other organisms including other rough strains of *E. coli*[56] and *S. minnesota*, as well as *K. pneumoniae* and *P. aeruginosa*.[52,57] In addition, SP-D binds with weak affinity to two smooth strains of *E. coli* that express the terminal O-antigen.[56]

SP-A and SP-D appear to bind to pathogens by multiple mechanisms. For example, SP-A binds to the lipopolysaccharide (LPS) component of the cell wall of gram negative

bacteria. At least part of this binding occurs via interaction with lipid A,[36,58] a glucosamine disaccharide with attached fatty acids that is also known as endotoxin.[35] SP-D does not bind to lipid A.[58] Available data suggest that SP-D interacts with *E. coli* through the core polysaccharides and/or the O-specific antigens.[56]

Whether or not SP-A binds to other components of LPS, such as the terminal polysaccharides, is controversial. It has been reported that SP-A does not bind to certain smooth strains of *E. coli*[58] and it was proposed that the terminal polysaccharides block the accessibility of SP-A to lipid A. In contrast, Kalina et al[36] reported that SP-A binds to both smooth and rough strains of *E. coli.* Interestingly, polymyxin, an antibiotic that binds to LPS, blocks the binding of SP-A to LPS. SP-A also binds to P2, an outer membrane protein of *Haemophilus,*[44] and to gram positive organisms via an unidentified mechanism.[42]

Both SP-A and SP-D interact, act with and affect the activity of viruses.[46,47,59] SP-A inactivates influenza virus in vitro[59] and the interaction of SP-A with the virus appears to be medicated by the sialic acid residues on the oligosaccharide residue of SP-A. Although SP-A inhibits the ability of influenza virus A to infect a monkey kidney cell line, it is ineffective at neutralizing the structurally related influenza B virus or the mumps virus. In addition, both SP-A and SP-D inhibit hemagglutination activity,[47] although SP-D is more effective than SP-A on a weight basis. While SP-D has no demonstrated role in enhancing phagocytosis, SP-D does enhance the binding of influenza type A virus to neutrophils and modulates the virus mediated downregulation of superoxide production.[47] The degree of multimerization of SP-D correlates with its ability to inhibit neutrophil inactivation by virus.[32,60] These interesting studies suggest that SP-A and SP-D (as well as other collectins) may enhance host defense responses by modulating the response of the immune cells without directly enhancing phagocytosis.

Several lines of evidence suggest that the mechanism by which SP-A and SP-D bind to bacteria and viruses may differ. For example, SP-A treated with endoglycosidase to remove oligosaccharides still binds LPS effectively.[58] In contrast, deglycosylated SP-A does not bind to the herpes simplex virus.[46] Because the binding of SP-D to influenza virus type A is dependent on calcium and inhibited by carbohydrates, it was concluded that the binding is mediated by the carbohydrate recognition domain.[47] Binding of SP-D to *P. carinii*[55]and *Cryptococcus neoformans*[61] is also inhibited by carbohydrates and is calcium-dependent.[55]

In addition to acting as an opsonin, SP-A can act as an activation ligand and enhance the uptake of particles coated with another opsonin, such as immunoglobulin (Ig) G.[30,62] The relative importance of the two pathways is not known. The structural homologues of SP-A, C1q and serum MBP, also act as activation ligands.[62,63]

It is generally assumed that the consequence of increased phagocytosis by immune cells is an increase in killing of the microorganisms (at least for nonopportunistic pathogens which often replicate inside phagocytic cells). However, relatively few studies have investigated the effects of SP-A on pathogen killing. Coonrod et al[64] have shown that there is an antibacterial activity in rat lavage fluid and that most of the activity could be attributed to phospholipids, including palmitoyl lysophospholipid. McNeely and Coonrod[44] reported that alveolar macrophages kill *H. influenza* type a (but not type b) that is opsonized with SP-A to a greater extent than unopsonized bacteria. SP-A enhances the killing of K21a strains of *K. pneumonia.*[48] Geertsma et al reported that whole surfactant inhibits,[65,66] and that SP-A does not enhance, killing of *S. aureus* by monocytes.[67] Whether these findings reflect different cellular responsiveness is not known.

