Gabrion, J. (1989) *J. Neurosci. 9*, 806-814.
Prasad, A. R. S., Prasad, V., Luduena, R. F., & Horowitz, P. M. (1987) *Biochem. Biophys. Res. Commun. 145*, 949-955.
Rasenick, M. M., & Bitensky, M. W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4628-4632.
Rasenick, M. M., & Wang, N. (1988) *J. Neurochem. 51*, 300-311.
Rasenick, M. M., & Childers, S. R. (1989) *J. Neurochem. 53*, 219-225.
Rasenick, M. M., Stein, P. J., & Bitensky, M. W. (1981) *Nature 294*, 560-562.
Rasenick, M. M., O'Callahan, C. M., Moore, C. A., & Kaplan, R. S. (1985) in *Microtubules and Microtubule Inhibitors* (DeBrabander, M., & DeMey, J., Eds.) pp 313-326, Elsevier, Amsterdam.
Rasenick, M. M., Yan, K., & Wang, N. (1989) in *The Guanidine Nucleotide Binding Proteins* (Bosch, L., Krall, B., & Parmeggiani, A., Eds.) pp 391-402, Plenum Publishing Corp., New York.
Salomon, Y. (1979) *Adv. Cyclic Nucleotide Res. 10*, 35-55.
Serrano, L., Avila, J., & Maccioni, R. B. (1984) *Biochemistry 23*, 4675-4681.
Shelanski, M., Gaskin, F., & Cantor, C. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 765-769.
Sternlicht, H., Yaffe, M., & Farr, G. (1987) *FEBS Lett. 214*, 226-235.
Vaillancourt, R., Dhanasekaran, N., Johnson, G. L., & Ruoho, A. E. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3645-3649.
Wang, N., Yan, K., & Rasenick, M. M. (1990) *J. Biol. Chem. 265*, 1239-1242.
Webb, B. C., & Wilson, L. (1980) *Biochemistry 19*, 1993-2001.
Yang, J. T., Laymon, R. A., & Goldstein, L. S. (1989) *Cell 56*, 879-889.
Zor, U. (1983) *Endocr. Rev. 4*, 1-21.

Characterization of Lipid Insertion into Monomolecular Layers Mediated by Lung Surfactant Proteins SP-B and SP-C†

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ABSTRACT: Pulmonary surfactant proteins, SP-B and SP-C, if present in preformed monolayers can induce lipid insertion from lipid vesicles into the monolayer after the addition of (divalent) cations [Oosterlaken-Dijksterhuis, M. A., Haagsman, H. P., van Golde, L. M. G., & Demel, R. A. (1991) *Biochemistry 30*, 8276-8287]. This model system was used to study the mechanisms by which SP-B and SP-C induce monolayer formation from vesicles. Lipid insertion proceeds irrespectively of the molecular class, and PG is not required for this process. In addition to lipids that are immediately inserted from vesicles into the monolayer, large amounts of vesicles are bound to the monolayer and their lipids eventually inserted when the surface area is expanded. SP-B and SP-C are directly responsible for the binding of vesicles to the monolayer. By weight, the vesicle binding capacity of SP-B is approximately 4 times that of SP-C. For vesicle binding and insertion, the formation of close contacts between monolayer and vesicles is essential. SP-B and SP-C show very similar surface properties. Both proteins form extremely stable monolayers (collapse pressures 36-37 mN/m) of α-helical structures oriented parallel to the interface. In monolayers consisting of DPPC and SP-B or SP-C, an increase in mean molecular area is observed, which is mainly attributed to the phospholipid. This will greatly enhance the insertion of new lipid material into the monolayer. The results of this study suggest that the surface properties and the hydrophobic nature of SP-B and SP-C are important for the protein-mediated monolayer formation.

Pulmonary surfactant, a complex lipid/protein mixture, is synthesized in alveolar type II cells, assembled into lamellar bodies, and secreted into the alveolar space (Haagsman & van Golde, 1991). Within the alveolar space, surfactant lipids are found in a number of different structural forms, including lamellar bodies, tubular myelin, and various vesicular structures (Manabe, 1979). Tubular myelin is thought to be the precursor of the monomolecular surface film that stabilizes the alveoli at end expiration (Goerke, 1974; Clements, 1977). Lipids comprise the majority (approximately 90%) of this surface-active material. The most abundant phospholipid components are dipalmitoylphosphatidylcholine (DPPC),[1] unsaturated phosphatidylcholine (PC), and phosphatidyl-

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[1] Abbreviations: SP-A, SP-B, and SP-C, surfactant proteins A, B, and C, respectively; PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid; MLV, multilamellar vesicle(s); SUV, small unilamellar vesicle(s); LUV, large unilamellar vesicle(s); CD, circular dichroism.

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glycerol (PG) (Haagsman & van Golde, 1991).

It has been reported that the hydrophobic surfactant proteins SP-B (Hawgood et al., 1987) and SP-C (Takahashi & Fujiwara, 1986; Warr et al., 1987; Arjomaa & Hallman, 1988) play an important role in the adsorption of phospholipids to an air/liquid interface. SP-B has a molecular weight of 18 000 under nonreducing conditions and a molecular weight of 5000–8000 under reducing conditions (Possmayer, 1988; Hawgood, 1989; Weaver & Whitsett, 1991). The reduced form of SP-B consists of 79 amino acids and has a high cysteine content (7 cysteines in a total of 79 residues). Two regions of 23 and 24 amino acids with a high content of both hydrophobic and cationic residues are possible sites for interaction with a phospholipid bilayer (Hawgood, 1989). SP-C has a molecular weight of 4500–8000 under both reducing and nonreducing conditions (Possmayer, 1988; Hawgood, 1989; Weaver & Whitsett, 1991). The monomeric form of SP-C consists of 35 amino acids and has 2 palmitoyl groups covalently linked to the polypeptide chain (Curstedt et al., 1990). A continuous hydrophobic domain of 23 residues with a high valine content probably plays an important role in the interaction with a phospholipid bilayer by forming a membrane-spanning domain (Hawgood, 1989).

