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REVIEW ARTICLE

Function and regulation of expression of pulmonary surfactant-associated proteins

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INTRODUCTION

The respiratory tree of the lung consists of a graduated series of conducting airways that terminate distally in the alveoli. Gas exchange occurs across the alveolar epithelium during alternate expansion (inspiration) and contraction (expiration) of the alveoli. The collapsing forces that narrow the alveolar diameter during expiration are the product of elastic tissues within the alveolar interstitial wall, and surface tension generated by a thin aqueous film on the surface of the alveolar epithelium. The dramatic increase in surface tension at end expiration poses a major obstacle to alveolar stability at low lung volumes and makes inflation of the lung much more difficult. This potential problem is overcome by the juxtaposition of a complex mixture of lipids and proteins, pulmonary surfactant, at the interface of the aqueous film and air within the alveolar lumen. The specialized structure of the surfactant allows dense packing of the lipid film during expiration, thereby opposing the surface tension generated by the aqueous subphase. In the absence of pulmonary surfactant, increased surface tension along the alveolar epithelium results in alveolar collapse and epithelial cell lysis culminating in respiratory distress syndrome (RDS), a major cause of morbidity and mortality in preterm infants. Treatment of RDS frequently requires respiratory support in order to achieve effective gas exchange. In recent years, the advent of replacement surfactant mixtures as a therapy for RDS has reduced the requirement for respiratory support and significantly improved the short-term outcome of infants suffering from RDS.

Pulmonary surfactant is composed of approximately 90% lipid, 10% protein and small amounts of carbohydrate. Dipalmitoylphosphatidylcholine (DPPC), which accounts for approximately half the lipid in surfactant, is primarily responsible for the surface tension reducing property of the surfactant complex. The synthesis, secretion and metabolism of DPPC and other surfactant lipids has been the subject of several recent reviews [1–5]. Specific surfactant proteins, SP-A, SP-B and SP-C, closely associated with surfactant lipids, contribute to the surfactant properties of the phospholipids. Recent studies suggest that surfactant-associated proteins may play other important roles in surfactant biology. The surfactant proteins have been the subject of several recent reviews [6–8] and the reader is directed to a review by Possmayer [8] for an historical perspective of the subject. The present review will focus on recent studies that provide insight into the regulation of expression and function of surfactant-associated proteins.

Identification of surfactant-associated proteins in broncheolar lavage fluid

Pulmonary surfactant is isolated by differential centrifugation of bronchoalveolar lavage fluid. The lavage procedure recovers a relatively large number of cells, predominantly alveolar macrophages, which are removed by centrifugation at low speed. The lipid-rich pellet, recovered by high-speed (10 000 *g*) centrifugation of the cell-free supernatant contains numerous proteins, many of which have been identified as serum proteins [9]. Extraction of the lipid components of the pellet into organic solvents leaves behind a major non-serum surfactant-associated protein referred as surfactant protein A (SP-A). In the human, SP-A consists of a major charge train of 9–13 proteins (M_r 34000–36000, pI 4.4–5.0) and a minor charge train of three proteins (M_r 28000–30000, pI 4.6–5.0) [10]. The charge and size heterogeneity of SP-A varies among species and primarily reflects the addition of one or two asparagine-linked oligosaccharide chains to a species-specific protein backbone of approx. 230 residues. SP-A exists in the airway as thiol-dependent and non-thiol-dependent [11] oligomers with sizes variously estimated as 1.6×10^6 kDa [11] and 650 kDa [12,13] by gel filtration analyses of rat SP-A. The latter estimation agrees well with a recent study [14] using electron microscopy and rotary shadowing, in which the structure of the native canine and recombinant human SP-A was resolved as an oligomer of 18 monomers arranged as six triple helices, similar to the structure reported for C1q [15].

Unlike SP-A, two other surfactant-associated proteins, SP-B and SP-C, are extremely hydrophobic. These peptides extract with lipids into ether/ethanol and coelute with phosphatidylglycerol (PG) and phosphatidylethanolamine during subsequent silicic acid column chromatography [16,17]. The larger of the two peptides, SP-B, is resolved as a protein of M_r 8000 following SDS/PAGE under reducing conditions. Consistent with this size estimate, amino acid sequence analyses identified SP-B as a peptide of 79 residues [17,18,19]. SP-B forms thiol-dependent oligomers with the dimer being the predominant form in most species studied to date. Although SP-B is somewhat soluble in aqueous solution and can be isolated in the presence of detergent [20], SP-C is soluble only in organic solvents. Amino acid sequence analyses of SP-C have identified three peptides of 33–35 residues differing only in the origin of the *N*-terminus [19,21,22]; the largest of these peptides is the predominant form in human surfactant [22]. SP-C aggregates *in vitro* to form non-thiol-dependent dimers [23].

A fourth surfactant-associated protein, SP-D, has recently been identified in rat bronchoalveolar lavage fluid [24,25]. SP-D consists of a charge train of proteins (M_r 43000, pI 6–8) that form disulphide-bonded trimers. SP-D is similar to SP-A in that it contains a bacterial collagenase-sensitive domain, hydroxyproline, asparagine-linked oligosaccharide and lectin-like activity [26], but differs in the presence of hydroxylysine and hydroxylysine glycosides.

Abbreviations used: CRE, cyclic AMP responsive element; DPPC, dipalmitoylphosphatidylcholine; DT-A, diphtheria toxin-A; EGF, epidermal growth factor; IFN-γ, interferon-γ; PC, phosphatidylcholine; PG, phosphatidylglycerol; RDS, respiratory distress syndrome; SP, surfactant protein; TPA, phorbol 12-myristate 13-acetate.

STRUCTURE OF SURFACTANT PROTEINS

Genomic clones encoding SP-A, SP-B and SP-C have been identified. Although no nucleic acid sequence is yet available for SP-D, processing studies and limited amino acid sequence data suggest that the organization of this protein may be very similar to that of SP-A. In this section, the primary structure of surfactant proteins, as deduced from cDNA and genomic clones, will be discussed and potential functional domains identified on the basis of homology with other proteins.

SP-A

Human SP-A (see Fig. 1) is encoded by approx. 4.5 kb of DNA on the long arm of chromosome 10 [27,28]; the structure of the mouse SP-A gene is very similar with an overall nucleotide similarity of 65% with the human gene [29]. The organization of the SP-A gene and the protein it encodes is remarkably similar to the gene for the mannose-binding protein [30,31], which has also been localized to the long arm of chromosome 10 [31]. The SP-A gene consists of five exons with the coding sequence distributed among four exons. The 5′-untranslated region consists of less than 100 nucleotides, on exons 1 and 2, while the 3′-untranslated region is approx. 1300 nucleotides in length and is entirely encoded by exon 5 [32]. Several lines of evidence suggest that there may be more than one SP-A gene in the human. Three human SP-A cDNAs have been isolated that differ by up to 3% in the coding regions and up to 15% in the non-coding sequence [32,33]. The cDNAs differ from the published human genomic

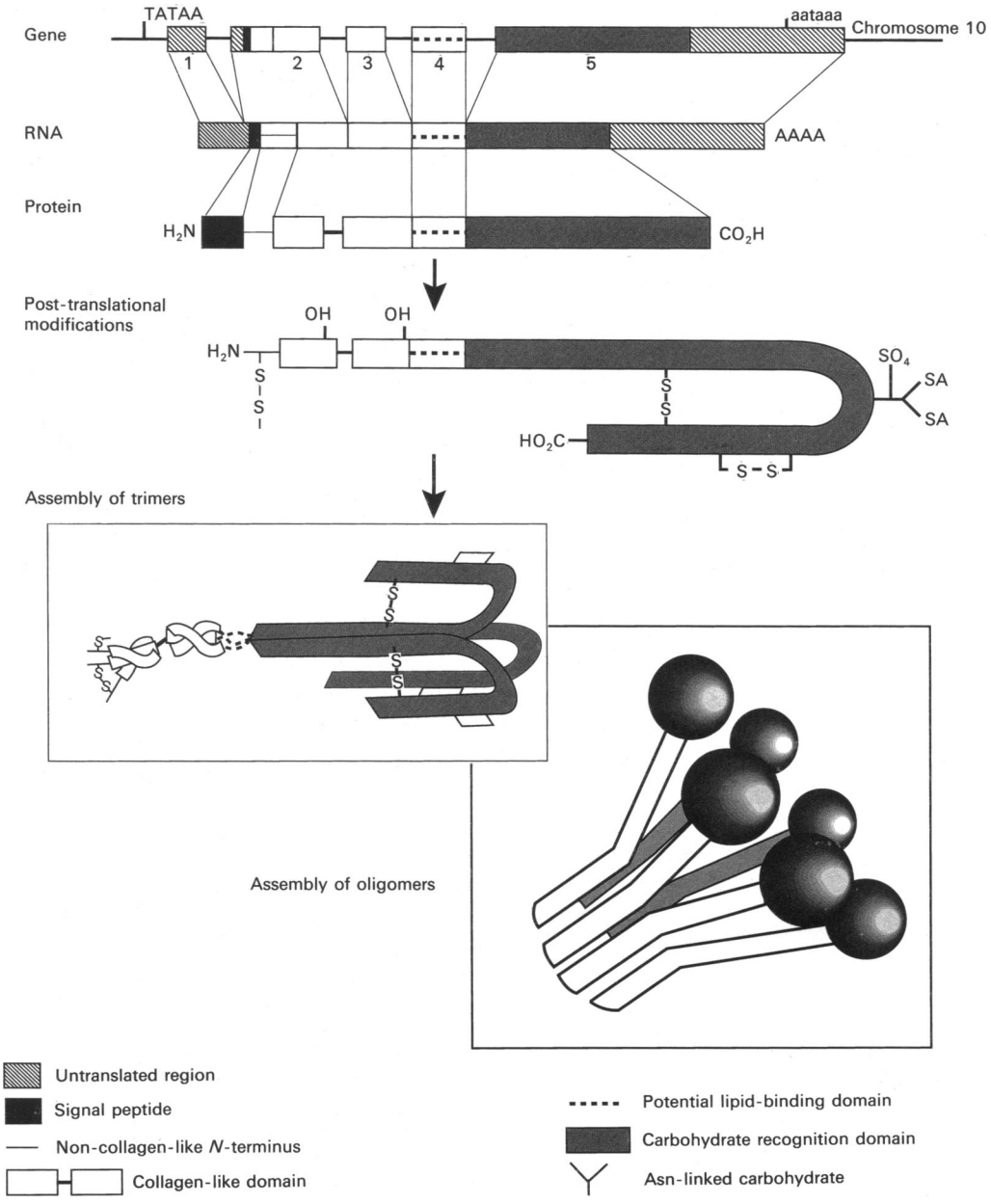

Fig. 1. Gene, RNA and proposed protein structure of human SP-A

The human SP-A gene is encoded by five exons (filled boxes) on chromosome 10. The approx. 2.2 kb SP-A mRNA encodes a protein of 248 amino acids including a 20-amino-acid signal peptide. Co- or post-translational modifications include signal peptide cleavage, inter- and intrachain disulphide bond formation, hydroxylation of specific proline residues and the formation of a triple helix in the collagen-like region, the addition of Asn-linked carbohydrate and sulphation of the carbohydrate moiety. Mature SP-A consists of six trimers of SP-A similar to the structure proposed for Clq.

