Pulmonary Surfactant Protein B (SP-B): Structure-Function Relationships

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SP-B is a protein in pulmonary surfactant that is, in greatest part, responsible for resistance to surface tension and prevention of collapse of pulmonary alveoli. Peptides of 21 residues, synthesized following the sequence of SP-B or resembling the hydrophobic and hydrophilic domains of SP-B (such as RLLLLRLLLLRLLLLRLLLLR, R, Arg, and L, Leu), enhanced the abilities of phospholipids to reduce surface tension both in vitro and in vivo. Intermittent positively charged residues were essential for this activity. The SP-B–like peptides were found by tryptophan fluorescence to partition within the phospholipid layer in contact with both polar head groups and acyl side chains. These data, together with findings that the SP-B–related peptides increase inter- and intramolecular order of the phospholipid layer, suggest that SP-B resists surface tension by increasing lateral stability of the phospholipid layer.

PULMONARY SURFACTANT, ESSENTIAL for survival, is a complex of lipids and proteins positioned on the air-water interface of the alveolus. The lipid consists principally of two major phospholipids (PLs), phosphatidyl choline, principally dipalmitoyl phosphatidyl choline (DPPC, 80% of total lipid), and phosphatidyl glycerol (PG) (7%). Four different proteins have been described in the composition of lung surfactant, termed SP-A, -B, -C, and -D.

SP-B and -C are distinct, low molecular weight (9 and 3.5 kD, respectively), hydrophobic protein components, the amino acid sequences of which have been deduced from the cDNA and partially or completely confirmed by sequence analysis of the proteins (*1*). These proteins are thought to enhance the rate of spreading of surfactant from the aqueous phase to a monolayer along the air-water interface of the alveolus, in part by destabilizing the lipid bilayer (*2*). We (*3*) and others [Curstedt *et al.* in (*2*) and Yu and Possmayer (*4*), for example] have observed that SP-B, when reconstituted into a PL layer, improves surface activity to a greater extent than does SP-C. A major structural difference between SP-B and -C is that SP-B bears intermittent highly charged residues, 9 of 12 of which are basic (Fig. 1).

We synthesized peptides taken from the native sequence of human SP-B that we (*5*) and Waring *et al.* (*6*) have found possess activity; these peptides resemble the hydrophobic-hydrophilic domains of SP-B. These peptides were then used to determine the relative effects of hydrophilic and hydrophobic properties of the peptides in surfactant activity and to examine the charge requirements of the peptides as determinants of activity. The data provide the basis of a theory regarding the mechanism of action of SP-B in surfactant layers (*7*).

A peptide consisting of residues 59 to 80 of the native sequence of SP-B (SP-B 59-80), known to increase surfactant activity of the phospholipids DPPC:PG (3:1) (*5*), was compared in activity with the same peptide having leucine residues substituted for its hydrophilic residues: DTLLGRMLPQLVC-RLVLRCSMD (SP-B 59-80) and LTLL-GLMLPLLVCLLVLLCLML (L-substituted SP-B 59-80) (*8*). The two peptides, dissolved in CH_3Cl–CH_3OH, were combined with DPPC:PG and tested for surfactant activity in the pulsating bubble and fetal rabbit assays. The data (Figs. 2A and 3A) indicate that substitution of the leucine for hydrophilic residues was associated with markedly diminished activity of the surfactant.

Simplified peptides containing hydrophilic and hydrophobic regions similar to those of the native SP-B were then assessed for activity. These peptides consisted of single arginines and stretches of hydrophobic leucines: RLLLLRLLLLRLLLLRLLLLR (RL_4). For comparison, lysines (KL_4) and leucines (poly-L) were substituted for arginine. After combination with DPPC:PG, activities of these peptides were assessed as above. Peptides containing arginine or lysine had activity equal to that of SP-B 59-80, whereas the poly-L peptide was considerably deficient in activity (Fig. 2B). The RL_4 and KL_4 peptides in PL dispersions had strong surfactant activity in 27-day-old fetal rabbits (Fig. 3B). Thus, peptides synthesized with the native sequence of SP-B did not have a unique capacity to convey surfactant activity to the DPPC:PG.