The mechanism by which SP-B and SP-C enhance phospholipid adsorption to the air/water interface is not known. In a previous paper (Oosterlaken-Dijksterhuis et al., 1991b), we described a new model system to study the interactions between the hydrophobic surfactant proteins and (phospho)-lipids at the air/water interface using the Wilhelmy plate method. In this model system, the surfactant proteins are included in a preformed monolayer, which leads to a higher rate of monolayer formation from lipid vesicles than if the proteins are solely present in the lipid vesicles (Oosterlaken-Dijksterhuis et al., 1991b). SP-B or SP-C present in preformed phospholipid monolayers of DPPC/PG (7:3 w/w) at an initial surface pressure of 20 mN/m induces the insertion of phospholipids from SUV or LUV of DPPC/PG (7:3 w/w) into the monolayer until a maximum surface pressure of 51 ± 1 mN/m is reached. This protein-mediated phospholipid insertion into the monolayer is dependent on (1) the presence of (divalent) cations, (2) the vesicle size and concentration in the subphase, (3) the protein concentration in the preformed monolayer, and (4) the initial surface pressure of the preformed monolayer (Oosterlaken-Dijksterhuis et al., 1991b).

In this study, we investigated the mechanism by which the hydrophobic surfactant proteins SP-B and SP-C induce monolayer formation from phospholipid vesicles. SP-B or SP-C was spread at the air/water interface to facilitate the study of the properties of the proteins and to quantify the involvement of the proteins in lipid insertion into the monolayer. The results presented in this paper show that the following aspects are important during protein-mediated monolayer formation: (1) binding of the phospholipid vesicles to SP-B or SP-C present in the monolayer; (2) induction of close contacts between monolayer and vesicles by (divalent) cations, involving dehydration of the polar head-group region of the phospholipids and reduction of the repulsive forces; and (3) insertion of lipid into the monolayer facilitated by the surface properties of the protein and an increase in mean molecular area of the mixed lipid/protein monolayer.

Materials and Methods

Materials. Dipalmitoylphosphatidylcholine (DPPC) was obtained from Roth (Karlsruhe, Germany). Cholesterol and phosphatidylglycerol (PG; sodium salt, prepared from egg yolk PC) were purchased from Merck (Darmstadt, Germany) and Sigma Chemical Co. (St. Louis, MO), respectively. Phosphatidylcholine from egg yolk (egg PC) was purified by high-performance liquid chromatography and judged chromatographically pure by the use of thin-layer chromatography. L-3-Phosphatidyl[U-^{14}C]inositol was purchased from Amersham ('s-Hertogenbosch, The Netherlands). [4-^{14}C]-Cholesterol and L-α-dipalmitoyl[1-^{14}C]phosphatidylcholine were purchased from New England Nuclear ('s-Hertogenbosch, The Netherlands).

Isolation of SP-B and SP-C. SP-B and SP-C were isolated from porcine lung lavage. Porcine lungs were obtained from the slaughterhouse and lavaged 3–5 times with a solution of 154 mM NaCl. Pulmonary surfactant was prepared from the bronchoalveolar lavage by the method of Hawgood et al. (1985). Lung surfactant was extracted with 1-butanol (Haagsman et al., 1987). Butanol was dried by rotary evaporation, and the residue was dissolved in chloroform/methanol/0.1 M HCl (1:1:0.05 v/v). Insoluble material was removed by centrifugation. SP-B and SP-C were separated from lipids and purified to homogeneity by Sephadex LH-60 chromatography as described earlier (Oosterlaken-Dijksterhuis et al., 1991a). Protein was determined by the fluorescamine procedure using bovine serum albumin as a standard (Böhlen et al., 1973). This procedure gave similar estimates to those obtained by quantitative amino acid analysis. Phospholipid phosphorus was estimated according to the method of Bartlett (1959). The SP-B (M_r 18 000) and SP-C (M_r 4500) fractions contained 1–2 and 0.5–1 mol of phospholipid/mol of protein, respectively.

Multilamellar Vesicles (MLV). Lipids dissolved in chloroform/methanol (1:1 v/v) were dried under a stream of nitrogen at 37 °C. The lipid films were hydrated in 25 mM Hepes (pH 7.0) (2 μmol of lipid/mL). The suspensions were vortexed for 5 min.

Small Unilamellar Vesicles (SUV). Small unilamellar vesicles were prepared from MLV by sonication with a Branson B12 sonifier equipped with a 0.5-in. flat-top disrupter tip for 1 min at 50 W.

Vesicles were prepared each day at 45 °C and kept at 37 °C.

Monolayer Studies. Monolayer experiments were performed in a thermostatically controlled box at 37.0 ± 0.1 °C unless otherwise stated. Surface pressure was determined by the Wilhelmy plate method, using a Cahn 2000 electrobalance (Demel, 1982). For constant-area monolayer experiments, a 5.5-mL Teflon trough (r = 1.25 cm) was used, and for monolayer experiments involving radiolabeled lipids, a 20-mL Teflon trough (5.3 cm × 5.7 cm) was used. The subphase buffer was 25 mM Hepes (pH 7.0) and was stirred continuously with a magnetic bar. Lipids and/or SP-B or SP-C dissolved in chloroform/methanol (3:1 v/v) were spread at the air/water interface with a glass capillary. Vesicles and salt solutions were injected into the subphase through an injection hole. Radiolabeled lipid vesicles were used to determine the amount of lipid associated with the monolayer. The surface radioactivity was detected continuously using a gas-type detector (Demel, 1974). In addition, the monolayers were collected from the air/water interface by sucking them into a counting vial (Rietsch et al., 1977) after the subphase had been flushed with 25 mM Hepes (pH 7.0) containing 10 mM EGTA for 10 min at a rate of 10 mL/min.

This procedure did not change the surface pressure. The radioactivity in the subphase dropped to nearly background values; interface radioactivity was corrected for the radioactivity of subphase collected (Sasaki & Demel, 1985).