sequence [32] by 4, 7 and 18 nucleotides that result in 3, 4, and 9 amino acid changes, respectively [32,33]. One of these cDNAs indicates the presence of another exon encoding approx. 30 nucleotides of 5′-untranslated region [33]. Restriction maps of human genomic DNA are also consistent with the presence of more than one SP-A gene [28]. Recent work by Korfhagen *et al.* [34] identified a human SP-A pseudogene, consisting of sequences with 84% homology to intron 4 and exon 5 of the human SP-A gene, which also maps to chromosome 10. Interestingly, a rat mannose-binding protein pseudogene with 77% similarity to exons 3 and 4 of the mannose-binding protein gene has also been identified [30]. Collectively, these observations suggest that SP-A may belong to a larger family of closely related proteins. Although it appears that there is more than one SP-A gene in the human, similar studies in rat [35], rabbit [36] and mouse [29] suggest that there is only one SP-A gene in these species. The functional implication of multiple human SP-A genes therefore remains unclear.

The SP-A gene encodes a mRNA of 2.2 kb in the human [37] and the dog [38]. Multiple SP-A RNAs of 1.6 and 0.9 kb in the rat [35,39], 2.0 and 3.0 kb in the rabbit [36] and 3.0, 1.7 and 1.0 kb in the mouse [29] likely arise from utilization of alternative poly(A)$^+$ addition signals in the 3′-untranslated region of the mRNA. SP-A RNA encodes a protein of 248 amino acids in the human [32,33], rat [39] and dog [38] and 247 amino acids in the rabbit [36]; rat and rabbit SP-A are 64% and 74% similar to human SP-A. The structural organization of the proprotein is very similar to that for mannose-binding protein [30,31,40], an acute-phase serum protein belonging to the animal lectin family, and includes a short *N*-terminal domain, a collagen-like domain and a *C*-terminal carbohydrate recognition domain.

The *N*-terminal domain of human and rat SP-A includes a signal peptide of 20 amino acids followed by a short sequence of 7–10 residues. The cysteine in this latter sequence is invariant among the four species examined to date and is essential for interchain disulphide bond formation that leads to the multimerization of SP-A [38,41]. The comparable sequence in mannose-binding protein is somewhat larger (approx. 20 residues) and contains several cysteine residues that are also essential for oligomerization of the protein [31].

The *N*-terminal domain adjoins a collagen-like domain composed of 23–24 Gly-Xaa-Yaa repeats in which Yaa is frequently hydroxyproline. The results of circular dichroism studies are consistent with formation of a triple helix in this region of the molecule [13,14,42]. The 72-residue collagen-like domain is encoded on exons 2, 3 and 4 of the human gene [32]. The Gly-Xaa-Yaa repeats are interrupted after the 13th tripeptide by the sequence Pro-Cys-Pro-Pro; this irregularity nearly corresponds with the placement of the intron between exons 2 and 3, a pattern which also occurs in C1q [43], mannose-binding protein [30,31], and the non-fibrillar collagen genes [44]. By analogy with C1q [45] an interruption midway through the collagen-like region may result in a bend in the triple helix. Electron microscopy supports this prediction and suggests a structure composed of six triple helical molecules associated at their *N*-terminal ends to form a rod-like stem, which is presumably stabilized by the disulphide linkages of the *N*-terminal domain [14]. The bend in the middle of the collagen-like domain results in a funnel-shaped molecule that terminates in the globular non-collagenous domain.

The 148-residue non-collagenous region of SP-A (M_r 22000) is encoded on exons 4 and 5. This region includes a carbohydrate recognition domain of 130 amino acids (encoded on exon 5) which shows 30% similarity with the comparable domain in the mannose-binding protein [30,31]. These proteins belong to a larger family of carbohydrate-binding proteins which includes the asialoglycoprotein receptor [40]. The carbohydrate recognition domain of these proteins is characterized by 18 invariant residues including four cysteines, which are likely involved in ligand binding; divergence at other sites within the carbohydrate recognition domain may account for disaccharide specificity [40]. The cysteine residues in the non-collagenous region of SP-A have been shown to be involved in intrachain disulphide bonding [42,46] which presumably results in a hairpin fold in the *C*-terminus of SP-A. Binding of SP-A to mannose occurs in a calcium-dependent manner [47], as has been described for other carbohydrate-binding proteins and may play a role in the clearance of bacteria from the airway (discussed below). It has been suggested that the interaction of the carbohydrate recognition domain of mannose-binding protein with cell surface mannose produces a conformational change in the molecule that unmasks a cell attachment site leading to internalization of the complex [31].

Since SP-A clearly associates with lipid, the existence of a lipid-binding domain has also been predicted. The region immediately following the collagen-like domain, and consisting of almost all of exon 4, fits the model for a 21-residue, amphipathic helix [48]; this region is joined to the carbohydrate recognition domain by a 16-amino-acid, non-polar sequence. SP-A molecules lacking this region do not bind phospholipids [48]; however, direct evidence identifying this region as the lipid-binding domain is lacking.

SP-B

The human SP-B gene (see Fig. 2) encompasses approx. 9.5 kb and is located on chromosome 2 [49,50]. Restriction mapping of human genomic DNA is consistent with a single SP-B gene [49]. The gene consists of 11 exons including an exon encoding 823 bp of 3′-untranslated region containing a single consensus polyadenylation site [49]. The transcription start site has been mapped to a position 14 nucleotides upstream from the initiator ATG. The 5′-flanking sequence contains typical promoter elements including TATA and CAAT sequences at positions −33 and −67 respectively.

SP-B cDNA sequences for human [51–53], canine [54], rabbit [55] and rat [56] have been reported. Comparison of the coding region of human SP-B gene [49] with cDNA sequences for human SP-B, reported by Glasser *et al.* [51] and Jacobs *et al.* [52], indicate nucleotide differences resulting in one and two amino acid changes respectively. A single SP-B RNA of 2.0, 1.9 and 1.5 kb has been detected in human [51], rabbit [55], and rat [56] lung respectively. SP-B RNA encodes a protein of 381 amino acids in the human [49,51,52], 376 amino acids in rat [56], and 370 amino acids in rabbit [55]. The 79-residue active airway peptide, encoded by exons 6 and 7, is found within the sequence of this precursor protein. Across the entire precursor, approx. 67% of amino acid residues are conserved among species examined to date; the similarity increases to greater than 80% in the region of the mature peptide. The human SP-B precursor is a preproprotein consisting of a signal peptide of 20–23 residues, a propeptide of approx. 176 residues followed by the mature 79-residue SP-B peptide and a *C*-terminal peptide of 102 amino acids. The mature 79-amino-acid SP-B peptide contains three sequences that fit the model for an amphipathic helix and an extremely hydrophobic region that presumably confers close association with phospholipids [51,54]. There is also an 11-residue sequence that shares homology with the active site of mouse contrapsin proteinase inhibitor and bovine pancreatic trypsin inhibitor [56].

The secondary structure of the *N*-terminal propeptide of SP-B shares features with the sphingolipid activator proteins A and B [57,58]. Residues 66–148 of human SP-B can be aligned with