Curosurf® (Chiesi Farmaceutici, Parma, Italy), a replacement surfactant prepared as a chloroform/methanol extract of minced bovine lungs and containing approximately 1% surfactant protein B (SP-B) and surfactant protein C (SP-C) by weight but no detectable SP-A or SP-D, moderately decreases phagocytosis.[68]

In summary, both SP-A and SP-D appear to bind to virus and bacteria, although the mechanism of binding may differ for the different organisms and the different proteins. To date, only SP-A has been clearly shown to enhance phagocytosis of bacteria (Table 9.2). In general, replacement surfactants (Table 9.3) and some surfactant lipids (Table 9.4) tend to suppress phagocytosis. Both SP-A and SP-D inhibit influenza virus type A hemagglutination activity, and SP-D is more effective than SP-A on a weight basis. Only a few studies to date have demonstrated a functional consequence of the increased phagocytosis and binding, e.g., killing of the organism. Additional studies are required to further assess the functional consequences of the increased collectin-mediated association of organisms with immune cells.

Regulation of Cell Surface Receptors and Adhesion Molecules by Surfactant

Both surfactant lipids and proteins regulate cell surface receptors. Coonrod and coworkers found that treatment with whole surfactant reduces the number of Fc receptors on macrophages and that the majority of the inhibitory effect is due to lysophospholipids and free fatty acids found in surfactant.[64,69-71] Treatment of peritoneal macrophages with surfactant also reduces the number of complement receptors; thus, it was suggested that one reason that alveolar macrophages have few complement receptors is due to the inhibitory effects of alveolar surfactant to which they are exposed.[71] Surfactant was also found to decrease Fc receptor levels on murine macrophages; much of this inhibitory effect was attributed to free fatty acids in surfactant.

Survanta® (Ross Laboratories, Columbus, OH), another replacement surfactant, inhibits the expression of several adhesion molecules including CD2, ICAM-1, LFA-1 and LFA-3.[72]

Gaynor et al[30] reported that SP-A increases levels of uptake of *M. tuberculosis* by monocyte-derived macrophages and that this effect is abolished by an antibody against the mannose receptor, a member of the C-type lectin family, found in a variety of tissues and important in host defense against pathogens.[73] These interesting findings were interpreted as evidence for SP-A mediated up-regulation of the macrophage mannose receptor activity. This hypothesis is supported by the work of Kabha et al[48] which shows that SP-A increases phagocytosis of *K. pneumoniae* by two different mechanisms: opsonic phagocytosis and/or activation of macrophages resulting in an increase in activity of the mannose receptor.

Effects of Surfactant on Free Radical Production

Whole surfactant has been reported to have both stimulatory and inhibitory effects on production of free radicals. The variable composition of "purified surfactant" may account for at least part of these differences (see above). Both whole surfactant and surfactant lipids have been reported to inhibit the production of reactive oxygen species by alveolar macrophages and monocytes stimulated with zymosan and 12-O-tetradecanoylphorbol-13-acetate (TPA).[38,66,74] At least part of the inhibitory effect appears to be mediated by lipids, since a mixture of synthetic lipids similar to surfactant lipids inhibits chemiluminescence.[38] Interestingly, different individual lipids[75] have different effects. For example, dipalmitoylphosphatidylglycerol (DPPG) inhibits, but dipalmitoylphosphatidylcholine (DPPC) increases, the oxidative response induced by zymosan. In contrast, Webb and Jeska[75] reported that surfactant lipids increase the production of chemiluminescence induced by latex beads. Purified unsaturated fatty acids mimic the response, and DPPC is ineffective.

Ahuja et al[76] reported that native surfactant and KL_4, a synthetic surfactant that contains a 21 residue peptide with surface active properties similar to those of SP-B, inhibit the TPA-stimulated respiratory burst of neutrophils. A different replacement surfactant,

Table 9.2. Effects of surfactant proteins on immune cell function

Enhance chemotaxis
Enhance phagocytosis/binding
Aggregate microbes
Enhance killing of some bacteria
Stimulate immunoglobulin production
Alter lymphocyte proliferation
Exhibit antibacterial activity
Alter production of free radicals and cytokines

SP-A and SP-D affect a variety of immune cell functions. The table summarizes the reported effects of at least one of the surfactant proteins. In some cases, for example enhancement of phagocytosis, only one of the proteins, SP-A, is effective. In other cases the reported effects are controversial. For example, SP-A has been shown to both stimulate and inhibit lymphocyte proliferation and production of TNF-α by macrophages. See text for details and references.

Table 9.3. Effects of surfactant replacement preparations on immune cell function

Decrease phagocytosis
Decrease cytokine release
Inhibit lymphocyte proliferation
Inhibit adhesion molecule expression
Inhibit immunoglobulin production
Inhibit DNA synthesis

Many of the effects of replacement surfactants can be attributed to the lipid components. See text for references and details.