Table I: Effect of Monolayer Protein Concentration on Lipid Insertion[a]

monolayer composition at 20 mN/m	time to reach maximum surface pressure (min)
SP-B	2
(DPPC/PG)/SP-B, 10:1 (w/w)	2
(DPPC/PG)/SP-B, 25:1 (w/w)	6
(DPPC/PG)/SP-B, 50:1 (w/w)	>60
SP-C	10
(DPPC/PG)/SP-C, 5:1 (w/w)	10
(DPPC/PG)/SP-C, 10:1 (w/w)	16
(DPPC/PG)/SP-C, 25:1 (w/w)	25
(DPPC/PG)/SP-C, 50:1 (w/w)	>60
DPPC/PG	>60

[a] Time required to reach the maximum surface pressure (51 ± 1 mN/m) was measured as a function of the protein concentration in the preformed monolayers. The monolayers were spread on a subphase of 25 mM Hepes (pH 7.0) to an initial surface pressure of 20 mN/m at 37 °C. The vesicle concentration in the subphase was 20 nmol of lipid/mL. The phospholipid insertion into the monolayer was "triggered" by the addition of $CaCl_2$ (final concentration 3 mM). The results are representative of at least three separate experiments. The percentage of variability between individual experiments was less than 10%.

Table II: Phospholipid Insertion into SP-B- or SP-C-Containing Monolayers[a]

monolayer composition at 20 mN/m	vesicle composition	maximum pressure (mN/m)	time to reach maximum pressure (min)
(DPPC/PG)/SP-B, 10:1 (w/w)	DPPC/PG	51 ± 1	2
(DPPC/PG)/SP-C, 5:1 (w/w)	DPPC/PG	51 ± 1	10
SP-B	DPPC/PG	51 ± 1	2
SP-C	DPPC/PG	51 ± 1	10
egg PC/SP-B, 10:1 (w/w)	egg PC[b]	45 ± 1	2
egg PC/SP-C, 5:1 (w/w)	egg PC[b]	45 ± 1	10
cholesterol/SP-B, 10:1 (w/w)	DPPC/PG	41 ± 1	2
cholesterol/SP-C, 5:1 (w/w)	DPPC/PG	41 ± 1	10
DPPC/SP-B, 10:1 (w/w)	egg PC/PG	51 ± 1	2
DPPC/SP-B, 10:1 (w/w)[c]	egg PC/PG	51 ± 1	2

[a] The monolayers were spread on a subphase of 25 mM Hepes (pH 7.0) to an initial surface pressure of 20 mN/m at 37 °C. SUV (final concentration 20 nmol of lipid/mL) were injected into the subphase prior to the $CaCl_2$ addition (final concentration 3 mM). [b] Vesicle concentration in the subphase: 400 nmol of lipid/mL. [c] 20 °C.

For measurements at constant surface pressure, a Teflon trough measuring 15 cm × 5 cm × 0.5 cm was used. The monolayer (35 mN/m) was formed at an initial surface area of 26.5 cm^2. Lipid vesicles and $CaCl_2$ were injected through an injection hole. The insertion of lipid from the vesicles into the monolayer was compensated by a computer-controlled movable Teflon barrier, and the increase in surface area was recorded.

Pressure–area measurements were performed at the air/water interface in a Teflon trough (35.5 cm long × 17.3 cm wide) with a movable Teflon barrier. Known amounts of protein and/or lipid dissolved in chloroform/methanol (3:1 v/v) were carefully spread at the air/water interface with an Agla micrometer syringe. The compression rate was 97.16 cm^2/min.

Circular Dichroism (CD) Measurements. Monolayers for CD measurements were collected on seven quartz plates put in series into the subphase and lifted at 3 mm/min. When the monolayer was deposited, the surface pressure was kept constant (25 mN/m) with a computer-controlled stepper motor. Measurements were performed at room temperature with a Jasco-600 spectropolarimeter, connected to a Laser 386 computer. The cuvette chamber was flushed with nitrogen gas. For each sample, 18 spectra at an angle of 0°, 45°, and 90° were accumulated. After subtraction of the spectra of a protein-free sample, the spectrum was smoothed. The scan speed was 10 nm/min (185–260 nm), the time constant 0.5 s, and the bandwidth 1 nm.

Results

Biophysical Studies with Different Monolayer and/or Vesicle Compositions. SP-B or SP-C present in a preformed monolayer of DPPC/PG (7:3 w/w) can induce, in the presence of 3 mM Ca^{2+}, the insertion of phospholipids from SUV or LUV of DPPC/PG (7:3 w/w) into the monolayer to a maximum surface pressure of 51 ± 1 mN/m (Oosterlaken-Dijksterhuis et al., 1991b). For monolayers with an initial surface pressure of 20 mN/m and at a vesicle (SUV) concentration of 20 nmol of lipid/mL, we observed that the insertion rate of phospholipids into the monolayer was maximal when the lipid to protein ratio in the monolayer was ≤10:1 (w/w) and ≤5:1 (w/w) for SP-B and SP-C, respectively (Table I). At lipid to protein ratios higher than 50:1 (w/w), the time required to reach the maximum surface pressure at this vesicle concentration was more than 1 h.

To determine whether there is a preferential insertion of particular lipids and to study the role of (phospho)lipids in the protein-mediated insertion process in more detail, the lipid composition of the monolayer and vesicles was varied. In this way, we could also investigate the involvement of anionic lipids in the association between monolayer and vesicles. The results are presented in Table II. The lipid composition of the preformed SP-B- or SP-C-containing monolayers had no effect on the insertion rates into the monolayers, but did affect the maximum surface pressure attained. The maximum surface pressures measured after phospholipid insertion into protein-containing egg PC monolayers or protein-containing cholesterol monolayers (45 ± 1 and 41 ± 1 mN/m, respectively) corresponded with the collapse pressures of egg PC (Phillips, 1972) and cholesterol (Pethica, 1955). They were about 6–10 mN/m lower than the maximum pressure of 51 mN/m for the DPPC/PG-containing monolayer (Table II). This high maximum surface pressure was also reached with the pure protein monolayers, which can most likely be attributed to DPPC present in the vesicles. In order to investigate whether DPPC and/or PG are preferentially inserted into the monolayer, the vesicle composition was varied. Phospholipids from egg PC vesicles could be inserted into protein-containing egg PC monolayers, although 20-fold higher vesicle concentrations in the subphase were required to obtain the same insertion rates as with DPPC/PG vesicles. These results suggest that unsaturated phosphatidylcholine species can also be inserted into the monolayer and that anionic lipids are not essential for the protein-mediated lipid insertion. Interestingly, the insertion of phospholipids into protein-containing monolayers that lacked PG could proceed in the absence of cations, albeit at a lower rate. After injection of the phospholipid vesicles into the subphase underneath the preformed monolayers [protein only, protein and (DP)PC, protein and cholesterol], surface pressure immediately increased at a rate of 1.5 mN/m·min. The insertion rate was enhanced 10-fold by the addition of 3 mM $CaCl_2$.