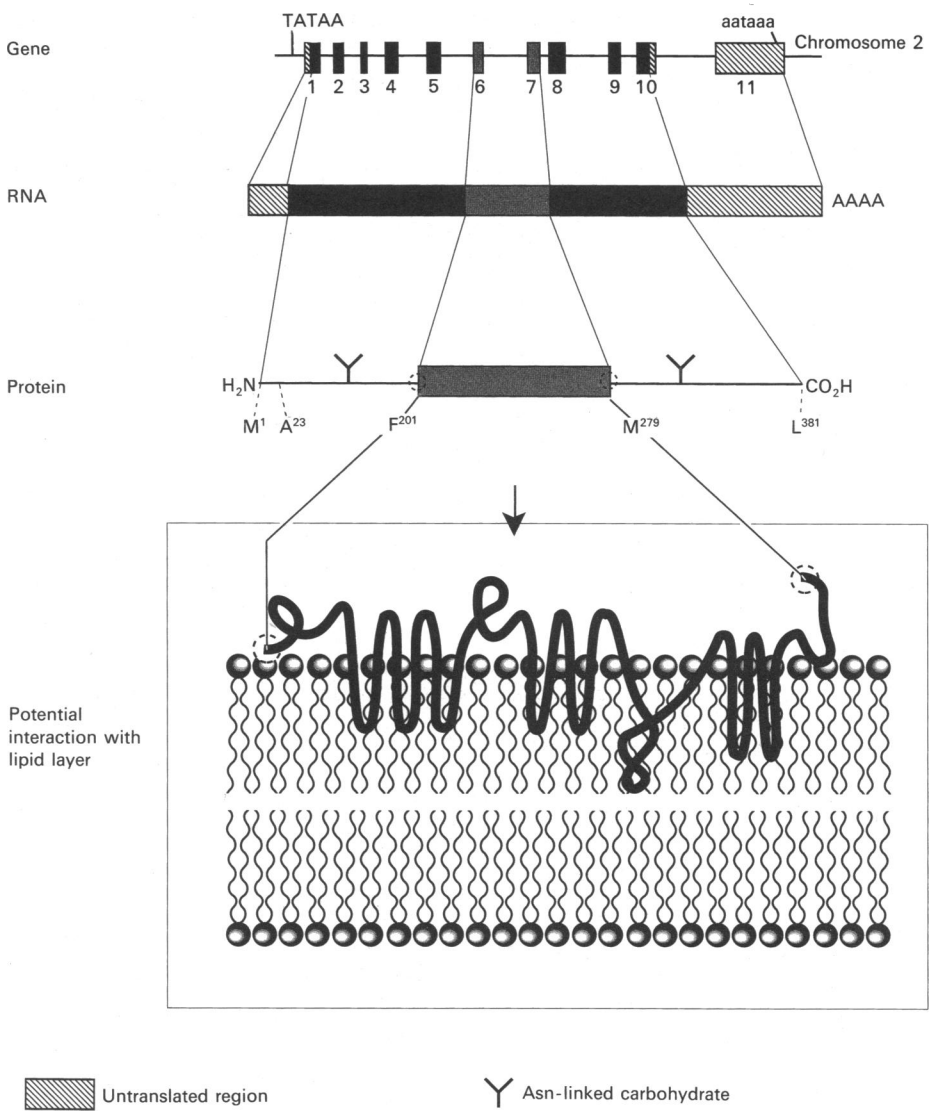

Fig. 2. Gene, RNA and proposed protein structure of SP-B

The human SP-B gene is encoded by 11 exons (filled boxes) on chromosome 2. The SP-B RNA of approx. 2 kb encodes a preproprotein of 381 amino acids. Processing of the precursor includes removal of a signal peptide of approximately 23 residues, and proteolytic cleavages between Gln^{200} and Phe^{201} and between Met^{279} and Asp^{280} to produce the 79-residue active airway peptide. A consensus sequence for the addition of complex-type Asn-linked carbohydrate is present in the *N*- and *C*-terminal peptides of the proprotein, but not the mature peptide. Potential amphipathic helices may facilitate interaction of the peptide with phospholipid.

sphingolipid activator protein A to show identical placement of the six cysteine residues. Helical wheel projections identify three potential amphipathic helices that could associate to form a cylindrical hydrophobic domain. The helices are separated by the helix breaker proline, whose position is also conserved between sphingolipid activator proteins A and B.

Other than the structural similarity with sphingolipid activator proteins there are no obvious similarities between the SP-B proprotein and other proteins of known function. It is assumed, but not proven, that the *N*- and *C*-terminal peptides of the SP-B proprotein provide a more polar environment for the extremely hydrophobic active peptide as it traverses the secretory pathway. It is unclear if these peptides also contain information for intracellular routing.

SP-C

The human SP-C gene (see Fig. 3) has been localized to the short arm of chromosome 8 [59,60]. The gene is organized into six exons encompassing approx. 2.7 kb [61]. The organization of the mouse SP-C gene is similar to that of the human but contains an insertion of 533 basepairs within intron 1 [62]. The 25 base pairs of 5′-untranslated sequence in the human SP-C gene is encoded on exon 1 while part of exon 5 and all of exon 6 encode the 3′-untranslated region. Sequences similar to the TATA and CAAT consensus promoter elements are located 34 and 62 base pairs upstream from the predicted transcription initiation site. Restriction mapping of human genomic DNA indicates the presence of two closely related genes which may be alleles [61]. Sequence comparison of the two genes shows no nucleotide differences within the first five exons indicating that they encode identical polypeptides. Nucleotide differences are restricted to exon 6, which contains two substitutions, and the introns and flanking regions of the genes. Both genes are transcribed, as indicated by detection of the corresponding cDNAs [60,61].

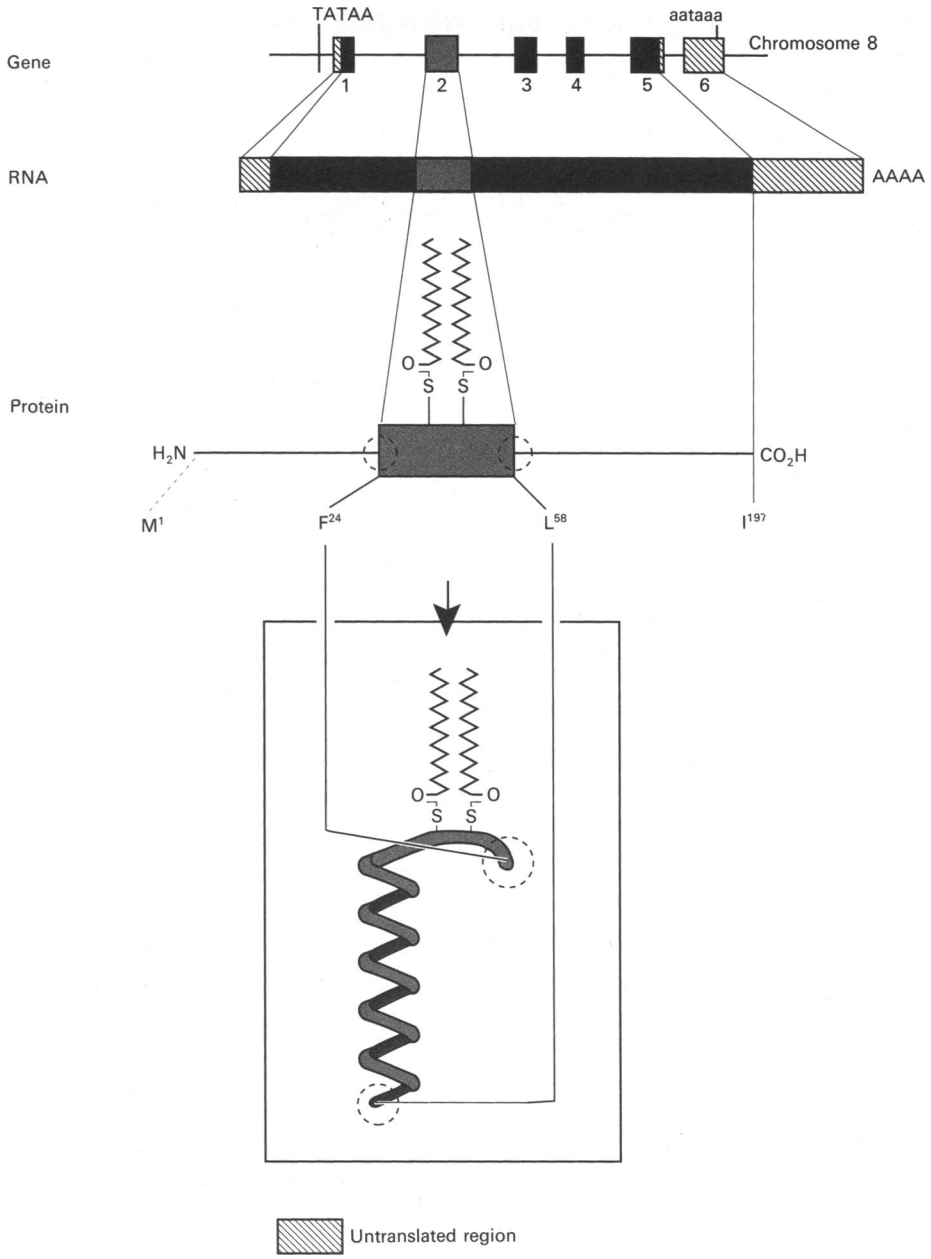

Fig. 3. Gene, RNA and proposed protein structure of SP-C

The human SP-C gene is encoded by six exons (filled boxes) on chromosome 2. SP-C RNAs of approx. 0.9 kb encode a proprotein of 191–197 residues which has neither a signal peptide or Asn-linked carbohydrate. Proteolytic cleavages between Arg^{23} and Phe^{24} and between Leu^{58} and His^{59} result in an active airway peptide of 35 residues; mature peptides with an *N*-terminus of Gly/Arg^{25} or Ile^{26} have also been detected. Palmitoylation of Cys^{28}–Cys^{29} (residues 5 and 6 of the mature peptide) has been recently reported.

Differential splicing of the primary transcript leads to several SP-C RNAs [60,61,63]. An 18-bp deletion at the beginning of exon 5 results in an mRNA encoding a protein which is reduced in size by six amino acids; it is unclear if this mRNA is actually translated. Another minor SP-C RNA species contains an eight-bp deletion, at the end of exon 5, which does not alter the size of the encoded protein. Since these deletions are not detected in the genes, different SP-C RNAs result from the use of alternate splice sites which were identified at 5′ and 3′ ends of exon 5. The SP-C RNA of approx. 0.9 kb encodes a protein of 197 amino acids (191 residues if the RNA with the 18-bp deletion is translated) in human [60,61,63], 194 amino acids in rat [64] and 190 amino acids in dog [64]. Unlike the vast majority of proteins destined to be secreted, the SP-C proprotein does not contain an *N*-terminal signal peptide. The sequence for the mature 35-residue SP-C peptide, encoded entirely on exon 2, begins at Phe^{24} (peptides with *N*-terminal residues of Gly/Arg^{25} and Ile^{26} have also been detected [22]) of the human proprotein indicating that, as for SP-B, cleavage of *N*- and *C*-terminal peptides is necessary to generate the active airway peptide. Hydropathy analysis of the proprotein identifies an extremely hydrophobic region of 23 amino acids corresponding to residues 13–33 of the mature peptide; this region, composed of 43% valine and 35% leucine or isoleucine, is predicted to form a rigid α-helix that is capable of spanning a membrane bilayer. Lysine and arginine residues, at positions 11 and 12 in the mature peptide, are conserved in all

five species examined to date [19,21,22,60,63,64]; overall conservation of amino acids in the active peptide is greater than 80%. Cysteine residues, at positions 5 and 6 in the active peptide, are palmitoylated in human and porcine SP-C [23]; it is predicted that the comparable cysteine residues in bovine and rat SP-C and serine residues at positions 8 and 9 in canine SP-C are also acylated, although the function of this modification is unclear.

