Lateral Phase Separation in Interfacial Films of Pulmonary Surfactant

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ABSTRACT To determine if lateral phase separation occurs in films of pulmonary surfactant, we used epifluorescence microscopy and Brewster angle microscopy (BAM) to study spread films of calf lung surfactant extract (CLSE). Both microscopic methods demonstrated that compression produced domains of liquid-condensed lipids surrounded by a liquid-expanded film. The temperature dependence of the pressure at which domains first emerged for CLSE paralleled the behavior of its most prevalent component, dipalmitoyl phosphatidylcholine (DPPC), although the domains appeared at pressures 8–10 mN/m higher than for DPPC over the range of 20–37°C. The total area occupied by the domains at room temperature increased to a maximum value at 35 mN/m during compression. The area of domains reached 25 ± 5% of the interface, which corresponds to the predicted area of DPPC in the monolayer. At pressures above 35 mN/m, however, both epifluorescence and BAM showed that the area of the domains decreased dramatically. These studies therefore demonstrate a pressure-dependent gap in the miscibility of surfactant constituents. The monolayers separate into two phases during compression but remain largely miscible at higher and lower surface pressures.

INTRODUCTION

The phase behavior in films of pulmonary surfactant may be critical for normal function of the lung. Pulmonary surfactant is the mix of lipids and proteins that covers the thin liquid layer which lines the alveolar air spaces and minimizes the interfacial tension of the curved surface, preventing alveolar collapse at the end of exhalation. Premature babies born with inadequate amounts of surfactant before the lungs have matured develop the life-threatening injury of the neonatal respiratory distress syndrome caused by repeated collapse and reopening of the small air spaces (Robertson, 1984). Measurements in the lung indicate that the surfactant films achieve extraordinarily dense structures that effectively eliminate interfacial tension when compressed by the shrinking surface area during normal exhalation. Surfactant films in the lung lower interfacial tension, which would be 70 mN/m for a clean air-water interface, to values less than 1 mN/m (Schürch, 1982). Only films with the highly ordered structure and rigidity of a condensed phase seem likely to withstand compression without collapse from the interface to the high surface pressures necessary to achieve this effect.

The compression isotherm of pulmonary surfactant, however, provides no evidence for the formation of liquid-condensed (LC) lipid. In films that contain a single compound, a first-order phase transition produces a discontinuity in the compression isotherm at the onset of the liquid expanded (LE)-LC coexistence region. For dipalmitoyl phosphatidylcholine (DPPC), the most prevalent component of pulmonary surfactant, this discontinuity occurs at 3–5 mN/m at 20°C and shifts to progressively higher surface pressures with increasing temperatures (Albrecht et al., 1978). The isotherms for lung surfactant do not show this characteristic feature and so provide no support for a phase transition. Prior studies, however, have shown that in mixed films, smooth isotherms do not rule out the presence of phase separation. Nag and Keough have shown with epifluorescence microscopy that binary films of DPPC and dioleoyl phosphatidylcholine form a condensed phase despite the absence of any discontinuity in the compression isotherm (Nag and Keough, 1993).

We have used two complementary microscopic techniques to establish that phase separation does occur in films with the much more complicated composition of pulmonary surfactant. Epifluorescence microscopy distinguishes regions with different packing densities in interfacial monolayers on the basis of differences in solubility of fluorescent lipid probes between ordered domains and the more fluid LE phase (Lösche et al., 1983; McConnell et al., 1984; Peters and Beck, 1983). Brewster angle microscopy provides an alternative method of detecting phase separation without the need to add exogenous probes, although at somewhat lower spatial resolution (Hénon and Meunier, 1991; Hönig and Möbius, 1991). Our experiments used spread monolayers of extracted calf surfactant (calf lung surfactant extract, CLSE) as a model of the surfactant film in the lung. The spread films provide a system in which the composition and surface concentration of the monolayer are known. The extracts of calf surfactant provide a well-characterized mixture that contains the complete mix of surfac-

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tant phospholipids, neutral lipids, and the two hydrophobic surfactant proteins SP-B and SP-C. It differs in composition from complete surfactant only in the absence of the glycoprotein SP-A (Kendig et al., 1989). The ability of extracted surfactant to adsorb to an air-liquid interface and then undergo compression to high surface pressure is comparable to the performance of complete surfactant (Hall et al., 1992), and it functions well as a therapeutic surfactant in the lung (Jobe, 1993). Our measurements extend over a range of temperatures, including physiological conditions, to determine the circumstances under which phase separation occurs in films of pulmonary surfactant.