Table 9.4. Effects of surfactant lipids on immune cell function

Enhance chemotaxis
Increase phagocytosis
Alter free radical production
Alter lymphocyte proliferation

Surfactant lipids affect a variety of immune cell functions. In some cases, conflicting data have been reported. For example, surfactant lipids have been reported to both enhance and inhibit lymphocyte proliferation and free radical production. Some of these discrepancies may be due to the variable composition of the surfactant preparations used. Some specific lipids stimulate lymphocyte proliferation, whereas others inhibit proliferation. See text for details and references.

Curosurf, which contains SP-B and SP-C but no SP-A, has no effect on chemiluminescence.[68]

Both SP-A and SP-D enhance the production of free radicals by alveolar macrophages[77,78] as measured by lucigen-dependent chemiluminescence. Weissbach et al[31] observed that SP-A attached to a surface is more effective in enhancing free radical production than SP-A in solution. These studies suggest that SP-A that is attached to a surface (e.g., a tissue culture plate or a bacterium or virus) may have enhanced functionality compared to SP-A in solution, thus raising the possibility that SP-A associated with pathogens such as bacteria and viruses may have an enhanced ability to stimulate immune cells.

In contrast, Weber and coworkers reported that SP-A isolated from dog lavage inhibits the respiratory burst of alveolar macrophages.[79] The lack of an effect of dog SP-A may be explained by the finding that canine SP-A appears to be susceptible to inactivation or denaturation by butanol extraction, which is routinely used in SP-A purification protocols.[26] However, Katsura et al[80] also found that rat SP-A inhibits superoxide production stimulated with zymosan and TPA. The reasons for these different findings are not known.

In contrast, neither SP-D nor SP-D associated with influenza virus stimulate production of superoxide anion by neutrophils.[47] Furthermore, SP-A does not stimulate the production of free radicals by peripheral monocytes.[31] These data are consistent with the possibility that different accessory cells respond differently to the surfactant proteins.

Effects of Surfactant on Chemotaxis

It has long been recognized that the postnatal influx of alveolar macrophages occurs concurrently with the intra-alveolar increase in surfactant that occurs at the same time. In order to test the possibility that surfactant induces the influx, Schwartz and Christiman[81] tested bronoalveolar lavage fluid for chemotactic activity and reported that it stimulates the migration of alveolar macrophages. The active component was separated on a Sepharose column and found to have a molecular mass of approximately 5 kDa. Purified surfactant stimulates migration and polarization of monkey alveolar macrophages and monocytes in vitro and the active component has a molecular mass greater than 10 kDa.[82] Hoffman et al[83] reported that a delipidated surfactant preparation increases migration of macrophages that are stimulated with endotoxin-activated serum. The data are consistent with the possibility that the active component is a protein.

Purified DPPC inhibits the directed migration of alveolar macrophages stimulated with activated serum.[84]

Both SP-A and SP-D enhance cell migration. SP-A stimulates chemotaxis of alveolar macrophages and peritoneal macrophages;[85] SP-D is a potent chemoattractant for neutrophils and monocytes.[86] Although a direct comparison was not made between the two proteins, SP-D appears to be a more effect stimulant. A concentration of approximately 10-11 M SP-D (5 ng/ml) induces a degree of migration comparable to that induced by a potent chemotactic stimulus, N-formyl-met-leu-phe. SP-A is active in the range of several μg/ml. The stimulation of chemotaxis by SP-D is inhibited by maltose, suggesting that the carbohydrate recognition domain of SP-D may be involved in the response.

Curosurf does not stimulate chemotaxis directly, nor does it augment zymosan-induced chemotaxis (or generation of chemiluminescence) by peripheral blood monocytes.[68]

Effects of Surfactant on Lymphocyte Proliferation

Lung lymphocytes and alveolar macrophages tend to be less responsive to mitogens than their serum counterparts. The possibility that exposure to surfactant may suppress lymphocyte function has been supported by studies in which treatment of circulating lymphocytes with surfactant results in a phenotype more typical of lung lymphocytes or

alveolar macrophages.[28,87-89] Both phosphatidylcholine (PC) and phosphatidylglycerol (PG) are potent inhibitors of lymphocyte proliferation. Surfactant and isolated surfactant lipids also suppress the functions of cytotoxic T cells and antibody-forming B cells in vitro.[90]

The findings of Wilsher and coworkers that some specific phospholipids stimulate and others inhibit the phytohemagglutinin (PHA)-induced proliferation of lymphocytes raises the interesting possibility that changes in lipid ratios that occur in disease states may result in altered states of lymphocyte activation.[91,92] They found that PC and PG inhibit lymphocyte proliferation, whereas other less abundant lipids, including phosphatidylethanolamine, sphingomyelin and cholesterol, stimulate proliferation.