As for SP-B, it is presumed that the 23-residue *N*-terminal and 133–139-residue *C*-terminal peptides of the SP-C proprotein maintains the extremely hydrophobic active peptide in a soluble state within the cell. At this time, there are no obvious similarities with other proteins of known function that might suggest alternative functions for these peptides.

THE LIFE CYCLE OF SURFACTANT PROTEINS

Sites of surfactant protein synthesis

The alveolar epithelium consists of thinly attenuated Type I cells, which occupy approx. 90% of the alveolar surface area, and an equal number of cuboidal Type II cells [65]. It is generally accepted that the Type II cell is the site of surfactant synthesis [66]. Within the Type II cell, surfactant is stored in secretory granules (lamellar bodies) whose lipid composition is similar to that of extracellular surfactant [65]. At least two surfactant proteins, SP-A and SP-B, have been localized to the lamellar body with monospecific antisera [67–70]; SP-C was also detected in a lamellar body enriched fraction of lung tissue by Western blotting [71]. The messenger RNAs encoding SP-A and SP-B have also been localized to the Type II cell by *in situ* hybridization [72]; SP-C mRNA has been detected in isolated, purified Type II epithelial cells by Northern analyses [64,73,74]. Immunoreactive SP-D has also been detected in rat Type II epithelial cells [24,25].

In addition to its localization to the Type II cell, surfactant proteins have been detected in non-ciliated bronchiolar cells (Clara cells). SP-B RNA was detected in Clara cells of both rat and human lung [72,75]. SP-A RNA [72,75] and protein [67,68] was detected in Clara cells of rat and canine lung but not human lung, suggesting species-specific expression of SP-A in the Clara cell. SP-A and SP-B RNA were not detected in other cells of the distal respiratory tree and there is no evidence for expression of SP-A [29,36,76], SP-B [56,77], or SP-C [62–64,77] in tissues other than lung.

The synthesis of and processing of surfactant proteins has been studied in a number of experimental systems including primary cultures of Type II epithelial cells, pulmonary adenocarcinoma cell lines, fetal lung explant culture and transfected cell lines. Although identification of intracellular forms of surfactant proteins has been accomplished in all of these systems, studies on the trafficking of surfactant proteins through the secretory pathway have employed pulse–chase experiments in pulmonary adenocarcinoma cell lines and primary cultures of purified Type II epithelial cells. Type II cells rapidly change their phenotype in primary culture, resulting in loss of SP-A, SP-B and SP-C expression within 24 h of isolation [73,74,78]; prolonged maintenance of Type II cell phenotype has been achieved by culturing these cells on components of extracellular matrix [74,79]. Despite the difficulties of maintaining Type II cell phenotype in primary culture, the results of pulse–chase studies in these cells are in good agreement with similar studies in pulmonary adenocarcinoma cell lines and thus likely constitute a representative experimental system.

Processing and secretion of surfactant proteins

SP-A. Based on the number of SP-A clones detected during cDNA library screening, the abundance of SP-A has been estimated to be between 0.1 and 1% [33,38,39]. The SP-A primary translation products in all species examined to date [78,80–85] are heterogeneous proteins, consisting of two or three proteins (M_r 29000) and one or two proteins (M_r 31000), pI 5–5.2, in the human [84,85]. The human M_r 29000 and 31000 proteins are apparently encoded by separate mRNAs [33]; anomalous migration of the similarly sized primary translation products may be related, in part, to post-translational hydroxylation [86]. Heterogeneity of the primary translation products may also be due in part to multiple gene products, although in rabbit [36], rat [35] and mouse [29] restriction mapping suggests only a single SP-A gene. Cotranslational acetylation, which has been demonstrated for rat [87] and human [33] SP-A *in vitro*, further contributes to the charge heterogeneity of the primary translation products. Acetylation likely occurs on the initiator methionine [88] which is cleaved with the signal peptide from the mature protein during translocation into the lumen of the endoplasmic reticulum; the role of *N*-terminal acetylation of SP-A and its occurrence *in vivo* have not been established. Comparison of the *N*-terminal sequence of mature canine [38], human [32], and rat [39] SP-A with amino acid sequences derived from their respective cDNAs indicates cleavage of a signal peptide of 17 residues in dog and 20 residues in human and rat. Signal peptide cleavage is accompanied by the transfer of high-mannose oligosaccharide from dolichol to asparagine residue 188 in human [32], rat [39], dog [38] and rabbit [36] SP-A; rat and canine SP-A have an additional site predicting asparagine-linked glycosylation near the *N*-terminus. The function of this asparagine-linked carbohydrate is unrelated to intracellular trafficking, since SP-A is correctly routed and secreted in the absence of glycosylation or in the presence of inhibitors of microsome-associated oligosaccharide-processing enzymes [89,90]. Exit of secretory proteins from the endoplasmic reticulum is dependent upon the correct folding and assembly of protein subunits within the lumen of the endoplasmic reticulum [91]. For SP-A, this involves formation of a collagen-like triple helix and thiol-dependent oligomerization of the oligomers. The assembly of thiol-dependent multimeric SP-A complexes is likely due in part to the action of protein disulphide isomerase [92]. Interestingly, this enzyme may also play a role in the formation of the collagen-like triple helix of SP-A. Protein disulphide isomerase forms the β-subunit of prolylhydroxylase [92], an enzyme involved in the post-translational hydroxylation of specific proline residues in the collagen-like domain of SP-A. Proline hydroxylation is essential for the formation of a stable triple helix in collagen [93] and inhibition of prolylhydroxylase, or incorporation of the proline analogue *cis*- or hydroxy-L-proline, results in accumulation of SP-A in the endoplasmic reticulum, presumably due to incorrect assembly of the SP-A multimer [25,90]. Mechanisms for the retention [94] and degradation [95] of incompletely or aberrantly assembled secretory proteins in the endoplasmic reticulum have recently been described. In addition to the above described modifications of SP-A occurring in the endoplasmic reticulum, it has been proposed [96,97] that rat SP-A also undergoes vitamin K-dependent carboxylation of glutamic acid residues (reviewed in [98]). These findings have recently been challenged by Wallin *et al.* [99] who failed to detect γ-carboxyglutamic acid in canine SP-A and also observed that the consensus recognition site for vitamin K-dependent carboxylation is not present in SP-A.

Transport of SP-A from the endoplasmic reticulum to the Golgi proceeds relatively slowly, resulting in an accumulation of endoglycosidase -H sensitive (high-mannose oligosaccharide) SP-A [89,100]. The time required to exit the endoplasmic reticulum may be related to the complex folding and assembly of multimeric SP-A. Processing of SP-A during transit through the

Golgi complex appears to be restricted to the high-mannose oligosaccharide tree and includes the addition of sialic acid and sulphate which significantly increase the charge heterogeneity of the molecule; the occurrence of SP-A sulphation *in vivo* has not been established. Inhibitors of Golgi-associated oligosaccharide processing enzymes, which reduce the charge heterogeneity of SP-A, do not affect the routing of SP-A to the secretory granule (lamellar body) or its secretion [89,90].

The exit of SP-A from the Golgi complex is probably similar to that described for exocrine cells (reviewed in [101]), involving both constitutive and stimulus-regulated secretion. In pulse–chase studies, small amounts of SP-A are detected in media at early chase time points consistent with a vesicular (constitutive) secretory pathway. Throughout the chase period, SP-A continues to accumulate in the media in the absence of secretagogues, suggesting a second, unstimulated secretory pathway involving basal level exocytosis of lamellar bodies. SP-A is secreted with the same time course as DPPC [102] and its secretion is significantly enhanced by the secretagogue TPA [103] consistent with a stimulus-regulated secretory pathway [103]. However, although it is likely, cosecretion of SP-A and surfactant phospholipids within the same secretory granule has not been conclusively demonstrated.

SP-D. The synthesis and post-translational processing of SP-D is likely very similar to that for SP-A, although very little information is currently available for this protein. SP-D contains asparagine-linked carbohydrate (the number of oligosaccharide moieties is unknown) which is modified by the addition of sialic acid residues [24,25]. Inhibition of hydroxylation of proline residues in the collagen-like region inhibits secretion of SP-D suggesting that, as for SP-A, assembly of the molecule into a stable oligomer is critical for trafficking through the secretory pathway.