The relative importance of hydrophilic and hydrophobic domains was assessed by comparing 21-residue peptides in which eight, four, two, or zero (poly-R) leucines separated arginines. In surfactometer tracings, equal activity was observed with each peptide dispersion, although with poly-R a longer latency period was required. The activities of these peptide surfactants compared favorably with that of SP-B and were far greater than that of SP-C (Fig. 2C). Peptides consisting solely of hydrophobic residues have been reported to have surfactant activity, somewhat less than that of native surfactant, when reconstituted with PLs (*9*). The data herein, which utilize both peptides of native SP-B in which hydrophobic leucines were substituted for hydrophilic residues of the native sequence and peptides consisting of poly-L, would support the reported observations. These hydrophobic peptides yielded activity between that of phospholipid alone and that of native human surfactant.

Studies were then conducted to determine if the basic residues, arginine and lysine, could potentially form a charge interaction with negatively charged phosphates and if aspartate or other acidic residues could form a charge interaction with the positively charged quaternary ammonium groups of the polar head group of phosphatidyl choline. The RL_4 peptides were compared with similar peptides synthesized with aspartic acid (DL_4). Surfactant with RL_4 reduced surface tension in the pulsating bubble to a greater degree than that with the DL_4 peptide (Fig. 2D). This difference at both minimal and maximal bubble radius was consistent in ten experiments. The RL_4 and DL_4 peptides in phospholipid dispersions were also assessed for surfactant activity in the Wilhelmy balance. Incorporation of RL_4 with DPPC:PG immediately lowered surface tension (increased resistance to surface tension) as the surface area in the trough was reduced (Fig. 4). The increased resistance was maintained on the descending limb of the curve as the surface area was increased. The DL_4 PL dispersion had approximately the same weak capacity to resist surface tension as that of the PL alone.

To determine if the weak activity of DL_4 peptide could be attributed to charge interaction between aspartic acid and the quaternary ammonium of DPPC, vesicles consisting of DL_4 and 1,2-dipalmitoyl PG: 1-palmitoyl-2-oleoyl PG (DPPG:POPG, 3:1) or DL_4 and DPPC:1-palmitoyl-2-oleoyl phosphatidyl choline (POPC) (3:1) were tested for activity in the pulsating bubble. The results of three experiments indicated that DL_4 with DPPC:POPC low-

Fig. 1. Amino acid sequences of SP-B and SP-C. Strongly hydrophilic residues are shaded.

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ered surface tension at minimal bubble radius to an average of 24.0 dyne/cm, whereas with DPPG:POPG the surface tension was decreased to only 38.0 dyne/cm at 5 min. RL_4 in the same mixtures of PLs gave comparable values of 15.5 and 19.0 dyne/cm, respectively. Both PL mixtures alone gave values of >49 dyne/cm, that is, undetectable activity. These results indicate that DL_4 may interact with the positive charge of the quaternary ammonium in phosphotidyl choline (PC), whereas RL_4 can interact with PC as well as PG.