MATERIALS AND METHODS

Materials

Extracts of surfactant from calf lungs provided by Dr. Edmund Egan of ONY, Inc. (Amherst, NY) and Dr. Robert Notter of the University of Rochester were prepared as described previously (Hall et al., 1992). Surfactant was removed from freshly excised calf lungs by repeated lavage with 150 mM NaCl. Centrifugation at 250 × *g* for 10 min removed cells and large debris. Higher speed centrifugation of the resulting supernatant at 12,500 × *g* for 30 min then pelleted the large surfactant aggregates. After resuspension of the pelleted surfactant, the hydrophobic constituents were extracted into chloroform (Bligh and Dyer, 1959) to yield CLSE.

The composition of the major phospholipid headgroups of this mixture is sphingomyelin, 1% (mol/mol); phosphatidylcholine (PC), 82%; phosphatidylinositol, 3%; phosphatidylethanolamine, 3%; phosphatidylglycerol, 6% (Hall et al., 1994). Ester-linked diacyl compounds constitute 97% of the PCs, of which the four major components are DPPC, 42%; palmitoyl-palmitoleoyl PC (16:0–16:1), 19%; palmitoyl-oleoyl PC (16:0–18:1), 14%; and palmitoyl-myristoyl PC (16:0–14:0), 13% (Kahn et al., 1995). CLSE also contains approximately 8% (mol/mol) neutral lipid, consisting almost entirely of free cholesterol, and approximately 1% (w/w) of the mixture of surfactant proteins SP-B and SP-C (Hall et al., 1994).

The data reported here represent results obtained with a single preparation of CLSE. Experiments with other preparations produced qualitatively equivalent results. Domains appeared, grew, and then abruptly decreased in total area during compression for all preparations. The surface pressure of the maximum area, however, varied by approximately 5 mN/m. We attributed this variation to measured differences in the composition, particularly in the content of neutral lipid.

DPPC purchased from Sigma (St. Louis, MO) was used without further purification.

Reverse-osmosis-grade water for these studies was obtained from purification systems purchased from either Millipore (Bedford, MA) or Barnsteadt (Dubuque, IA) and had a resistivity of approximately 18 MΩ-cm. All glassware was acid-cleaned. All solvents were at least reagent grade and contained no surface active stabilizing agents.

Methods

Biochemical assays

Phospholipid concentrations were determined by measuring the phosphate content (Ames, 1966) of measured aliquots of extracted material.

Compression isotherms

Surface pressure–area (π-A) isotherms of interfacial monolayers were measured on a commercially available trough (KSV-3000; KSV Instruments, Helsinki, Finland). Monolayers were compressed at a rate of 1.0 Å^2/phospholipid molecule/min. The temperature of the trough was regulated with a Lauda RCS circulating bath. Monolayers were created by spreading 80 μl of a 0.95 mM phospholipid stock solution in chloroform at the air-liquid interface. Only the phospholipid concentration of the surfactant solutions was measured, and consequently molecular areas were expressed only in terms of phospholipids, with no attempt made to correct for the presence of neutral lipid and protein. A 10-min waiting period before monolayer compression allowed for evaporation of the spreading solvent. The π-A curve reported here was selected from a group of three reproducible isotherms in which deviations in molecular area and surface pressure between different experiments were less than 2 Å^2/phospholipid molecule and 0.4 mN/m, respectively. All experiments, including epifluorescence and Brewster angle microscopy as well as compression isotherms, used monolayers spread on a solution of 10 mM HEPES (pH 7.0), 150 mM NaCl, and 1.5 mM $CaCl_2$ (HSC).

Epifluorescence microscopy

Epifluorescence microscopy used a Zeiss-ACM microscope (Meller, 1988) with a 50× objective to visualize lipid monolayers (Lösche et al., 1983; McConnell et al., 1984; Peters and Beck, 1983) on the surface of a previously described home-built Wilhelmy balance. The Teflon trough had a surface area of 108 cm^2 and a subphase volume of 100 ml (Maloney and Grainger, 1993), the temperature of which was regulated to ±1°C with water pumped through jackets surrounding the trough. Samples of surfactant preparations containing 1% (mol/mol surfactant phospholipid) of rhodamine-dipalmitoyl phosphatidylethanolamine (Rh-DPPE) labeled at the headgroup (Molecular Probes, Eugene, OR) were spread in approximately 80 μl of chloroform to give an initial molecular area of 150 Å^2/phospholipid molecule. Films were then compressed at 2.8 Å^2/phospholipid molecule/min either to specific surface pressures at which images were recorded on the static film, or until domains appeared in experiments at different temperatures. A Hamamatsu C2400 SIT camera recorded fluorescence images either to VHS videotape for later analysis or directly to a computer (Quadra 650, Apple, Cupertino, CA; with a LG-3 frame grabber, Scion Corp, Frederick, MD). A C-shaped Teflon mask placed directly in the trough and extending through the interface minimized movement of the monolayer (Grainger et al., 1989; Meller, 1988). Images obtained inside and outside the mask at frequent intervals ensured that the mask created no artifacts.