SP-B. Estimates of the abundance of SP-B based on the number of positive clones detected during screening of cDNA libraries range from 0.006–0.02% [51,54,55] to as high as 0.1% [56]. Comparison of the amino acid sequence of SP-B, derived from human cDNA [51–53] or genomic [49] clones, indicates that the 79-residue active airway peptide is contained within the sequence of a larger precursor of 381 amino acids. Consistent with this observation, the SP-B primary translation products were identified as proteins of M_r 40000 [51,100,104]. The primary translation products are heterogeneous with respect to charge, consisting of proteins with pI approx. 5.1–5.4 in the human [104] and pI approx. 5.8–7.0 in the rat [100]; the source of the charge heterogeneity is unknown. The SP-B precursor contains a signal peptide, estimated to be 23 residues in length, which is cotranslationally cleaved [104]; however, identification of the precise size of the signal peptide must await isolation and amino acid sequence analysis of the SP-B proprotein. Examination of the amino acid sequence of the preproprotein indicates the presence of a consensus sequence for asparagine-linked glycosylation, at positions 293, 301, 310 and 306 in canine, rabbit, human and rat SP-B respectively. Treatment of the SP-B proprotein with endoglycosidase-F, or prevention of the co-translational transfer of high-mannose oligosaccharide to the proprotein by tunicamycin, results in a shift in the electrophoretic mobility of SP-B, confirming the presence of asparagine-linked carbohydrate in the *C*-terminal peptide of the proprotein [100,104]. A second human SP-B cDNA [49,51,52] contains an additional site for asparagine-linked glycosylation at position 129 in the *N*-terminal peptide of the proprotein; it is not known if this site is glycosylated *in vivo*. As for SP-A, asparagine-linked glycosylation of SP-B is not required for intracellular routing and secretion [104]. Unlike SP-A, however, an endoglycosidase-H sensitive pool of SP-B is not detected in Type II cells, suggesting that transport from the endoplasmic reticulum to the Golgi is relatively rapid. The time course of secretion ($t_{\frac{1}{2}} < 2$ h) is consistent with release via a constitutive secretory pathway; this hypothesis is supported by the observation that the proprotein is not detected in the lamellar body fractions [100]. Transport of the SP-B proprotein through the Golgi is associated with extensive sialylation of the oligosaccharide tree in a human pulmonary adenocarcinoma cell line [104]; however, it is unclear whether sialylation is a normal, post-translational modification of SP-B or is a characteristic of the transformed cell phenotype [105].

Processing of the human SP-B proprotein to the mature 79-residue peptide involves removal of approx. 176 amino acids of *N*-terminal propeptide and 102 amino acids of the *C*-terminal peptide. Cleavage of a 16 kDa *N*-terminal propeptide to generate a processing intermediate, consisting of an active peptide and the *C*-terminal peptide of the proprotein, has been detected *in vitro* [69,100,104,106]; it is unclear if the *N*-terminus of the active mature peptide is generated by a single proteolytic cleavage between Gln^{200} and Phe^{201}. Processing of the proprotein proceeds to completion in primary cultures of Type II cells and fetal lung explant culture to generate the mature, M_r 8000, SP-B monomer [100]. Although the active peptide, M_r 18000, has been detected in lamellar bodies of Type II cells [69,71,81,100,107], the actual site(s) of processing remains uncertain. Most of the proprotein appears to be secreted and it is unclear if the small amount of intracellular proteolysis detected *in vitro* [100,104] represents a true processing pathway or basal degradation of newly synthesized precursor, as has been described for collagen [108], acetylcholinesterase [109] and other secretory proteins [110]. Proprotein processing does appear to occur in the media of Type II cell cultures, but intracellular processing which is closely coupled to secretion of the precursor, as has recently been described for atrial natriuretic factor [reviewed in 111], has not been excluded as an explanation for this observation.

SP-C. Like SP-B, SP-C is also contained within the sequence of a precursor protein [60,61,63,64]; generation of the 33–35-residue active peptide involves removal of 23–25 residues of *N*-terminal propeptide and 133–139 residues from the *C*-terminus of the proprotein. SP-C primary translation products of M_r 22000 have been detected in primary cultures of purified rat Type II cells [100] and by *in vitro* translation of human lung RNA [60,106]. The *N*-terminal propeptide of the precursor does not conform to a signal peptide and we have been unable to detect cotranslational cleavage in numerous attempts. As for SP-B, the sequences surrounding the sites of *N*- and *C*-terminal cleavage provide no clues as to the nature of the enzymes involved in proprotein processing. There are no sites for asparagine-linked glycosylation in the sequence of the SP-C proprotein. The only post-translational modification of SP-C reported to date is the palmitoylation of adjacent cysteine residues, at positions 5 and 6 of human and porcine mature SP-C [23], a modification which presumably occurs in the Golgi. Processing of SP-C has not been studied because of the difficulty of generating monospecific antisera to the extremely hydrophobic active peptide.

Summary of processing and secretion

Surfactant is stored in lamellar bodies for regulated release into the airway. Isolated lamellar body fractions containing phospholipids, SP-A, SP-B and SP-C are capable of forming tubular myelin (in the presence of calcium) and reducing surface tension (< 12 mN/m), suggesting that these secretory granules house the mature assembled phospholipid–protein complex [71].

A number of questions regarding the processing and assembly of surfactant proteins with surfactant lipids remain unanswered. Does the lamellar body represent the site of surfactant protein lipid association or does assembly occur prior to lamellar body incorporation? In this regard, assembly of triacylglycerol with apolipoproteins has recently been shown to occur in the Golgi [112]. What are the sites for processing of SP-B and SP-C proproteins? Are surfactant proteins targeted to the lamellar body and, if so, what are the signals for routing? How is secretion of the lamellar body regulated? Recent studies regarding the inhibition of surfactant secretion by SP-A have provided some insight into this last question and are discussed more fully below.

Fate of surfactant proteins

Relatively little is known about fate of secreted SP-A. SP-A is cleared from the airway more rapidly than surfactant phospholipids [113]. Surfactant lipids are cleared largely via reuptake by cells of the distal airway, particularly Type II cells; there is evidence that alveolar macrophages may also participate in this process (reviewed in [114]). Internalization of SP-A by alveolar macrophages [68,115,116] occurs by a mannose-dependent mechanism [117] suggesting a possible role for asparagine-linked carbohydrate or the lectin-like region of SP-A, and is associated with enhanced phagocytosis of opsonized targets [118,119]. SP-A internalized by this route is presumably degraded in lysosomes [117]. In contrast to macrophages, internalization of SP-A by Type II cells appears to occur by a mannose-independent mechanism. Binding and internalization of SP-A by Type II cells was found to be consistent with a receptor-mediated process [12,80,120,121]: binding was saturable, was inhibited by excess unlabelled SP-A and occurred with an estimated K_d of approx. $(5–6.4) \times 10^{-10}$ M. The receptor-binding domain of SP-A has not been identified, although the carbohydrate recognition domain and the carbohydrate moiety of SP-A do not appear to be candidates. The number of SP-A receptors and their nature (protein and/or glycolipid) is also unclear. Receptor-mediated internalization of SP-A apparently requires intact oligomers and occurs in a calcium-dependent manner. Internalization does not appear to be accompanied by significant degradation suggesting that SP-A may be recycled [120]. Consistent with this idea, internalized SP-A has been identified sequentially in coated pits, coated vesicles, endosomes and multivesicular bodies in close proximity to lamellar bodies [80,122]. However, the precise fate of internalized SP-A, i.e. degradation or resecretion and reutilization, remains to be determined.

Studies on the turnover of SP-C in the airway of neonatal rabbits suggest that SP-C also has a faster turnover rate than phosphatidylcholine (PC) [123]. Both Type II cells and alveolar macrophages appeared to participate in SP-C turnover, although the time course of association with SP-C was different for the two cell types, suggesting that they may represent sites of recycling and degradation respectively. SP-B and SP-C peptides have been shown to enhance the uptake of phospholipids by Type II cells in a dose-dependent manner [124]; however, unlike SP-A, this process does not appear to be receptor-mediated and it is unclear if it is accompanied by internalization of the peptides. SP-B and SP-C are completely resistant to protease digestion *in vitro* and have been successfully degraded only after extensive acid hydrolysis [21,22,51]. Thus, the mode and sites of surfactant protein turnover remain unresolved.

REGULATION OF EXPRESSION OF SURFACTANT PROTEINS

Developmental regulation of expression

SP-A protein [37,70,125–127] and RNA [37,127] are not detected or are detected at very low levels (less than 0.5% of adult lung SP-A) in second trimester human fetal lung. SP-B and SP-C proteins are also not detected in early second trimester lung; however, Northern analyses detected low levels of SP-B and SP-C RNAs that increased to 50% and 15% respectively of levels in adult lung between 13 and 24 weeks gestation [77]. Expression of surfactant proteins is rapidly induced when fetal lung tissue is cultured in the absence of hormones. Human SP-A protein [37,69,70,84,125,127] and RNA [37,127] are detected by day 2 of culture and continue to rise until day 5, reaching RNA levels about 30% of those in adult lung. Induction of SP-A expression in explant culture has been detected in lung tissues as early as 8 weeks of gestation [127]. SP-A in fetal lung explant culture displays the same charge and size heterogeneity as that detected in adult lung [37,84,127]. Expression of SP-B RNA and protein is also rapidly induced in explant culture and is detected as early as 12 h [77,106]. The 42 kDa SP-B proprotein as well as processed 25 kDa and 18 kDa forms of SP-B were detected, indicating that at least some of the proteins involved in SP-B processing are also expressed in explant culture [69,106]. Expression of SP-C is also induced, although to a lesser extent than SP-A and SP-B [77,106]. Thus, although expression of pulmonary surfactant proteins is very low during the second trimester of pregnancy, lung tissues during this developmental period are capable of rapidly increasing expression of these proteins *in vitro* similar to the dramatic elevation of expression that occurs *in vivo* immediately prior to birth.

The synthesis and secretion of SP-A, SP-B and SP-C increases during the last trimester of pregnancy, as indicated by the appearance of these proteins in amniotic fluid [128–135]. During the last 20% of gestation multiple SP-A RNAs in rat [35,136], rabbit [36] and mouse [29] are induced co-ordinately, rising from barely detectable levels to levels that are about 50% of those detected in adult lung and decreasing slightly in newborn lung. The developmental increase in SP-A mRNA is due at least in part to an increase in the rate of transcription of the SP-A gene [137]. SP-A protein also increases in developing rat [71,136,138,139], rabbit [83], and mouse [140] lung. SP-B [55,136] and SP-C [62,136,141] RNAs also increase during this period, reaching levels that exceed those in adult lung, and are accompanied by an increase in SP-B and SP-C airway peptides [71]. The developmental increase in SP-A, SP-B and SP-C parallels the increase in surfactant phospholipids (reviewed in [2]) and is associated with a developmental increase in the surface tension reducing properties of fetal lung surfactant [71].