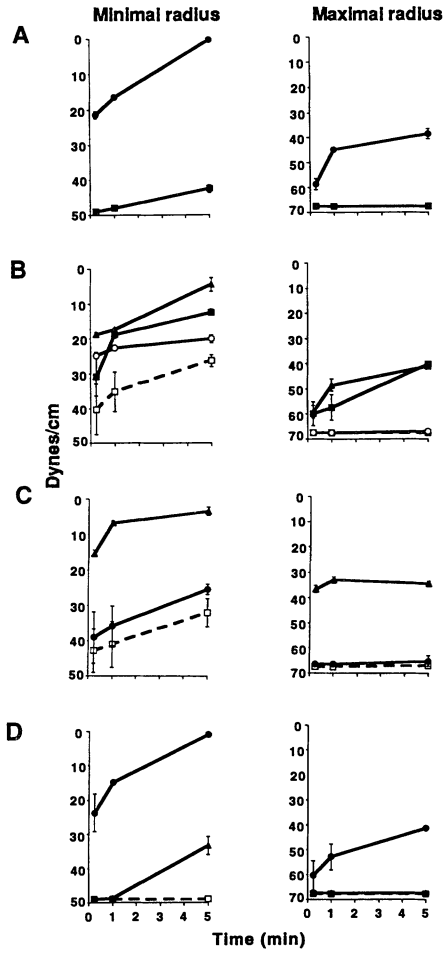

Fig. 2. Comparative activities of proteins and peptides in PL dispersions (*15, 16*). The effect of the various lipid-peptide mixtures on surface tension at an air-liquid interface was measured on a pulsating bubble surfactometer (*17*). SEM of $n \geq 3$ experiments is given for each data point. (**A**) PL (DPPC:PG, 3:1) dispersions containing 5% peptide SP-B 59-80 (●) or L-substituted SP-B 59-80 (■). (**B**) PL (DPPC:POPG, 3:1) dispersions containing 3% RL_4 (▲), poly-R (■), poly-L (○), or PL alone (□). Similar results were obtained with 1% RL_4 dispersions. (**C**) SP-B (▲) and SP-C (●) in DPPC:PG (3:1), and PL alone (□) (0.5% protein by weight). PL was at 4 mg/ml in these studies. Similar comparative results were obtained with SP-B and SP-C proteins at 0.1, 0.25, and 1.0% protein by weight. Three different preparations of SP-B and SP-C provided similar data. (**D**) PL (DPPC:POPG, 3:1) dispersions containing RL_4 (●), DL_4 (▲), each at 1% by weight peptide, and PL alone (□).

To determine the position of peptides with increasing relative concentrations of hydrophilicity in the DPPC:PG lipid layer, we synthesized peptides having a single tryptophan residue in each molecule: RLLLLR-LLLLWLLLLRLLLLR (RL_4-W); RLLR-LLRLWLRLLRLLRLLR (RL_2-W); and RRRRRRRRWRRRRRRRRRR (poly R-W). These peptides (50 μM) were incorporated into DPPC:PG, and the fluorescence emission spectra were obtained at an excitation wavelength of 295 nm. The emission maxima of tryptophan in various media (Table 1) suggest that the tryptophan in the RL_2 and RL_4 peptides was exposed to a relatively hydrophobic environment, whereas the tryptophan in poly-R was restricted to a hydrophilic environment. Because tryptophan in a completely nonpolar environment develops an emission maximum of 308 nm [for example, see (*10*)], these data suggest that the RL_4 and RL_2 peptides lie within the hydrophilic influence of the polar head groups. The hypothesis that SP-B acts by binding multiple PL polar head groups is supported by Raman

Table 1. Tryptophan emission maxima of surfactant peptides in various media. Peptide concentrations were 50 μM, and the excitation wavelength was 295 nm. These results are typical of two to four experiments for each point.

Peptide or amino acid	Medium	Emission maximum (nm)
Poly-R	DPPC:PG	346
RL_2-W	DPPC:PG	322
RL_4-W	DPPC:PG	326
Poly R-W	0.15 M NaCl	351
W	0.15 M NaCl	352
RL_2-W	0.15 M NaCl: CH_3OH (50:50)	345