To study whether Ca^{2+} bound functionally to the monolayer, a monolayer consisting of DPPC/PG (7:3 w/w) and SP-B (lipid to protein ratio 10:1 w/w) was spread at the interface. First, Ca^{2+} was added to the subphase to a final concentration of 3 mM. After 6 min, the subphase was depleted of Ca^{2+}

by flushing with Ca^{2+}-free buffer prior to vesicle injection; phospholipids were not inserted into the protein-containing monolayer. The insertion of phospholipid into the monolayer could be induced by the addition of 3 mM $CaCl_2$. This indicates that no strong functional Ca^{2+} binding to the monolayer occurs.

To determine the effect of monolayer lipid fluidity on the protein-mediated lipid insertion, the influence of temperature was studied. The insertion rates into an SP-B-containing DPPC monolayer lacking PG (initial surface pressure 20 mN/m) were determined at 37 and at 20 °C. At 37 and at 20 °C, a pure DPPC monolayer is in gel phase at pressures >35 and >6 mN/m, respectively. In this experiment, egg PC/PG (7:3 w/w) vesicles were used to preclude an influence of temperature on the phospholipid vesicles. The insertion rates into the SP-B-containing DPPC monolayers were the same at both temperatures (Table II), indicating that monolayer fluidity is influenced by the presence of the protein.

Vesicle Binding Studies. To study the binding of vesicles to the monolayer, tracer amounts of [^{14}C]DPPC were incorporated into the phospholipid vesicles. Surface radioactivity reflects both incorporation into the monolayer and adsorption to the monolayer, while radioactivity in the subphase is not detected. Surface pressure and surface radioactivity were measured simultaneously with time. Figure 1A shows that in experiments with monolayers containing DPPC/PG (7:3 w/w) and SP-B (10:1 w/w) and DPPC/PG (7:3 w/w) vesicles no increase in surface radioactivity was measured in the absence of Ca^{2+}. After the addition of $CaCl_2$ to the subphase to a final concentration of 3 mM, phospholipids were inserted into the preformed monolayer, as indicated by a rapid change in surface pressure and an increase in surface radioactivity. Surface radioactivity continued to increase even after the maximum surface pressure was reached (2 min), and reached a plateau after about 1 h. It can be calculated [on the basis of the molecular areas (see below)] that about 2.5 nmol of lipid has to be inserted into the monolayer to increase the surface pressure from 20 to 51 mN/m. The total amount of lipid finally bound to the monolayer, measured after flushing of the subphase with buffer, was 30 nmol and, therefore, has to be ascribed largely to vesicle adsorption to the monolayer.

In order to discriminate between insertion of lipids into the monolayer and adsorption of lipid vesicles to the monolayer, we attempted to remove the latter lipids. In a previous paper, we showed that lipid insertion could be inhibited or terminated by EGTA (Oosterlaken-Dijksterhuis et al., 1991b). Therefore, after the surface radioactivity had reached a plateau, the subphase was flushed with buffer containing 10 mM EGTA to remove phospholipid vesicles. In this way, the subphase radioactivity dropped to nearly background values, but only about one-third of the radioactivity associated with the monolayer was removed. This indicated that phospholipid vesicles were tightly bound to the SP-B-containing monolayers. In fact, 8 times as much phospholipid was bound to the monolayers as was inserted into the monolayer. This means that once the vesicles are bound to the protein-containing monolayer there is no fast desorption, not even after Ca^{2+} depletion of the subphase.

When phospholipid insertion into the monolayer was terminated just before the maximum pressure was reached by adding EGTA to the subphase and immediately flushing the subphase with EGTA-containing buffer, the amount of phospholipid associated with the SP-B-containing monolayer was close to the calculated amount of phospholipid that could be inserted into the monolayer (Figure 1B), indicating that vesicle adsorption was inhibited. Comparable results were