Tissue-specific regulation of expression

As noted above, surfactant proteins A, B and C are expressed only in lung tissues. *Cis*-acting sequences, sufficient to direct expression of SP-C in the distal epithelium of developing mouse lung, have recently been identified [141]. A fusion gene consisting of a diphtheria toxin A (DT-A) gene, under control of 3.6 kb of 5′-flanking sequence from the human SP-C gene, was injected into fertilized mouse eggs to produce transgenic mice. Expression of the DT-A fusion gene resulted in ablation of the host cell. Among transgenic mice expressing the DT-A construct, only lung tissues were affected and these animals developed respiratory failure in the immediate postnatal period. In moderately-affected pups, only the distal respiratory epithelium was ablated. Thus, the 5′-flanking sequence of the SP-C gene contains elements that direct the appropriate tissue and developmental expression of the SP-C protein.

Regulation of expression by humoral and cellular factors

Disruption of the normal ontogenic pattern of surfactant

Table 1. Effects of various agents on expression of surfactant proteins in human fetal lung explant culture

See the text for details. ↑, Increase; ↓, decrease; ↔, no effect.

	Expression of:		
	SP-A	SP-B	SP-C
Glucocorticoids	↑↓	↑	↑
Cyclic AMP	↑	↔	↔
EGF	↑	?	?
IFN-γ	↑	↔	↔
T_3	↔	?	?
TGF-β	↓	?	?
Insulin	↓	?	?
TPA*	↓	↓	?
TNF-α*	↓	?	?
Androgens	↔	↔	↔
Oestrogen	↔	↔	↔

* Effects reported for the H441 adult human pulmonary adenocarcinoma cell line.

synthesis by premature birth results in surfactant deficiency at delivery which rapidly leads to RDS, the leading cause of morbidity and mortality in neonates. In this section the effect of humoral and locally-derived factors on the expression of surfactant proteins in developing lung will be examined. This aspect of regulation of expression has recently been reviewed by Ballard [3].

Glucocorticoids

The sequence (G/C)GGT(A/T)CA(A/C)NNTGT(C/T)CT comprises a consensus sequence for binding of the glucocorticoid receptor (reviewed in [142]). The presence of elements with homology to the glucocorticoid responsive element (GRE) in the flanking region or within the introns of the gene implies but does not necessarily prove regulation by glucocorticoids. The SP-A gene contains a GRE approx. 100 bp upstream from the transcription initiation site [32] and the SP-B gene contains four such elements within 700 bp of 5′-flanking sequence [49].

SP-A. The regulation of expression of SP-A by glucocorticoids is extremely complex and varies as a function of dose, developmental age and species. In fetal lung explant culture, glucocorticoids were generally found to stimulate expression of SP-A RNA and protein at concentrations $\leqslant 10$ nM [37,83,125,143–145] and to inhibit SP-A expression at concentrations $\geqslant 1$ μM [127,143–146]. A biphasic response to glucocorticoids was also detected in an adult human pulmonary adenocarcinoma cell line (H820) [147]. In contrast, 10 nM-dexamethasone decrease RNA and protein in a separate adult human pulmonary adenocarcinoma cell line (H441) [148]. In human fetal lung explant culture, the stimulatory effects of 10 nM-dexamethasone on SP-A RNA levels were detected as early as 10 h after addition, were maximal by 30–55 h and then decreased below control levels [144,145]; comparable effects were produced by cortisol, cortisone and corticosterone [145]. Similar doses of glucocorticoids have previously been shown to maximally stimulate synthesis of DPPC [37,125,149]. Higher concentrations of dexamethasone produced a much more rapid onset of the inhibitory effect, leading to reduced SP-A RNA levels as early as 2 h after addition [143,145]. Despite reduced SP-A RNA levels, however, dexamethasone caused a dose-dependent increase in SP-A gene transcription (EC_{50} 10–100 nM), suggesting that decreased SP-A RNA levels were the result of altered RNA stability [143]. In contrast to the inhibitory effects of 1 μM dexamethasone in human fetal lung explants, similar treatment in rabbit lung explants resulted in a transient (0–24 h) decrease in SP-A RNA followed by a modest increase in SP-A RNA levels; these alterations in SP-A RNA levels could be fully accounted for by changes in SP-A gene transcription [137]. From these limited data it appears that modulation of SP-A expression by glucocorticoid is not only dose-dependent but also species-specific.

Developmental age may also moderate the effects of glucocorticoids. Postnatal administration of dexamethasone to rats resulted in a dose-dependent increase in both newly synthesized and secreted SP-A as well as a modest increase in SP-A RNA at all ages, but appeared to be somewhat more effective at earlier postnatal ages [150]. Prenatal maternal administration of dexamethasone also increased SP-A RNA and protein in fetal lung although no effect was detected before day 19 of gestation [139]. Others have also reported that maternal treatment on day 18 of gestation did not result in increased SP-A protein and RNA in fetal lung on day 19 [151]; however, earlier treatment (days 14–18) resulted in accelerated expression of SP-A. These observations are consistent with a developmental change in the glucocorticoid sensitivity of the SP-A gene and suggest a role for endogenous glucocorticoids in the regulation of SP-A expression during the perinatal period. However, although plasma-free corticosteroid levels rise at the onset of surfactant synthesis [152,153], it appears unlikely that this event initiates SP-A expression in developing rat lung. Numerous studies have shown that the developmental increase in surfactant lipids and proteins (including SP-A), proceeds in fetal lung explant culture in the absence of hormones; further, pretreatment of explants with the glucocorticoid antagonist RU486, which abolished cytoplasmic and nuclear binding of glucocorticoids, did not prevent the developmental increase in SP-A RNA [154]. Thus, a role for endogenous glucocorticoids in the modulation of surfactant protein expression remains to be determined.

SP-B and SP-C. Stimulation of the expression of SP-B and SP-C RNA in human fetal lung explant culture occurs at concentrations of dexamethasone that clearly inhibit the accumulation of SP-A RNA [77,106]. Dexamethasone increased SP-B and SP-C RNA in a dose-dependent manner, with an $EC_{50} \sim 1$ nM for SP-B and ~ 5 nM for SP-C [77]. The effects of dexamethasone were also time-dependent, with half-maximal stimulation occurring at 14 h for SP-B and 19 h for SP-C [77]. Dexamethasone also produced a dose- and time-dependent increase in SP-B RNA and protein in the H441 cell line [148,155]; similar effects on SP-B and SP-C expression were observed in the H820 cell line [147]. The approx. 100-fold increase in SP-B RNA in response to dexamethasone treatment was due in part of a 4-fold increase in transcription of the SP-B gene [155]; however, the increase in SP-B RNA accumulation was primarily related to increased RNA stability in the presence of glucocorticoid. SP-B RNA accumulation was dependent not only upon continued gene transcription but also upon continued protein synthesis, suggesting that a relatively labile protein(s) is required for the induction and maintenance of high levels of SP-B RNA following glucocorticoid treatment [155]. Thus, in the H441 cell line, glucocorticoids alter levels of SP-B RNA by increasing transcription and decreasing RNA turnover.

Summary of glucocorticoid effects on surfactant protein expression

Although glucocorticoids have been shown to affect the rate of transcription of both SP-A and SP-B, direct interaction of the promoter elements of the genes encoding these proteins with the

glucocorticoid receptor remains to be demonstrated. In addition to transcriptional effects, glucocorticoids altered the turnover of human SP-A and SP-B RNAs, in the former case to decrease mRNA levels and in the latter to increase mRNA levels. The effects of glucocorticoids may be positive or negative depending on the particular protein, dose, developmental age and species. The role of endogenous glucocorticoids in the regulation of surfactant protein expression remains to be established.

Cyclic AMP

α-Adrenergic agonists stimulate the synthesis and secretion of pulmonary surfactant; these agents exert their effects through increased intracellular levels of cyclic AMP. The sequence CTGACGTCAG comprises a consensus sequence associated with transcriptional, regulation by cyclic AMP (reviewed in [156]). Cyclic AMP response elements (CRE) have been detected in the 5′-flanking sequences of the SP-B and SP-C genes and within the first intron of the SP-A and SP-B genes [32,49,61,157].

The cyclic AMP analogues dibutyryl cyclic AMP, 8-bromo cyclic AMP and dibromo cyclic AMP, at concentrations of 0.1–1 mM, have been shown to stimulate SP-A expression by 5–15-fold. The stimulatory effect of cyclic AMP on SP-A RNA and protein was detected in both human [127,143,145,158] and rabbit [36,83,137] fetal lung explants. Agents that increased endogenous cyclic AMP levels, by activating adenylate cyclase (forskolin and terbutaline) or inhibiting phosphodiesterase (isobutylmethylxanthine) also increased SP-A RNA and protein [83,145,158]. The stimulatory effect of these agents on expression of SP-B and SP-C RNA was much smaller than for SP-A and was not associated with increased protein [77,106]. The cyclic AMP-stimulated increase in SP-A RNA levels was detected as early as 6 h and could be accounted for by enhanced transcription of the SP-A gene [137,143]. Cyclic AMP stimulation was inhibited by cycloheximide, suggesting that ongoing protein synthesis was necessary for the effects of cyclic AMP [36,137]; however, this conclusion is tenuous since cycloheximide also decreased SP-A RNA in the absence of cyclic AMP in these studies. Dexamethasone (1 nM) in combination with cyclic AMP further increased the rate of transcription and the levels of SP-A RNA and protein above that of either agent alone [83,143,145]; higher concentrations of dexamethasone (1 μM) reduced the cyclic AMP stimulated accumulation of SP-A RNA and protein similar to the effects of dexamethasone alone [127,137,143–145].