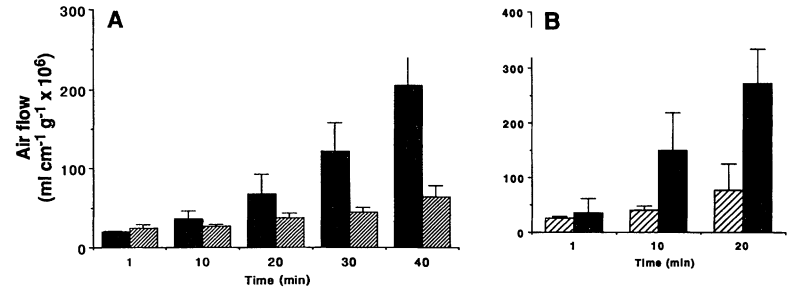

Fig. 3. (**A**) Capacity of peptide SP-B 59-80 (solid bars) and SP-B 59-80 L-substituted (crosshatched bars) to increase compliance (air flow in milliliters of air per centimeter of H_2O per gram of body weight) in 27-day-old gestation fetal rabbit lungs. Studies were performed as described (*5*). Error bars represent SEM of $n \geq 3$ experiments. (**B**) Capacity of SP-B–like peptide KL_4 (21 residues), dispersed at 3% concentration in PL, DPPC:POPG (3:1), and palmitic acid (15%) to increase compliance in 27-day-old fetal rabbits (solid bars, PL + KL_4; crosshatched bars, PL). RL_4 peptides gave similar results. Peak inspiratory and expiratory pressures were 28 and 0 cm H_2O, respectively. Error bars represent SEM of $n \geq 3$ experiments.

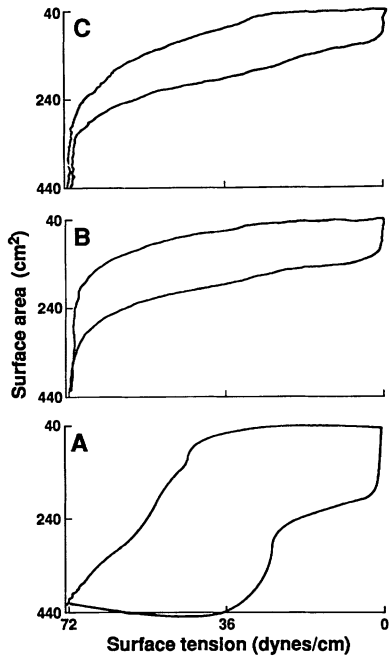

Fig. 4. Wilhelmy balance tracings of PL (DPPC:PG, 3:1) dispersions containing 5% RL_4 (**A**), or DL_4 (**B**), or PL alone (**C**) (*18*). The surface area is shown in the ordinate, with the top position representing minimal surface area formed by the moving baffle. Surface tension is on the abscissa, with lowest surface tension (greatest resistance to surface tension produced by the surfactant layers) at the right. The tracing proceeds from the lower left (at maximal area of the trough and greatest surface tension) to the right along the lower portion of the curve. The descending limb, formed as the surface area increases, begins at the upper right and moves directly to the left. The RL_4 dispersion, but not the DL_4 dispersion, lowers surface tension immediately as the surface area is reduced and, on the descending limb, maintains resistance to surface tension to a degree far greater than DL_4 or PL alone. The RL_4-PL tracings maintained activity in subsequent cycles to a much greater degree than DL_4-PL and PL alone.

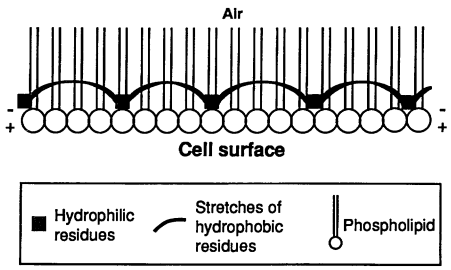

Fig. 5. Two-dimensional theoretical representation of the mechanism of function of SP-B protein in the PL monolayer. The hydrophilic, charged residues interact with polar head groups of the PL, whereas the hydrophobic stretches of the peptide interact with the acyl side chains. These interacting forces induce stabilization of the PL layer, providing greater capacity to resist surface tension or alveolar collapse.

spectroscopic data, published separately (*11*), showing that RL_4 and SP-B peptide 59-81 complex with PL to form a denser head group packing order than that demonstrated by PL alone and provide increases in intermolecular and intrachain order of the PLs. These data are consistent with the observations of Baatz *et al.* (*12*) showing that bovine SP-B induces an increased ordering of the polar surface of DPPC-DPPG membrane bilayers.

The data support a concept that electrostatic charges of the intermittent hydrophilic residues of SP-B interact with the PL polar head groups and that stretches of hydrophobic residues interact with acyl side chains of the PLs to induce increased lateral intermolecular order. The electrostatic interactions are essential for surfactant function. These relationships are depicted in Fig. 5. The combination of these simplified peptides and DPPC-POPG provide excellent surfactant in 27-day-old fetal rabbits and 130-day-old fetal rhesus monkeys (*13*). The simplified peptide surfactant could serve as an excellent material for replacement therapy in respiratory distress syndromes.