Brewster angle microscopy

Brewster angle microscopy (BAM) allowed examination of surfactant monolayers without fluorescent probes. Contrast in BAM results from differences in reflectivity to p-polarized light produced by variation in the optical thickness within an interfacial film. The background intensity is suppressed by choosing the angle of incidence to be Brewster's angle for the bare interface, at which no p-polarized light is reflected (Hénon and Meunier, 1991; Hönig and Möbius, 1991).

The BAM was a custom-built apparatus configured to image the surface of a Wilhelmy balance. Light from a 100-mW diode-pumped Nd-YAG laser (Coherent, Santa Clara, CA) was set to p-polarization by a Glan-Thompson polarizer (CVI, Albuquerque, NM; extinction 10^{-6}) before incidence on the water surface at 53.12° with respect to the surface normal. A lens system (Spindler and Hoyer, Milford, MA) collected the reflected light and formed an image on a CCD camera (Dage-MTI, Michigan City, IN). The images were noise-filtered for display. Because the light collection of the BAM is highly sensitive to the height of the water in the trough, the water level was kept constant by the regular addition of water to counter the effect of evaporation. Details of this optical setup will be published elsewhere (Schief et al., manuscript in preparation). The image from the CCD was captured by a SG-9 Scion capture card (Scion, Frederick, MD), using the program Image from the National Institutes of Health, and viewed directly on the screen of a MacIntosh IIci computer (Apple). Images can be recorded either on line or on S-VHS (Mitsubishi, Tokyo, Japan). A graphical programming interface (LabView; National Instruments, Austin, TX) also provided computer control of the Wilhelmy

balance. The balance for these studies used a continuous ribbon barrier (Labcon, Darlington, England) inserted vertically through the air-liquid interface of buffer contained in a custom-built temperature-regulated Teflon trough.

Image analysis

The total area of the domains in epifluorescence images was analyzed using the program Image (National Institutes of Health, Bethesda, MD). The marked contrast between dark domains and the surrounding fluorescent film allowed direct measurements of the size of all domains in any given microscopic field based on digitally assigned pixel grayscale values. Each data point represents the analysis of a minimum of 12 recorded images from four independent experiments. Images were recorded in each experiment from at least three different regions of the monolayer. The fraction of the monolayer occupied by solid domains was calculated by expressing the sum of all domain areas as a percentage of the total area analyzed for each experiment and then averaging the results. Data are expressed as a mean ± SD.

RESULTS

Epifluorescence microscopy demonstrated that compression of films of pulmonary surfactant produces lateral phase separation (Fig. 1). Interfacial films composed of the hydrophobic constituents of calf surfactant contained discrete dark domains in an otherwise brightly fluorescent film. Depletion of the fluorescent probe Rh-DPPE in the domains implies different molecular packing from that of the surrounding film. Domains were present on the scale of a few microns at surface pressures of 9 mN/m and above. Formation of these domains produced no discontinuity in the π-A isotherm for CLSE or in its first or second derivative. Plots of the logarithm of the bulk modulus ($-A \cdot \partial\pi/\partial A$) as a function of molecular area were linear, with no evidence of the changes in slope that indicate phase separation (Hirshfeld and Seul, 1990). The isotherm remained entirely smooth with compression speeds as low as 0.1 Å^2/phospho-