Summary of cyclic AMP effects on surfactant protein expression

The effects of cyclic AMP on SP-A expression are similar to those of low doses of dexamethasone and include an increase in the rate of transcription of the SP-A gene, leading to increased accumulation of SP-A RNA and protein. Unlike the response to glucocorticoids, however, SP-A expression stimulated by cyclic AMP is sustained with time and does not show species differences. It is unknown if enhanced transcription underlies the much smaller increase in SP-B and SP-C RNAs in response to cyclic AMP and whether increased transcription of surfactant protein genes is regulated through *cis*-acting CRE. Transcriptional regulation by CRE requires activation of cyclic AMP-dependent protein kinase and phosphorylation of a nuclear protein (CREB) which binds to the regulatory element (reviewed in [159]). Finally, it remains unclear if agents that act to increase endogenous cyclic AMP play a role in the ontogenic increase in surfactant protein expression.

Other factors that stimulate expression of surfactant proteins

Among agents that increase the expression of SP-A, epidermal growth factor (EGF) was found to be particularly effective. Treatment of human fetal lung explants with EGF resulted in a dose-dependent (0.1–10 ng/ml, EC_{50} 1 ng/ml) increase in SP-A protein and RNA [146]. The magnitude of the increase was similar to that induced by cyclic AMP and was greatly reduced by coadministration of dexamethasone. The effects of EGF on the expression of SP-B and SP-C have not been investigated. Although there is some uncertainty regarding the origin and sites of action of endogenous EGF in fetal lung [160–162] there is evidence for a role for EGF, or an EGF-like molecule, in lung maturation. EGF receptors have been identified in fetal lung membranes and exhibit maximal binding around the time of the onset of surfactant synthesis [163]. Further, in addition to its stimulatory effects on SP-A expression, EGF also increases pulmonary DPPC synthesis and secretion [163,164]; however, the molecular mechanism whereby EGF exerts its affect on SP-A and DPPC and its relevance in developing lung remain to be elucidated.

Interferon γ (IFN-γ) has also been shown to stimulate expression of SP-A in human fetal lung explant culture [165]. IFN-γ increased SP-A protein and RNA approx. 3-fold in a dose-dependent manner (0.5–100 ng/ml, $EC_{50} \leqslant 5$ ng/ml). Low concentrations of dexamethasone (1 nM) in combination with IFN-γ increased SP-A protein by 10-fold. The stimulatory effect of IFN-γ was specific for SP-A in that SP-B and SP-C RNA and phosphatidylcholine synthesis were unaffected. The effects of IFN-γ may be mediated through an IFN regulatory element [GGAAAN(N)GAAACT] (reviewed in [166,167]); a sequence with homology to IFN-γ regulatory element is present within the first intron of human SP-A gene [32]. It is unclear if this sequence confers sensitivity to IFN-γ on the SP-A gene and if IFN-γ plays a role in modulating SP-A expression *in vivo*.

Other factors that inhibit surfactant protein expression

Several factors have been shown to specifically inhibit expression of surfactant proteins. TGF-β (1 ng/ml) decreases SP-A RNA and protein in fetal lung explant cultures [146]; effects on SP-B and SP-C were not investigated. Insulin has also been shown to cause a dose-dependent (2.5–250 ng/ml) decrease in SP-A protein in human fetal lung explants [70]. The effects of insulin on SP-B and SP-C, which greatly enhance the biophysical activity of surfactant phospholipids, are unknown. Phorbol 12-myristate 13-acetate (TPA) causes a dose-dependent decrease in SP-A and SP-B RNA (IC_{50} 0.5 nM) and protein (IC_{50} 0.1 nM) in the H441 tumor cell line [168]. The inhibitory effect of TPA was detected within 2 h and resulted in a significant decrease in the half-lives of SP-A and SP-B RNAs. The effect of TPA on SP-A and SP-B RNA stability required ongoing transcriptional activity; it was unclear if TPA also exerted transcriptional effects on these genes. The time course of inhibition of SP-A RNA by TNFα [169] is similar to that reported for TPA [168]. TNFα decreased SP-A protein and RNA in a dose-dependent manner (1–25 ng/ml) while increasing expression of manganese superoxide dismutase RNA. The inhibitory effects of TNFα and TPA are consistent with involvement of a protein kinase C-dependent pathway and may ultimately be mediated through AP1 binding sites [reviewed in 167,170] which have been identified in the first intron and the 5′-flanking sequence of the SP-A [32] and SP-B [49] genes respectively.

Male infants are at an increased risk of RDS. The basis for this increased risk has been attributed to an androgen-mediated delay in the developmental accumulation of DPPC and PG (reviewed in [171]). This maturational delay does not extend to surfactant proteins. Levels of RNAs encoding all three surfactant proteins were found to be similar in males and females during lung development [35,136]; moreover, dihydroxytestosterone, 17-hydroxyprogesterone and oestradiol were found to have no

effect on SP-A [145] or SP-B and SP-C [77] RNAs in human fetal lung explant culture.

Summary of effects of other agents on surfactant protein expression

The development of fetal lung *in vitro* in the absence of hormonal cues suggests a role for locally-derived factors in the control of lung maturation and surfactant protein expression in particular; cellular factors which specifically stimulate (EGF, IFN-γ) or inhibit (TGF-β, TNF-α) surfactant protein expression have been identified. Alternatively, rapid expression of surfactant proteins *in vitro* may be the result of removal of an inhibitory influence such as insulin. Clearly, surfactant protein expression is subject to regulation by a large number of factors; identification of these agents and the molecular mechanisms whereby they exert their effects will greatly facilitate our understanding of the regulation of surfactant protein expression.

Expression of surfactant proteins in RDS

Premature birth interrupts the normal pattern of lung development frequently resulting in surfactant insufficiency and RDS. Infants with severe acute RDS have low or undetectable amounts of SP-A, SP-B and SP-C [172–175]. It has also been reported that these infants have no detectable tubular myelin, the putative precursor to the surface-active alveolar film [176]; interestingly, SP-A and SP-B have been shown to be requisites for tubular myelin formation *in vitro* [177]. Resolution of acute RDS is accompanied by an increase in surfactant protein levels [173–175].

RDS is frequently treated with high concentrations of inspired oxygen. Hyperoxia has been shown to stimulate a time-dependent, 20-fold increase in SP-A accumulation in adult rat lungs [178,179]; increased accumulation of SP-B and SP-C was also detected [180]. Elevated expression of surfactant proteins accounted for at least part of the increase in accumulation; SP-A and SP-B RNA were similarly increased in hyperoxic animals while SP-C RNA was increased to a lesser extent [180]. Increased levels of SP-A RNA in response to hyperoxia have also been detected in neonatal rabbit lung [181,182]. It remains unclear if these effects are mediated through enhanced gene transcription or mRNA stability. In contrast to these results, increased SP-A levels associated with silicosis were not accompanied by increased SP-A RNA levels, consistent with altered metabolism rather than synthesis in silica-treated rats [183].

Ventilatory support, which often accompanies oxygen treatment, also affects the levels of surfactant proteins. Airway SP-A levels have been shown to vary with the mode of ventilation [175]. Consistent with this observation, stretching of Type II cells *in vitro* has been shown to stimulate DPPC secretion [184]. The extent to which stretch-induced secretion contributes to surfactant protein levels in normal lung function remains unknown.

Summary of regulation of surfactant protein expression

Surfactant protein expression is under cell-specific, developmental and humoral controls. Sequences directing tissue-specific and developmental expression have been identified in the 5′-flanking region of the SP-C gene; studies to identify the precise elements that account for cell-specific and developmental regulation of transcription, as well as the *trans*-acting factors that bind to these sites, are now underway. Potential *cis*-acting regulatory elements involved in humoral regulation have also been identified within or flanking the genes encoding the SP-A, SP-B and SP-C. Agents that have been shown to bind these regulatory domains in other systems modulate the expression of surfactant proteins in a dose-, time- and species-dependent manner; however, it remains unclear if these agents exert their effects directly on surfactant protein expression through interaction with *cis*-acting elements. The varied extent to which these agents regulate expression of SP-A, SP-B and SP-C suggest that these genes can be independently regulated. Finally, the level of surfactant protein expression involves not only altered transcription but also altered mRNA stability suggesting the involvement of other, as yet uncharacterized, proteins in the regulation of surfactant protein expression. Thus, the appropriate cell and developmental expression of SP-A, SP-B and SP-C is likely the result of the integration of a number of factors impinging upon different levels of control.

FUNCTIONS OF SURFACTANT PROTEINS

Functions related to the interaction of surfactant proteins and lipids

Surfactant proteins directly affect the biophysical properties of surfactant lipids both *in vivo* and *in vitro*. Rapid adsorption of surfactant phospholipid to the air–liquid interface is thought to be critical for maintaining the morphological integrity of the alveolus [53,185]. Purified SP-B [53,54,186], SP-C [53,63,187] or mixtures of the two proteins [16,188–191] markedly enhanced the rate of formation of a phospholipid surface film at an air–liquid interface *in vitro*; this activity was further enhanced by the addition of SP-A [54,192]. Preparations of phospholipids containing SP-B alone were more effective than similar preparations containing SP-C in reducing surface tension in a pulsating bubble surfactometer or Wilhelmy balance [16,53,193,194]. Injection of mice with hybridomas secreting monoclonal antibodies directed against SP-B resulted in respiratory failure, further supporting the role of SP-B in surfactant function [195,196]. Most significantly preparations of SP-B, SP-C and surfactant lipids were shown to increase lung compliance and preserve the morphological integrity of the distal airways in prematurely delivered ventilated fetal rabbits [53,185,193,194,196] and in surfactant-deficient isolated rat lungs [194]. Preparations of surfactant lipids containing mixtures of SP-B and SP-C have been widely tested in clinical trials and shown to significantly improve oxygenation and reduce the need for respiratory support in infants suffering from RDS (reviewed in [199,200]). Although the results of these studies are encouraging, the precise assignment of specific roles for SP-A, SP-B and SP-C in conferring biophysical activity to surfactant lipids will require a comprehensive assessment of the interactions of the purified surfactant protein with individual surfactant lipid components.