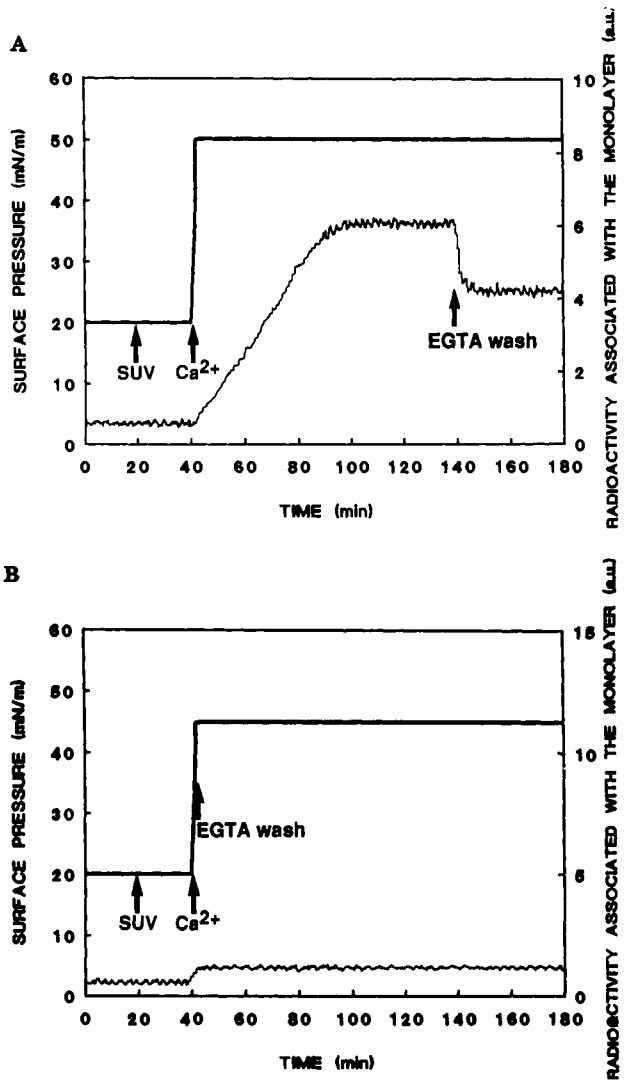

FIGURE 1: Vesicles of DPPC/PG containing tracer amounts of [^{14}C]DPPC. A monolayer consisting of DPPC/PG (7:3 w/w) and SP-B (lipid to protein ratio 10:1 w/w) was spread on a subphase of 25 mM Hepes (pH 7.0) to an initial surface pressure of 20 mN/m at 37 °C. The surface pressure (solid curve) and the surface radioactivity (wavy curve) were measured simultaneously with time. Vesicles [SUV of DPPC/PG (7:3 w/w) containing tracer amounts of [^{14}C]DPPC; final concentration 20 nmol of lipid/mL], $CaCl_2$ (final concentration 3 mM), and EGTA (final concentration 10 mM) were injected into the subphase at the indicated points. After the addition of EGTA, the subphase was flushed with buffer containing 10 mM EGTA. (A) EGTA was added after the surface radioactivity reached a plateau. (B) EGTA was added just before the maximum surface pressure was reached. The results are representative of two separate experiments.

obtained when phospholipid vesicles (DPPC/PG, 7:3 w/w) were labeled with tracer amounts of [^{14}C]PI or [^{14}C]cholesterol instead of [^{14}C]DPPC, indicating that all lipids were inserted into the protein-containing monolayer. [^{14}C]PI was used as a representative of anionic surfactant lipids since [^{14}C]PG was not readily available.

To determine whether the lung surfactant protein in the monolayer is directly responsible for binding of vesicles to the monolayer, vesicle binding was measured in relation to the SP-B concentration in the monolayer. In the absence of protein, about 2 nmol of lipid adsorbed to a monolayer of 7.4 nmol of lipid (Table III). Under these conditions, there was an increase in surface pressure of about 3 mN/m. For the protein concentrations used, the amount of phospholipid bound to the monolayer, measured after flushing of the subphase with

Table III: Total Amount of Lipid Bound to a Protein-Containing Monolayer[a]

monolayer composition	amount of lipid bound to monolayer (nmol)
DPPC/PG	2
(DPPC/PG)/SP-B, 10:1 (w/w)	20
(DPPC/PG)/SP-B, 20:1 (w/w)	10
(DPPC/PG)/SP-B, 50:1 (w/w)	7
(DPPC/PG)/SP-C, 5:1 (w/w)	11

[a] Monolayers of DPPC/PG (7:3) with different concentrations of SP-B or SP-C were spread on a subphase of 25 mM Hepes (pH 7.0) to an initial surface pressure of 20 mN/m at 37 °C. SUV of DPPC/PG (7:3 w/w) containing tracer amounts of [^{14}C]DPPC were injected into the subphase (final concentration 20 nmol of lipid/mL). After $CaCl_2$ was added to the subphase (final concentration 3 mM), the surface radioactivity and the surface pressure were simultaneously measured with time. After the surface radioactivity reached a plateau, the subphase was flushed with buffer containing 10 mM EGTA. The monolayers were collected, and the amount of lipid bound to the monolayer was calculated from the amount of radioactivity associated to the monolayer. The results are given as a representative of two separate experiments. The variability in the amount of lipid bound to the monolayer between individual experiments was less than 1 nmol of lipid.

10 mM EGTA, was linear with the SP-B concentration in the preformed monolayer. At the same vesicle concentrations in the subphase, the amount of phospholipid bound to monolayers containing DPPC/PG (7:3 w/w) and SP-C (5:1 w/w) was a factor of 2 less than the amount of phospholipid bound to monolayers of DPPC/PG (7:3 w/w) and SP-B (10:1 w/w) (Table III). The values obtained by counting the radioactivity of collected monolayers corresponded directly to the values of surface radioactivity registered by the gas-type detector.

The experiments described so far were performed at a constant surface area. Hence, the insertion of lipid into the monolayer will be terminated by the increase in the surface pressure. In order to determine the surface-covering capacity of vesicle insertion, the surface pressure was kept constant at 35 mN/m while the increase in surface area was measured. At a DPPC/PG vesicle concentration of 20 nmol of lipid/mL and an initial lipid to SP-B ratio in the monolayer of 25:1 (w/w), the surface area increased by 300%. The final lipid to SP-B ratio was about 100:1 (w/w) (data not shown). Under these conditions, this protein concentration in the monolayer would be too low to mediate lipid insertion (Table I). This means that the vesicles initially bound to the monolayer, as presented in Table III, were inserted at expanding surface area.

Monolayer Characteristics. The area per molecule and the area per residue of SP-B or SP-C monolayers were determined by surface compression of an exact quantity of protein. Figure 2 shows the pressure–area characteristics of pure SP-B and SP-C monolayers. The collapse pressures of SP-B and SP-C monolayers were 36 and 37 mN/m, respectively. The results were independent of the compression rate and were completely reproducible after expansion, indicating that there was no desorption from the interface. The pressure–area curve was not affected by the presence of 3 mM $CaCl_2$ or 150 mM NaCl in the subphase. At a surface pressure of 35 mN/m, the areas per residue were 0.16 and 0.17 nm^2 for SP-B and SP-C, respectively, corresponding with molecular areas for SP-B (M_r 18 000) and SP-C (M_r 4500) of 25.09 and 5.85 nm^2, respectively. The molecular areas at a surface pressure of 0.5 mN/m were 125.77 nm^2 for SP-B and 31.43 nm^2 for SP-C.