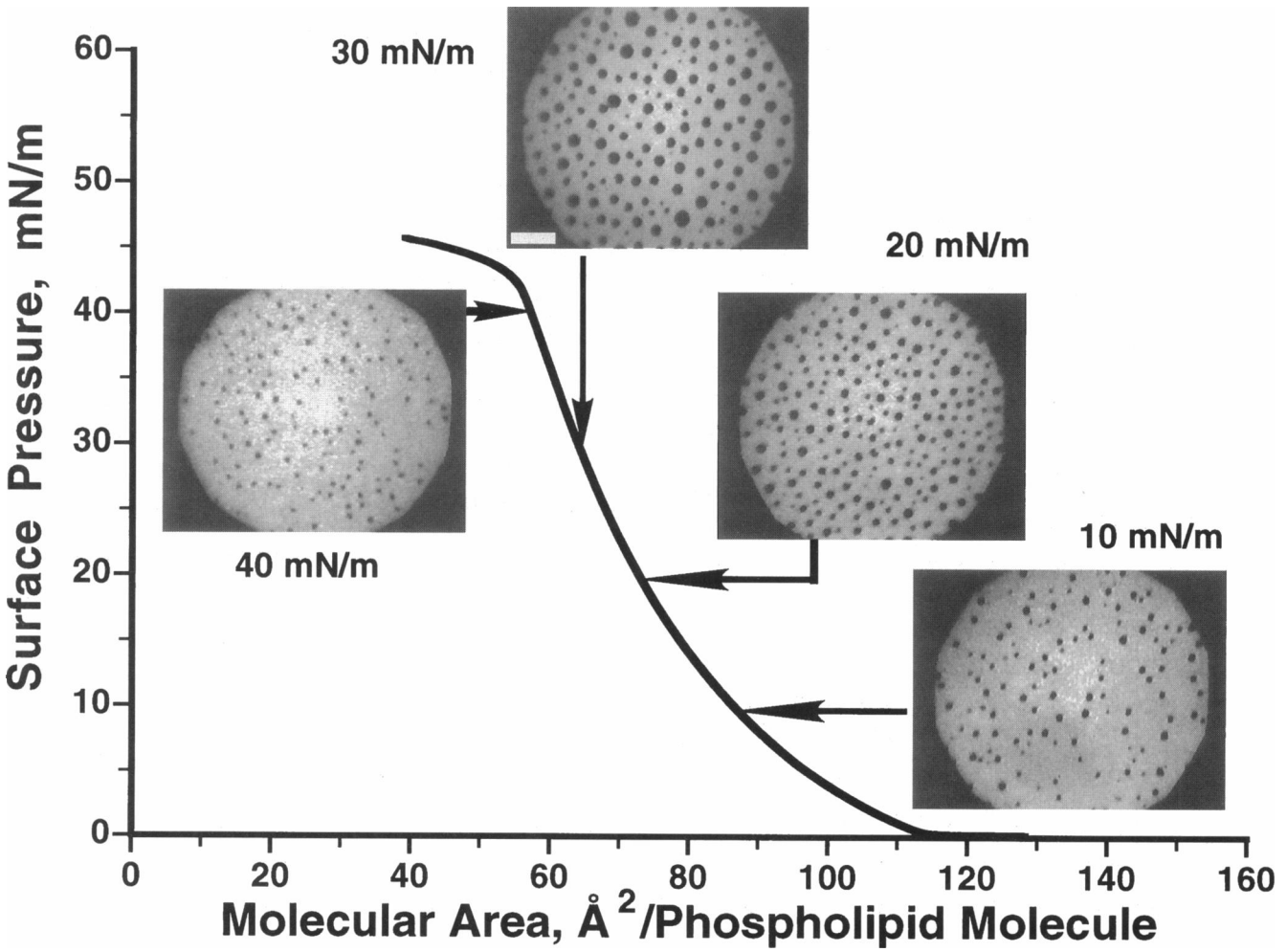

FIGURE 1 Surface pressure-area isotherm with epifluorescence micrographs of calf lung surfactant extract (CLSE). Chloroform solutions of preparations containing 1 mol% rhodamine-DPPE were spread to an initial area of 150 Å^2/phospholipid molecule at 20°C on HSC-buffered electrolyte. Isotherms were recorded during compression at 1 Å^2/phospholipid molecule/min. Images were obtained in separate experiments from static films after compression at 2.8 Å^2/phospholipid molecule/min to the desired pressure. Representative images are given at 10, 20, 30, and 40 mN/m. Scale bar, 50 μm.

lipid molecule/min, reduction in temperature to 5°C, or the use of water as subphase rather than buffered electrolyte.

The total area occupied by the domains increased progressively over a broad range of pressures during the initial stages of compression (Fig. 2). This behavior is distinct from that of pure DPPC, for which nonfluorescent domains grow to dominate the interfacial film over the narrow range of surface pressure of the LE-LC coexistence region evident in the compression isotherm (Meller, 1988; Weis and McConnell, 1984). The behavior of the surfactant films was also distinct in that at room temperature, their total area passed through a maximum and then declined rapidly at higher surface pressures (Fig. 2). The domains reached a maximum of 25 ± 5% of the total interfacial area at a surface pressure of 35 mN/m, but then fell to 4 ± 1% by 40 mN/m. Most of the individual domains vanished, although a small fraction persisted after decreasing in size. These changes in total area of the domains appeared to represent a true decrease in area rather than simply a loss of fluorescent probe from the interface. The surface film remained brightly fluorescent during these changes, making it unlikely that the decrease in area resulted from a loss of probe from the interface and insufficient contrast to distinguish the domains.