Surfactant phospholipids in the airway exist in several physical forms which presumably represent various metabolic intermediates (reviewed in [114]). It has been proposed [201] that one of these fractions, the highly-ordered, calcium-dependent [202] lattice structure referred to as tubular myelin, is the precursor to the surface-active phospholipid monolayer. SP-A has been localized by immunocytochemistry to the tubular myelin lattice [68,203]. Further, both SP-A and SP-B are necessary to reconstitute tubular myelin from synthetic phospholipids [177]

Table 2. Functions of surfactant proteins

Function	SP-A	SP-B	SP-C
1. Contribute to surfactant properties of phospholipids	+	+	+
2. Formation of tubular myelin	+	+	−
3. Facilitate turnover of surfactant phospholipids	+	+	+
4. Inhibition of pulmonary surfactant secretion	+	−	−
5. Facilitate phagocytosis of opsonized particles	+	−	−

suggesting that these proteins may play an important role in the organization of lipids in the airway. However, the precise role of tubular myelin in surfactant remains uncertain. Preparations of surfactant phospholipids containing SP-B and SP-C, but not SP-A, do not form tubular myelin yet reconstitute the full biophysical properties of surfactant [204]. Tubular myelin has also been shown to be a component of a surfactant fraction which adsorbs relatively slowly to the air–liquid interface *in vitro* [205]. Others [202], however, have reported that the presence of tubular myelin is correlated with an increased rate of adsorption. Thus the role of tubular myelin and its association with the surface-active or inactive fraction of pulmonary surfactant remains to be elucidated.

Although SP-A, SP-B and SP-C are clearly associated with surfactant phospholipids, the nature of this interaction is not clear. SP-A associates with phospholipids [206–208] and promotes the calcium-dependent aggregation of phospholipid vesicles [209]; this function is dependent upon glycosylation of SP-A [210]. SP-A also participates with SP-B in the ordering of tubular myelin [177]. Although the precise role of these proteins in the generation of tubular myelin figures is unclear, mixtures of SP-B and SP-C have been shown to induce the fusion of PG vesicles [211], consistent with the idea that SP-B may be involved in the formation of the characteristic tubular myelin membrane crossings. Studies using fluorescence anisotropy indicate that SP-B interacts selectively with PG in model membrane lipids composed of 7:1 DPPC:DPPG [192]. These studies also suggest that SP-B resides primarily in the fluid phase domains, where it markedly orders the surface of the membrane bilayer, consistent with the amphipathic helical structure previously predicted for SP-B [51]. These observations are intriguing because PG in pulmonary surfactant is second in abundance only to PC, a concentration which represents a disproportionately high content relative to other mammalian tissues [2].

Surfactant proteins may also be involved in the turnover of surfactant phospholipids. The major route of clearance of surfactant from the alveoli occurs via reuptake by the Type II epithelial cell (reviewed in [114]). SP-A enhances the uptake of phospholipids by Type II cells and may play a role in directing phospholipids to the lamellar body [212–215]. Hydrophobic surfactant proteins, likely to contain both SP-B and SP-C, have also been shown to enhance the uptake of phospholipid liposomes by Type II cells [216,217]. Although these results have recently been ascribed to the phospholipids coisolated with SP-B and SP-C [218], Rice *et al.* [124] have recently shown that synthetic peptides of SP-B and SP-C also enhance the uptake of PC by rat alveolar Type II cells and chinese hamster lung fibroblasts. The uptake of PC occurred in a dose-dependent manner but was not saturable and occurred at both 4 °C and 37 °C, suggesting a mechanism of internalization independent of a cell-surface receptor. Similar to results with SP-A [212,213], SP-B- and SP-C-mediated uptake of PC by Type II cells was associated with an increase in labelled phospholipid in lamellar body fractions. It remains to be shown if SP-B and SP-C facilitate surfactant phospholipid turnover *in vivo*.

Summary of surfactant protein–lipid interactions

Very little is known about the association of surfactant proteins and lipids. Progress in this area has been hampered by the solubility characteristics of the proteins, particularly SP-C. Interaction of SP-A, SP-B and SP-C with surfactant lipids appears to be essential for the complete biophysical activity of the complex. Whether the formation of a surface active film *in vivo* is related to the ordering of phospholipid head groups by SP-B, or the SP-A/SP-B-dependent formation of tubular myelin remains unclear. It is also unclear whether the peptide domains that mediate these properties are distinct from peptide domains that participate in surfactant protein-stimulated uptake of phospholipids by Type II cells. Several other questions regarding the role of surfactant proteins in the turnover of surfactant lipids remain unanswered. Do surfactant proteins target alveolar phospholipids to the lamellar body, and if so, how is directionality conferred? Does binding of SP-A to lipid moderate interaction with its cell surface receptor? And, perhaps most importantly, do these properties of surfactant proteins *in vitro* have functional correlates *in vivo*?

Other functions of surfactant proteins

SP-A has been shown to inhibit the secretion of surfactant phospholipids by rat Type II cells *in vitro* [11,121,219,220]. SP-A inhibited both basal and secretagogue-induced release of PC, with an IC_{50} 0.1 μg/ml for rat SP-A [11,220] and 1 μg/ml for canine SP-A [219]. The ability of SP-A to inhibit PC secretion was dependent upon the intact oligomeric form of SP-A and calcium. Prior interaction of SP-A with phospholipids was found to reduce the ability of SP-A to inhibit phospholipid secretion, suggesting that the inhibitory effect was mediated by unbound SP-A [219,220]. The precise domain involved in inhibitory activity has not been identified, although the lectin-like activity of SP-A, localized to the *C*-terminal portion of the molecule, and asparagine-linked carbohydrate are apparently not essential [11,121,219]. Evidence linking SP-A-mediated inhibition of surfactant phospholipid secretion to receptor occupancy has recently been reported [121].

SP-A may also play an important role in the immune defence system of the lung. SP-A has been shown to increase phagocytosis of opsinized erythrocytes and bacteria by macrophages and monocytes [118,119]. Preincubation of bacteria with SP-A did not enhance phagocytosis, suggesting the SP-A itself did not act as an opsinin but facilitated uptake of opsinized targets by phagocytes [119]. Specific binding of SP-A occurred in a time-, temperature- and concentration-dependent manner [118,119,221] and was localized to coated pits and vesicles [117], consistent with a receptor-mediated process. Binding of SP-A was also found to be mannose-dependent [117], probably involving the carbohydrate-binding region of SP-A. SP-D has also recently been shown to be a calcium-dependent carbohydrate binding protein with a specificity for α-glucosyl residues [26]; however, its effects on phagocytosis have not yet been examined. Internalization of SP-A by macrophages was accompanied by an increase in the production of superoxide radicals; this effect was found to be specific for alveolar macrophages [119]. Thus, SP-A may play a role in facilitating the clearance of bacteria from the distal airway via enhancement of uptake and stimulation of oxygen radical production by alveolar macrophages.

Summary of surfactant protein functions

Surfactant proteins may be important regulators of surfactant phospholipid metabolism. SP-A, SP-B and SP-C have all been shown to promote the uptake of phospholipids by Type II cells; the mechanisms involved in surfactant protein-mediated internalization of phospholipids, however, remains unknown. SP-A has also been shown to inhibit surfactant secretion. Although experimental evidence is currently lacking, it is interesting to speculate that SP-A which leaves the Type II cell via the constitutive secretory pathway, and is therefore presumably not associated with phospholipids, may play a role in the feedback inhibition of surfactant phospholipid secretion. The SP-A domain(s) involved in the inhibition of phospholipid secretion and cell surface binding remains to be identified. Cell surface binding of SP-A by macrophages and monocytes also plays an important role in mediating the immune function of SP-A; the

receptor and the SP-A domain involved in the receptor binding are likely different from those mediating the inhibition of surfactant secretion. The isolation and characterization of expression of receptors for SP-A is therefore crucial for a better understanding of the functions of this molecule.

CONCLUSIONS

RDS is a leading cause of morbidity and mortality in newborn infants. Current treatment of the disease involves tracheal administration of multiple doses of a surfactant replacement mixture. In most instances, these mixtures contain phospholipid and the surfactant proteins, particularly the hydrophobic peptides SP-B and SP-C. Understanding the metabolism of surfactant proteins is therefore of paramount importance in the overall design of an effective treatment regimen. An alternative or adjunct therapy for RDS involves pharmacological intervention. This approach will require a thorough understanding of the regulation of expression of surfactant proteins (and enzymes involved in surfactant phospholipid synthesis) including the identification of *cis*- and *trans*-acting factors regulating transcription of the surfactant protein genes, the post-transcriptional regulation of surfactant protein RNA levels, the regulation of the synthesis of the protein products and their assembly with phospholipids and the regulation of secretion of the surfactant complex. Although it is still unclear if expression of surfactant proteins is essential for ventilation, this question can be addressed with currently available technology. Transgenic animals in which expression of one or more surfactant proteins is suppressed (by gene ablation or antisense expression) may allow inferences as to the functions of surfactant proteins from the resulting pathology. Further progress in identifying the functions of surfactant proteins will be greatly enhanced by elucidation of their three-dimensional structures and analysis of potential functional domains. Collectively the results of studies on the regulation of surfactant protein expression may aid in the design of strategies for the treatment of other pulmonary diseases. Pulmonary cell-specific developmental and other regulatory elements can be used in conjunction with transgenic technology to design new animal models of lung injury and repair. Identification of regulatory elements may also facilitate gene therapy for diseases such as cystic fibrosis and pulmonary fibrosis by directing cell-specific expression of a selected protein(s) *in vivo*. These and other potential applications will permit novel approaches to the treatment of pulmonary diseases.

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