Protein/lipid mixtures were studied by spreading premixed monolayers of surfactant proteins and DPPC or cholesterol at a ratio as used in the experiments described above. For SP-B, the lipid to protein ratios of 10:1 (w/w) and 5:1 (w/w) correspond with molar ratios of 240:1 and 120:1. For SP-C,

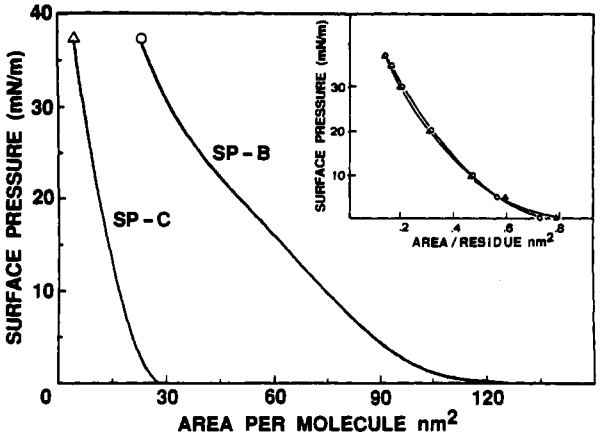

FIGURE 2: Pressure–area characteristics of SP-B (○) and SP-C (△). The symbols indicate the maximally obtainable stable monolayer pressures.

Table IV: Area per Molecule in SP-B- or SP-C-Containing Monolayers[a]

lipid	protein	lipid to protein ratio (w/w)	area per molecule (nm^2) at π = 35 mN/m: mean area	area per molecule (nm^2) at π = 35 mN/m: calculated area
DPPC			0.505	
DPPC	SP-B	10:1	0.674	0.606
DPPC	SP-B	5:1	0.804	0.707
	SP-B		25.09	
DPPC	SP-C	10:1	0.670	0.590
DPPC	SP-C	5:1	0.788	0.674
	SP-C		5.85	
cholesterol			0.380	
cholesterol	SP-B	5:1	0.471	0.454
cholesterol	SP-C	5:1	0.464	0.440

[a] Monolayers (50 nmol of lipid) were spread on a subphase of 25 mM Hepes (pH 7.0) at 37 °C.

the lipid to protein ratios of 10:1 (w/w) and 5:1 (w/w) correspond with molar ratios of 60:1 and 30:1. Calculations were based on at least two measurements. The deviation was generally ±0.01 nm^2. After compression of the mixed monolayers to 51 mN/m, the expansion curves showed no hysteresis. The pressure–area curves were completely reproducible after recompression. In monolayers of DPPC and SP-B or SP-C at a surface pressure of 35 mN/m, the mean area per molecule was 0.06–0.11 nm^2 larger than the value calculated by the additively rule (Table IV). The phase transition of DPPC vanished in the presence of the surfactant proteins (results not shown). At higher lipid to protein ratios, calculations of the mean molecular area are less accurate due to the large difference in the molecular areas of the compounds. In monolayers of cholesterol and SP-B or SP-C, however, the mean area per molecule at a surface pressure of 35 mN/m was hardly increased compared to the value calculated by the additively rule (Table IV). The mixed monolayers were not affected by the presence of 3 mM $CaCl_2$ or 150 mM NaCl in the subphase.

The secondary structures of SP-B and SP-C present in pure protein monolayers and mixed protein/lipid monolayers were determined by circular dichroism. The monolayers were collected at a surface pressure of 25 mN/m. The CD spectra of SP-B and SP-C monolayers (Figure 3) show minima at approximately 209 and 221 nm, indicating that SP-B and SP-C in pure protein monolayers have a high content of α-helical structure. The presence of phospholipids in the monolayer and/or 3 mM $CaCl_2$ or 150 mM NaCl in the subphase had

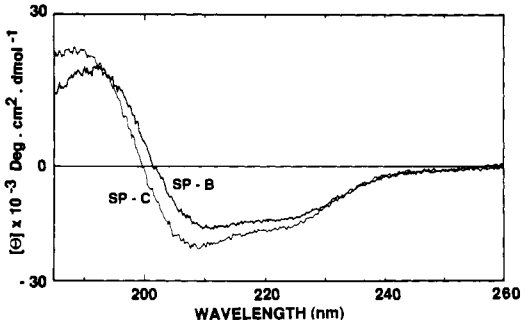

FIGURE 3: Circular dichroism spectra of SP-B and SP-C monolayers collected at a surface pressure of 25 mN/m and measured at room temperature.

no significant effect on the secondary structure of either protein.

Discussion

In a previous paper, we described a model system in which the interactions between the hydrophobic surfactant proteins (SP-B and SP-C) and (phospho)lipids can be studied. SP-B and SP-C, present in preformed monolayers of DPPC/PG (7:3 w/w), can induce the insertion of phospholipids from phospholipid vesicles (SUV or LUV of DPPC/PG, 7:3 w/w) into the monolayer to a maximum surface pressure of 51 ± 1 mN/m after the addition of (divalent) cations (Oosterlaken-Dijksterhuis et al., 1991b).

In this study, we showed that the protein-mediated phospholipid insertion was independent of the lipid composition of the preformed monolayer. Although the maximum surface pressures were determined by the collapse pressures of the lipid constituents of the monolayer, the insertion rates were not influenced by the composition of the monolayer (Table II). Phospholipids could also be inserted, at maximal rates, into monolayers consisting of only SP-B or SP-C (Tables I and II). These results suggest that in this model system the initial presence of (phospho)lipids in the preformed monolayer is not a prerequisite for protein-induced monolayer formation. However, the (phospho)lipids in the monolayer do influence the maximum surface pressure that is attained. The use of vesicles with different lipid compositions showed that unsaturated phospholipid species like egg PC can also be inserted into the monolayer, indicating that there is no specific lipid insertion (Table II).