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7. A preliminary account has been published in abstract form; see C. G. Cochrane and S. D. Revak, *Pediatr. Res.* **27**, 297A (1990).
8. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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15. Peptides were synthesized by solid-phase synthesis (*14*) as noted (*5*). Peptides were analyzed by high-performance liquid chromatography (HPLC) on a Vydac C18 column and by fast-atom bombardment mass spectrometry. In each case a single major peak was observed in HPLC chromatograms, and mass determinations were less than 1 mass unit from calculated molecular masses. The PLs L-α-DPPC, POPC, PG (egg), DPPG, and POPG were purchased from Avanti Polar-Lipids, Inc. (Pelham, AL).
16. Synthetic peptides in chloroform-methanol (generally 50% chloroform and 50% methanol) were added to mixtures of DPPC and PG (3:1) in glass tubes. Relative to the total amount of PL, the peptide was 1%, 3%, or 5% by weight as noted. After a 10-min incubation at 43°C, the samples were dried under N_2 and held for 15 min under vacuum. Water was added, and the tubes were incubated at 43°C with periodic vortexing for 1 hour. Small volumes (usually 10% of final volume) of concentrated NaCl solutions were added to yield a final salt concentration of 0.9%. The final concentration of PL was 10 mg/ml for studies performed in the pulsating bubble surfactometer and 20 mg/ml for fetal rabbit studies. Vesicle preparations used for tryptophan fluorescence studies differ slightly; the dried lipid-peptide mixtures were rehydrated directly in saline (0.9% NaCl), and the final peptide concentration was held constant at 50 μM with a peptide to lipid ratio of 1:20, resulting in PL concentrations of 2.6 to 3.2 mg/ml.
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28 May 1991; accepted 20 August 1991

Phosphorylation of c-Src on Tyrosine 527 by Another Protein Tyrosine Kinase

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The protein tyrosine kinase activity of the cellular Src protein is negatively regulated by phosphorylation at tyrosine residue 527 (Tyr^{527}). It has not been established whether this regulatory modification of Src is mediated by autophosphorylation or by another cellular protein kinase. The phosphorylation of a modified form of c-Src that lacks kinase activity was examined in mouse cells that do not express endogenous Src (because of the targeted disruption of both *src* alleles). Phosphorylation of the inactive form of Src on Tyr^{527} occurred to a similar extent in cells lacking endogenous Src as it did in cells expressing Src. Therefore, Tyr^{527} phosphorylation, and thus negative control of Src kinase activity, is mediated by another cellular protein tyrosine kinase.

THE C-*SRC* PROTEIN IS A 60-KD CELLULAR protein tyrosine kinase that has served as the prototype member of a family of cytoplasmic protein tyrosine kinases. The c-*src* protein is the cellular homolog of the v-*src* oncogene product encoded by the Rous sarcoma virus. The specific activity of c-Src is 4 to 50 times lower than that of v-Src and other oncogenic variants of Src (*1, 2*). Wild-type c-Src is phosphorylated on Tyr^{527} (*3*), whereas oncogenic, activated variants of c-Src show severely reduced or undetectable phosphorylation at this site (*2, 4, 5*). The kinase activity of c-Src appears to be negatively regulated by phosphorylation at Tyr^{527}. Dephosphorylation at this site causes a 10- to 20-fold enhancement of catalytic activity (*6, 7*), and amino acid substitutions at residue 527 cause an increase in kinase activity and an activation of the transforming potential of c-Src (*8*). Lastly, the activated form of c-Src that is associated with middle T antigen of polyoma virus is not phosphorylated on Tyr^{527} (*9*).

It is not clear whether Tyr^{527} is phosphorylated by an autophosphorylation event or by another tyrosine kinase. Partially purified c-Src is inefficiently phosphorylated on Tyr^{527} in vitro (even after removal of the endogenous phosphate from Tyr^{527}), indicating that Tyr^{527} may be phosphorylated by another cellular enzyme (*7, 10*). Modified forms of c-Src lacking kinase activity were

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