Studies by Lesur et al[93] support this hypothesis. They found that the ability of surfactant lipids to affect lymphocyte proliferation is altered in disease states. For example, the lavage fluid from patients with alveolar proteinosis is a less effective inhibitor of lymphocyte proliferation than that from control patients. Lavage fluid from patients with hypersensitivity pneumonitis and sarcoidosis stimulate lymphocyte proliferation. Changes in the ratios of the surfactant lipids may account for some of the altered ability to enhance or inhibit proliferation.

The mechanisms by which lipids inhibit lymphocyte proliferation have not been elucidated. However, it has been shown in other systems that changes in cell membrane fluidity occur when cells are incubated with phospholipids and that these changes correlate with the mitogenic response of blood lymphocytes to concanavlin A (ConA).[94]

Survanta inhibits ConA-induced lymphocyte proliferation.[28] Survanta also inhibits the proliferative response of human peripheral blood lymphocytes, as well as purified $CD56^+/CD3^-$lymphocytes that are stimulated with interleukin (IL)-2. In addition, the killing of two tumor cell lines is also inhibited.[72] Although the precise mechanism of the inhibition is not known, it was shown that surfactant decreases binding to the tumors of lymphocytes enriched in killer cells.

A surfactant replacement preparation known as SF-RI 1, which is derived from bovine lungs and contains small amounts of SP-B and SP-C, inhibits the PHA-induced proliferation of human lymphocytes.[94] Purified phospholipids including DPPC, DPPG, PI and sphingomyelin also inhibit proliferation.

A recent study by Kremlev et al[28] provides evidence that surfactant lipids and proteins may have counter-regulatory effects. For example both DPPC and Survanta inhibit proliferation of lymphocytes stimulated with Con A; in contrast, SP-A enhances Con A-mediated proliferation. Interestingly, when they are combined, SP-A reverses the inhibitory effect of Survanta. The findings are consistent with the hypothesis that changes in the ratios of surfactant lipids and protein that occur in disease states may be related to alterations in immune cell function.

Borron and coworkers reported that SP-A inhibits the proliferation of lymphocytes stimulated with PHA and anti-CD3.[95] The inhibition correlates with a reduced production of IL-2. The proliferation stimulated with Con A was slightly enhanced by SP-A, consistent with the findings of Kremlev et al.[28]

In summary, surfactant lipids (Table 9.4) and surfactant replacement preparations (Table 9.3) tend to inhibit lymphocyte proliferation, although specific lipids may stimulate proliferation. SP-A both inhibits and stimulates proliferation (Table 9.2). The reasons for these apparent inconsistencies are not clear, but may be related to differences in experimental design or preparations of surfactant.

Effects of Surfactant on Cytokine Production and Release

The regulation of cytokine production by replacement surfactant preparations has been investigated in some detail. In general, the replacement surfactants inhibit cytokine release

from immune cells. For example, Curosurf inhibits secretion of tumor necrosis factor-α (TNF-α) by LPS-stimulated monocytes,[68] and Exosurf® (Burroughs Wellcome, Research Triangle Park, NC) and Survanta both reduce the production of IL-1β, pro-IL-1β and IL-1 receptor antagonist by human alveolar macrophages stimulated with LPS.[96] In addition, Exosurf and Survanta inhibit release of TNF-α, IL-1β and IL-6[97] as well as the message levels for these cytokines in human alveolar macrophages stimulated with endotoxin.[98] The finding that IL-8 levels are not reduced suggests that this effect is not due to an overall inhibition of cytokine release.[97] Tyloxapol, a nonionic detergent, appears to be the active ingredient in Exosurf.[99] The inhibitory component of the Survanta preparation has not been identified; however, it cannot be tyloxapol, as this is not an ingredient of Survanta.

Both Survanta and Exosurf decrease the activation of NF-κB, a factor that activates the transcription of multiple inflammatory cytokines in the monocytic cell line THP-1.[100] Both surfactant preparations also inhibit the IL-1 stimulated release of IL-6 and prostaglandin E2 by fibroblasts.[101] These studies suggest that the inhibition of cytokine expression is not specific to monocytes and macrophages and that the suppression may be regulated at the transcriptional level via NF-κB activation.