It is generally believed that anionic lipid–Ca^{2+} interactions play an important role in fusion reactions. Such interactions could also play a role in vesicle binding to monolayers. However, as we demonstrated that vesicle binding to the monolayer was determined by SP-B and SP-C, and that there was no absolute requirement for PG in the monolayer and/or phospholipid vesicles for the protein-mediated insertion process (Table II), PG–Ca–PG bridges play no absolute role in monolayer formation. Vesicle diameter and structure are determining factors for the rate of vesicle insertion into the monolayer (Oosterlaken-Dijksterhuis et al., 1991b). SUV prepared from MLV of DPPC and PG will be different from those prepared from MLV of egg PC. This could explain the observation that 20 times higher vesicle concentrations are required for the insertion of egg PC in the absence of PG. The possibility that proteins bearing a net positive charge have a higher affinity for anionic lipid-containing vesicles is not supported by the results shown in Figure 1A, since vesicle binding in this experiment [monolayer of (DPPC/PG)/SP-B (10:1 w/w) and vesicles of DPPC/PG] was initiated only after Ca^{2+} addition. Lipid insertion from DPPC/PG (7:3 w/w) vesicles into monolayers consisting of only SP-B was noted in the absence of Ca^{2+}, but the addition of Ca^{2+} was required to obtain the maximal insertion rate.

When PG was present in both the monolayer and the vesicles, Ca^{2+} was required for the protein-mediated insertion process. However, the insertion process proceeded in the absence of Ca^{2+} when PG was absent in the preformed monolayer, albeit at a lower rate. Furthermore, there were no indications for strong functional Ca^{2+} binding to the monolayer, and in addition, Ca^{2+} can be replaced by other (divalent) cations (Oosterlaken-Dijksterhuis et al., 1991b). This may suggest that (divalent) cations play a role in inducing close contacts between the monolayer and phospholipid vesicles by (1) reducing repulsive forces between negatively charged lipids present in both phospholipid vesicles and the monolayer and (2) dehydration of the phospholipid head-group region of phospholipids directly involved in the insertion process. Aruga et al. (1985) and Kataoka et al. (1985) demonstrated the binding of Ca^{2+} to DPPC vesicles, resulting in a long-range attractive interaction between bound Ca^{2+} and the polar head groups of distant phosphatidylcholine molecules.

In constant surface pressure experiments at a surface pressure below the maximum surface pressure, vesicles initially bound to the preformed monolayer with a lipid to SP-B ratio of 25:1 (w/w) inserted into the monolayer even when the SP-B to lipid ratio decreased to 1:100 (w/w). This demonstrates that vesicles initially bound to the monolayer are inserted into the monolayer rather than released from the interface when the surface area expands. The use of vesicles containing [^{14}C]DPPC, [^{14}C]PI, and [^{14}C]cholesterol showed that in experiments in which vesicle binding was prevented by 10 mM EGTA, all lipids were inserted into the monolayers. Therefore, it can be concluded that SP-B and SP-C mediate the insertion of all lipids present in the vesicles. The amount of lipid bound to a monolayer consisting of DPPC/PG (7:3 w/w) and SP-B (10:1 w/w) was 2 times the amount of lipid bound to a monolayer consisting of DPPC/PG (7:3) and SP-C (5:1 w/w). This difference in binding capacity between SP-B and SP-C may largely explain the fact that SP-B is more effective in inserting phospholipids from phospholipid vesicles into the monolayer than SP-C is (Table I; Oosterlaken-Dijksterhuis et al., 1991b). The difference in binding capacity might be due to differences in the surface properties of the proteins. Although there is hardly any homology in the sequence of the proteins, it is striking that the surface area per residue and the surface stability of SP-B and SP-C are very similar.

Spread monolayers of homopolypeptides which are α-helical assume areas of 0.13–0.19 nm^2 per residue (Malcolm, 1973). The variation in area depends upon the length of the amino acid side chain, with larger groups causing greater separation of the α-helical rods lying in the surface. The collapse pressures of pure SP-B and SP-C monolayers were 36 and 37 mN/m, respectively, which are extremely high for pure protein monolayers and resemble those of lipids such as cholesterol (Pethica, 1955). The molecular areas at a surface pressure of 35 mN/m correspond to an area per residue of 0.16 nm^2 for SP-B (158 residues) and 0.17 nm^2 for SP-C (35 residues), which is consistent with the notion that hardly any segments of the protein molecules are squeezed out of the plane of the interface. These packing densities are consistent with monolayers consisting largely of α-helical structures lying with the long axes of the helical segments in the plane of the interface (Malcolm, 1973). This is in agreement with the CD spectra (Figure 3) which showed that both SP-B and SP-C monolayers have a high content of α-helical structure. Comparable surface

properties are described for the apoproteins A-I and A-II which form amphipathic α-helices and have molecular areas at maximum surface pressure of 0.15 and 0.13 nm^2/residue, respectively (Krebs et al., 1988). Although hardly any segments of the proteins are squeezed out of the plane of the interface, a large change in molecular area was observed as a function of surface pressure. This indicates that both proteins have the ability to fold extensively in a plane parallel to the interface.

The fact that mixed lipid/protein monolayers can be compressed to a surface pressure of 51 mN/m without loss of protein from the monolayer indicates that there is an interaction between protein and lipid. The increase in mean area per molecule in mixed lipid/protein monolayers provides further evidence for protein/lipid interactions. The change in mean molecular area was minimal in mixtures with a rigid molecule such as cholesterol. This could indicate that the lipid surface area is mainly affected. Increased membrane fluidity would increase the molecular area of DPPC and would enhance lipid insertion into the monolayer. The insertion rates into SP-B-containing DPPC monolayers (initial surface pressure 20 mN/m) at 37 and 20 °C were the same (Table II). At a surface pressure of 20 mN/m, a pure DPPC monolayer is in liquid phase at 37 °C and in gel phase at 20 °C. In general, insertion into gel-phase monolayers is strongly reduced. The conclusion that DPPC fluidity is increased is further supported by the observation that the phase transition in pure DPPC monolayers vanished in the presence of SP-B. Differential scanning calorimetric studies (Shiffer et al., 1988; Simatos et al., 1990; Oosterlaken-Dijksterhuis, unpublished results) and Fourier-transform infrared spectroscopy studies (Reilly et al., 1989) have also shown that SP-B and SP-C affect the gel- to liquid-phase transition of phospholipids by reducing the cooperativity of the transition. In addition, the presence of phospholipids in the monolayer and/or (divalent) cations in the subphase had no significant effect on the secondary structure of SP-B or SP-C. Although a preferred orientation of the proteins with respect to the lipid monolayer or the air/water interface could greatly influence the shape of the CD spectra (Vogel, 1987), circular dichroism experiments with SP-C present in phospholipid vesicles also showed a high proportion of α-helical structures (Elledge et al., 1987; Haagsman, unpublished results).