Surfactant proteins both stimulate and inhibit cytokine production. Kremlev and Phelps[102] found that SP-A stimulates the production of TNF-α, IL-1α, IL-1β and IL-6 by human peripheral blood mononuclear cells. The enhanced production of TNF-α was also demonstrated with rat peripheral blood mononuclear cells, alveolar macrophages and splenocytes. In addition, SP-A enhances the production of IgG, IgA and IgM by splenocytes. Blau et al[103] showed that SP-A stimulates the release of colony stimulating factor (CSF) from both isolated alveolar type II cells and macrophages in vitro. Recent studies have implicated granulocyte-macrophage CSF (GM-CSF) in the regulation of surfactant metabolism.[104]

SP-A also inhibits cytokine production. For example, McIntosh et al[105] found that SP-A inhibits TNF-α release by alveolar macrophages stimulated with LPS. Borron et al[95] reported that SP-A inhibits production of IL-2 in PHA-stimulated mononuclear cells. The serum collectin MBP also inhibits release of TNF-α from human monocytes stimulated by rhamnose-glucose polymers from streptococcal cell wall polysaccharides.[106]

Receptors for Surfactant Proteins

The finding that SP-A and SP-D affect immune cell function is consistent with the possibility that immune cells express specific receptors for these proteins, a possibility supported by binding data showing that SP-A[107-109] and SP-D[110,111] bind specifically and with high affinity to immune cell surfaces. Indirect evidence from all of these studies suggest that there may be more than one, if not several, receptors for both SP-A and SP-D.

SP-A binds to C1q receptors of approximately 56 kDa,[112-114] and the collagen-like domains of the proteins appear to mediate this interaction. Chroneos and coworkers have purified a 210 kDa protein by SP-A affinity chromatography using lung membranes as a starting material.[115] Antibodies against the protein recognize immunoreactive material in alveolar macrophages, type II cells, rat bone marrow-derived macrophages and U937 cells. The receptor also binds MBP. Alveolar type II cells also appear to express other distinct SP-A binding proteins of 30 kDa[116] and 55 kDa[117] molecular size. Neither of these proteins is found on alveolar macrophages. Although SP-A binds to the 56 kDa C1q receptor, SP-D does not appear to do so.

SP-A Deficient Mice Show Altered Susceptibility to Infection

Until recently, most of the evidence supporting host defense functions of surfactant was obtained from in vitro studies. Recently, however, LeVine et al[13] demonstrated that

mice made deficient in SP-A by homologous recombination have an increased susceptibility to infection with Group B streptococcus, a pulmonary pathogen of extreme clinical importance in the newborn infant.

The SP-A deficient mice (SP-A -/-) received an intratracheal instillation of a clinical isolate of Group B streptococcus. At various later times, the mice were sacrificed, the lungs lavaged and samples of the lungs and spleen were cultured to measure the persistence and dissemination of the Group B streptococcus. The number of colony forming units of Group B streptococcus that remained in the lung 6 hours after instillation was greater in the SP-A -/- mice than in SP-A +/+ mice. Clearance was decreased in the SP-A -/- mice, as there were fewer bacteria associated with alveolar macrophages from the SP-A -/- than from the SP-A +/+ group (Fig. 9.2). Interestingly, the number of colony forming units were also increased in the spleens of the SP-A -/- mice, suggesting that in the absence of SP-A the bacteria may more readily disseminate into other organs.

It is of note that the SP-D levels in SP-A -/- mice are normal. The fact that Group B streptococci are still cleared from the lungs of SP-A -/- mice, albeit more slowly than from those of SP-A +/+ mice, suggests that other factors, possibly SP-D, may also be important in the clearance of pulmonary pathogens.

Changes in Surfactant in Disease States

It seems reasonable to speculate that changes in surfactant levels may alter susceptibility to disease or infection. This possibility, although unproven and difficult to test, is consistent with the data obtained from the SP-A deficient mice, discussed above. Although, in many cases, changes in surfactant could cause a predisposition to disease it also seems likely that the disease itself may lead to changes in surfactant levels if, for example, the alveolar epithelium is directly damaged or there is a large influx of serum proteins that inhibit surfactant function. Furthermore, changes in surfactant may occur secondary to changes in steroids, growth factors or cytokines, the levels of which may be altered in disease states.

The changes in levels of various surfactant components in diseases and in animal models are summarized in Table 9.5 and the changes in individual components are discussed separately below.

Infant RDS is a direct result of low levels of surfactant lipids and proteins secondary to immaturity of the surfactant synthetic system. The causes and/or consequences of alterations in lipid levels and composition in many other disease states are not as clear. Surfactant lipid levels are decreased and/or are abnormal in composition in adult or acute RDS (ARDS).[118,119] The low levels of lipids and proteins in ARDS are not due to an immature surfactant system but rather to an inactivation of surfactant or an inhibition of its ability to reduce surface tension (reviewed in ref. 120). Lipid levels are also reduced and altered in composition in idiopathic pulmonary fibrosis[121] and some types of pneumonia,[22-125] although the mechanism of these changes is not defined. Exposure to high concentrations of oxygen has been reported to both decrease[126] and increase[124,125] surfactant lipid levels.

Both SP-A and SP-D, as well as surfactant lipids, are increased in silicosis[127-129] and alveolar proteinosis.[130] It is not known whether the increase in surfactant is a result of increased production, decreased clearance or a combination of the two. Miller and coworkers have shown that type II cells isolated from silica-treated rats synthesize DPPC and SP-A at increased rates.[127] Silica treatment of animals also increases both secretion and clearance of surfactant.[131] The activation of the type II cell may be related to the state of inflammation.[129] Likewise, the cause of the accumulation of surfactant in alveolar proteinosis is not clearly established. Recent and surprising studies[104,132] have implicated GM-CSF in this disease. GM-CSF, a factor long thought to be involved in hematopoiesis, was ablated by recombinant technology. The only observable phenotype, which was unexpected, was an alveolar

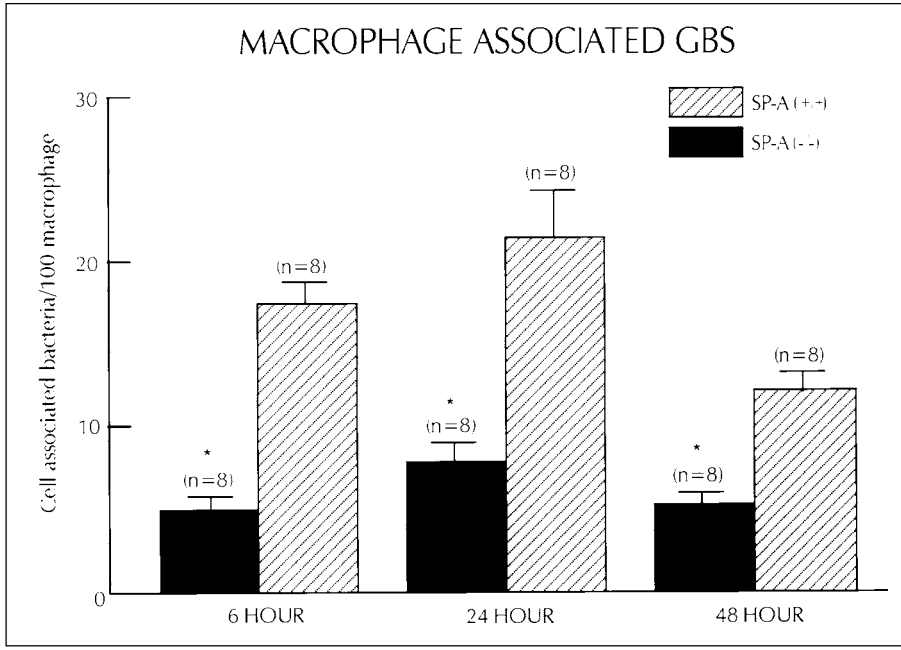


Fig. 9.2. Association of Group B streptococcus with alveolar macrophages from SP-A deficient (SP-A -/-) and control (SP-A +/+) mice. Group B streptococcus was instilled intratracheally to control and SP-A deficient mice and the lungs were subsequently lavaged at the times indicated. Cytospin preparations were stained with Diff-Quik, and bacteria associated and within the perimeter of the cell counted. Reproduced with permission from LeVine AM, Bruno MD, Huelsman KM et al. Surfactant protein-A deficient mice are susceptible to group B streptococcal infection. J Immunol 1997;158:4336-4340. Copyright 1997. The American Association of Immunologists.

proteinosis-like accumulation of surfactant in the lungs. Although the relationship of GM-CSF to this proteinosis-like state is not clear, additional studies with this interesting animal model should provide important new information about both the disease of alveolar proteinosis and surfactant turnover and metabolism.

The instillation of intratracheal LPS in rats increases surfactant lipid levels.[129] The increase at time points greater than 24 hours is probably due at least partly to an increased proliferation of type II cells seen in this model.

The production of surfactant proteins in a variety of in vivo conditions and in vitro models is affected by a variety of steroids, cytokines and growth factors, the levels of which may all change dramatically in disease. For example, SP-A expression levels are affected by dexamethasone,[133-139] insulin,[140] prostaglandins,[141] cyclic AMP,[142,143] TPA,[144] TNF-α,[145] transforming growth factor-β[146] and epidermal growth factor.[147] Ballard and coworkers reported the provocative finding that interferon-γ upregulates SP-A production in vitro.[148] The effects of cytokines and growth factors on regulation of SP-D have not been thoroughly investigated; however, Dulkerian et al[149] recently reported the somewhat surprising finding that interferon-γ does not increase the production of SP-D in cultured explants of fetal lung. Other agents also differentially regulate SP-D and SP-A in this culture system. For example, both LPS and TNF-α decrease levels of SP-A mRNA and have no effect on levels of SP-D.