The surface properties of SP-B and SP-C and the different steps by which SP-B and SP-C induce monolayer formation from (phospho)lipid vesicles are closely related. Both SP-B and SP-C are extremely hydrophobic, but their amino acid sequences differ markedly. This may imply that the function of both proteins is related to their overall structure rather than to highly specific amino acid sequences. Recently, Venkitaraman et al. (1990) described that hydrophobic homopolymeric peptides enhance the biophysical activity of synthetic lung phospholipids. Further studies on the mechanism of monolayer formation will involve a more specific determination of the structure–function relationship of SP-B and SP-C.

Registry No. DPPC, 2644-64-6; Ca^{2+}, 7440-70-2; cholesterol, 57-88-5.

References

Arjomaa, P., & Hallman, M. (1988) *Anal. Biochem. 171*, 207–212.

Aruga, S., Kataoka, R., & Mitaku, S. (1985) *Biophys. Chem. 21*, 265–275.

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.

Böhlen, P., Stein, S., Dairman, W., & Udenfriend, S. (1973) *Arch. Biochem. Biophys. 155*, 213–220.

Clements, J. A. (1977) *Am. Rev. Respir. Dis. 115*, 67–71.

Curstedt, T., Johansson, J., Persson, P., Eklund, A., Robertson, B., Löwenadler, B., & Jörnvall, H. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2985–2989.

Demel, R. A. (1974) *Methods Enzymol. 32*, 539–545.

Demel, R. A. (1982) in *Membranes and Transport* (Martonosi, A. N., Ed.) Vol. 1, pp 159–164, Plenum Press, New York.

Elledge, B. W., Whitsett, J. A., Kreishman, G. P., & Baatz, J. E. (1987) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 46*, 2097A.

Goerke, J. (1974) *Biochim. Biophys. Acta 344*, 241–261.

Haagsman, H. P., & van Golde, L. M. G. (1991) *Annu. Rev. Physiol. 53*, 441–464.

Haagsman, H. P., Hawgood, S., Sargeant, T., Buckley, D., White, R. T., Drickamer, K., & Benson, B. J. (1987) *J. Biol. Chem. 262*, 13877–13880.

Hawgood, S. (1989) *Am. J. Physiol. 257*, L13–L22.

Hawgood, S., Benson, B. J., & Hamilton, R. L. (1985) *Biochemistry 24*, 184–190.

Hawgood, S., Benson, B. J., Schilling, J., Damm, D., Clements, J. A., & White, R. T. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 66–70.

Kataoka, R., Aruga, S., Mitaku, S., Kinosita, K., & Ikegami, A. (1985) *Biophys. Chem. 21*, 277–284.

Krebs, K. E., Ibdah, J. A., & Phillips, M. C. (1988) *Biochim. Biophys. Acta 959*, 229–237.

Malcolm, B. R. (1973) *Prog. Surf. Membr. Sci. 7*, 183–229.

Manabe, T. (1979) *J. Ultrastruct. Res. 69*, 86–97.

Oosterlaken-Dijksterhuis, M. A., van Eijk, M., van Buel, B. L. M., van Golde, L. M. G., & Haagsman, H. P. (1991a) *Biochem. J. 274*, 115–119.

Oosterlaken-Dijksterhuis, M. A., Haagsman, H. P., van Golde, L. M. G., & Demel, R. A. (1991b) *Biochemistry 30*, 8276–8287.

Pethica, B. A. (1955) *Trans Faraday Soc. 50*, 1402–1411.

Phillips, M. C. (1972) in *Progress in surface and membrane science* (Danielli, J. F., Rosenberg, M. D., & Cadenhead, D. A., Eds.) Vol. 5, pp 139–221, Academic Press, New York.

Possmayer, F. (1988) *Am. Rev. Respir. Dis. 138*, 990–998.

Reilly, K. E., Mautone, A. J., & Mendelsohn, R. (1989) *Biochemistry 28*, 7368–7373.

Rietsch, J., Pattus, F., Desnuelle, P., & Verger, R. (1977) *J. Biol. Chem. 252*, 4313–4318.

Sasaki, T., & Demel, R. A. (1985) *Biochemistry 24*, 1079–1083.

Shiffer, K., Hawgood, S., Düzgünes, N., & Goerke, J. (1988) Federation of American Societies for Experimental Biology 72nd Annual Meeting, Las Vegas, NV, May 1–5, 1988, A132, p A319.

Simatos, G. A., Forward, K. B., Morrow, M. R., & Keough, K. M. W. (1990) *Biochemistry 29*, 5807–5814.

Takahashi, A., & Fujiwara, T. (1986) *Biochem. Biophys. Res. Commun. 135*, 527–532.

Venkitaraman, A. R., Hall, S. B., & Notter, R. H. (1990) *Chem. Phys. Lipids 53*, 157–164.

Vogel, H. (1987) *Biochemistry 26*, 4562–4572.

Warr, R. G., Hawgood, S., Buckley, D. I., Crisp, T. M., Schilling, J., Benson, B. J., Ballard, P. L. Clements, J. A., & White, R. T. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7915–7919.

Weaver, T. E., & Whitsett, J. A. (1991) *Biochem. J. 273*, 249–264.