Table 9.5. Changes in surfactant components in disease states and in animal models of lung disease

Condition	SP-A Levels	SP-D Levels	Lipid Levels
AIDS-related pneumonia	↑[151,152,168]	↓[135,153,169]	↓[123,170]; altered composition[171]
Alveolar proteinosis	↑[172]	↑[130]	↑[173]
ARDS (polytrauma)	↓[118,155]		↓[18,155]; altered composition[119]
Bacterial pneumonia	↓[151]		↓[122]
Bleomycin-induced pulmonary fibrosis	↔[174]		↓ then ↑[129,174]
BPD (animal model) + infection	↓[159]		
BPD (animal model)	↓[159]		
HIV-infection without *P. carinii* pneumonia	↓[168]		
Hypersensitivity pneumonitis	↑[165,166]		↔[175]
Idiopathic pulmonary fibrosis	↓[121]	↔[130]	↓ and altered composition[121,176]
Infant RDS	↓[177-180]		↓[177,180,181]
Intratracheal LPS	↑[129]	↑[129]	↑[129]
Oxygen exposure*	↑[125,162]		↓[126,182]; ↑[124,125,129,183]
Silicosis	↑[129,184]	↑[128]	↑[129,184]
Smokers	↓[167]	↓[167]	↔[167]; altered composition[185]

*The studies on lipid levels include different exposure times and animal species.

Patients and animals with *P. carinii* infections both have elevated levels of SP-A.[49,150-152] SP-A levels are increased in human immunodeficiency virus (HIV)-positive patients with *P. carinii* infections compared to HIV-positive *P. carinii*-negative patients.[49] The SP-A levels in HIV patients are significantly lower then in healthy, uninfected, normal volunteers. Interestingly, there is a significant correlation between the levels of SP-A and the abundance of *P. carinii* in the lavage. Limper et al[153] reported that SP-D levels are increased in an immunosuppression model of *P. carinii* infection in rats.

SP-A levels are decreased in patients with both gram positive and gram negative pneumonia, although the reduction is greater in the gram positive group.[151] LeVine et al[154] have shown that SP-A levels are decreased relative to total protein in tracheal aspirates from children with bacterial pneumonia, viral pneumonitis and ARDS. Children on cardiopulmonary bypass have normal SP-A levels.[155]

SP-A levels are also decreased in adults with ARDS.[118,155] In patients with the most severe lung injury, the levels of SP-A remain low. In patients with moderate lung injury, SP-A returns to normal levels and, in fact, increases to greater than control values by 3-6 days after injury. That the changes in the SP-A levels do not correlate with the time-dependent changes in lipid levels[118] is consistent with the possibility that lipid and SP-A levels are independently regulated. Recently, Doyle et al[156] found that SP-A levels are increased in sera of patients with acute cardiogenic pulmonary edema and proposed that SP-A leaks through the damaged alveolar/capillary endothelial barrier into the blood. SP-A levels are also increased in the sera[157] and decreased in the lavage[121] of patients with idiopathic pulmonary fibrosis. SP-D levels are unchanged in the lavage fluid of patients with idiopathic pulmonary fibrosis, but there is a detectable increase in immunoreactive material in the sera of

patients with fibrosis,[158] as well as pulmonary alveolar proteinosis, pulmonary sarcoidosis and tuberculosis.[130]

Coalson et al[159,160] reported that surfactant protein levels are altered in a primate model of bronchopulmonary dysplasia (BPD) induced by hyperoxia and in a BPD model with a superimposed infection. Levels of SP-A mRNA in alveolar cells are lower in the BPD and BPD plus infection groups compared to controls. Interestingly, the levels of SP-A in the BPD plus infection animals are greater than in the simple BPD without infection model. These findings are consistent with the possibility that the superimposed infection may affect SP-A levels. The message levels for SP-B are increased in both the BPD and BPD plus infection animals and the levels for SP-C are similar to control in both models. Hyperoxia increases levels of SP-A in a number of animal models,[125,161,162] and Horowitz et al[125,161,162] reported the interesting finding that the relative increase in SP-A and SP-B message levels is much greater in Clara cells than in type II cells, suggesting that expression of the proteins in these two cell types may be regulated differently.

The intratracheal instillation of LPS into rats results in increased SP-A levels in lung tissues as soon as 6 hours after instillation, increased lavage levels of SP-A by 24 hours and increased levels of SP-D in lavage fluid by 72 hours.[163] Message levels for SP-A and SP-D are increased at 24 hours, whereas levels for SP-B are unchanged. Sughara et al[164] used higher doses of LPS and reported increases in SP-A, SP-B and SP-C 3-7 days after instillation and concluded that the increase at that time is due largely to proliferation of type II cells. Vivano et al[129] showed that intratracheal instillation of LPS into rats results in increased intra- and extra-cellular surfactant phospholipids. These studies taken together suggest that LPS and possibly other acute inflammatory stimuli may alter surfactant levels via a variety of mechanisms, including increases in protein synthesis at short times after instillation as well as changes in type II cell numbers at longer times after instillation. It seems likely that alterations in surfactant turnover and clearance may also contribute to the observed changes.

SP-A levels are increased in patients with hypersensitivity pneumonitis[165,166] and sarcoidosis,[166] and decreased in the lavage of smoker's lungs.[167] SP-D levels are unchanged in the lavage fluid recovered from patients with sarcoidosis as well as interstitial pneumonia with collagen disease.[130]

Summary and Future Directions

Although the details of a role for surfactant in host defense are in many ways still undefined, the studies described above support the following model (Fig. 9.3). Lipids, in general, tend to suppress immune cell function, in particular lymphocyte proliferation. Thus, when the levels of surfactant lipids are reduced, the inflammatory response may be enhanced by increases in lymphocyte proliferation. The surfactant proteins SP-A and SP-D appear to interact with carbohydrates and other molecules on the surface of inhaled pathogens. These pathogens, thus opsonized, are targeted to effector cells, such as alveolar macrophages, which are attracted to the site of infection/inflammation. SP-A and SP-D may be upregulated in acute infection or inflammation, thus serving to help protect the lungs by facilitating the clearance of inhaled pathogens to which they have bound. Phagocytosis of some pathogens is enhanced by SP-A (and possibly SP-D, although this has not been demonstrated) and the internalized pathogen may, in some cases, be killed by the immune cell. Some studies show that SP-A upregulates the production of inflammatory cytokines, such as TNF-α, and reactive intermediates. Other studies show that these processes are inhibited. Additional investigation will be required to resolve these inconsistencies.

Surfactant lipids and proteins are altered in a variety of diseases states; however, it is generally impossible to determine if these alterations are a cause or an effect of the disease.

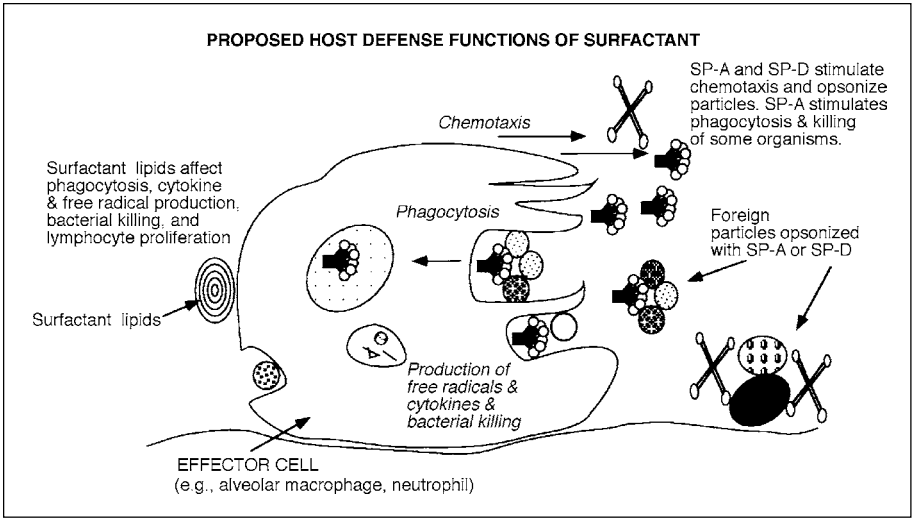


Fig. 9.3 Hypothetical host defense functions of surfactant. Many of the effects of surfactant on immune cell function have been described only in vitro, and thus many aspects of this model remain to be confirmed. See text for details and references.

Recent, exciting studies showing that mice in which the SP-A gene is ablated have an increased susceptibility to infection with Group B streptococcus provide compelling evidence for a role of surfactant proteins in the host defense response. Additional studies are required to define the mechanism by which protection is conferred, and additional studies with mice in which the SP-D gene and both the SP-A and SP-D genes are ablated will help clarify further the role of these proteins in the host defense response.

Acknowledgments

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