BAM demonstrated that the domains visualized by epifluorescence microscopy also exist in the absence of added fluorescent probe and confirmed that the domains are not caused by the presence of the probe (Fig. 3). The domains viewed by BAM were bright against a darker background, demonstrating that the optical thickness of the domains was greater than that of the surrounding film. This appearance again suggests a higher packing density in the domains. During compression to high pressures, the contrast between domains and the surrounding film diminished, in large part because of increased reflectivity from the surrounding film. The domains disappeared in BAM images at pressures approaching 45 mN/m.

The nonfluorescent domains appeared at surface pressures that increased progressively with temperature (Fig. 4).

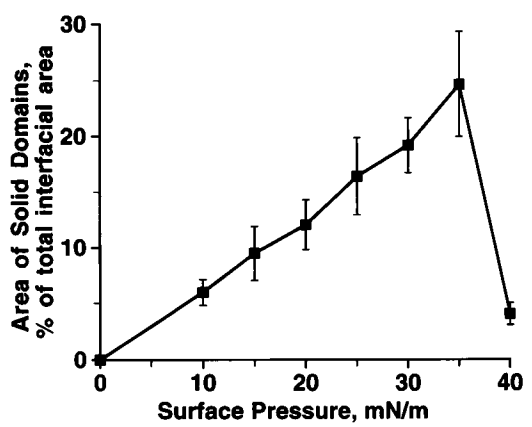

FIGURE 2 Variation of total area of condensed domains with surface pressure. Each data point represents mean ± SD from the analysis of 12 epifluorescence images generated from four independent experiments.

The emergence of the domains in CLSE paralleled the appearance of condensed phase observed in separate experiments with pure DPPC (Fig. 4). The slope of the surface pressure at which domains first appeared as a function of temperature was essentially the same for CLSE and DPPC. The curve for CLSE was shifted to pressures approximately 8–10 mN/m higher than for DPPC. The first appearance of condensed phase in DPPC detected by epifluorescence corresponded exactly to the onset of the LE-LC phase transition in the compression isotherms published previously for this range of temperatures by Albrecht et al. (1978) (Fig. 4).

The behavior of both the fluorescent probe and the surfactant limited the range of pressures over which our experiments could be performed. Bright fluorescent spots appeared at pressures above 45 mN/m, suggesting the separation of a distinct phase that is enriched in Rh-DPPE under those conditions. The compression isotherm for pulmonary surfactant also undergoes a prolonged plateau beginning just above 45 mN/m (Keough, 1984), consistent with the upper limit of pressures at which surfactant films exist under equilibrium conditions. These constraints established 45 mN/m as the approximate upper end of the range of pressures currently accessible in our experiments.

The surface pressure at which the domains reach their maximum size also varied with temperature. Increasing the temperature from 20° to 24°C shifted the maximum of the total area of the domains above 35 mN/m. At temperatures greater than 24°C, the domains no longer achieved a maximum area within our range of accessible pressures. The total area of the domains at these temperatures continued to increase during compression up to 45 mN/m. Domains persisted up to 37°C, but they appeared just below 45 mN/m at that temperature. Consequently they remained quite small (Fig. 5) in our experiments. Domains were not seen at temperatures above 37°C.

DISCUSSION

The compression of interfacial monolayers of extracted calf surfactant induces lateral separation of two phases. Both epifluorescence (Fig. 1) and BAM (Fig. 3) demonstrate the presence of discrete domains that are distinct from the surrounding film. The domains increase in size over a broad range of surface pressures (Fig. 2). At room temperature, however, their area achieves a maximum value and then rapidly declines with further compression (Figs. 1–3). The surface pressure at which the domains first emerge increases with temperature (Fig. 4). Domains persist to 37°C but at that temperature they are quite small (Fig. 5).

Although we have not provided detailed structural information on the molecular arrangement of components in the domains, we have distinguished regions of more tightly packed constituents. Both methods of microscopy used in these studies rely on structural differences in the surface monolayer to produce contrast. Epifluorescence distinguishes regions of the film that differ in the solubility of a

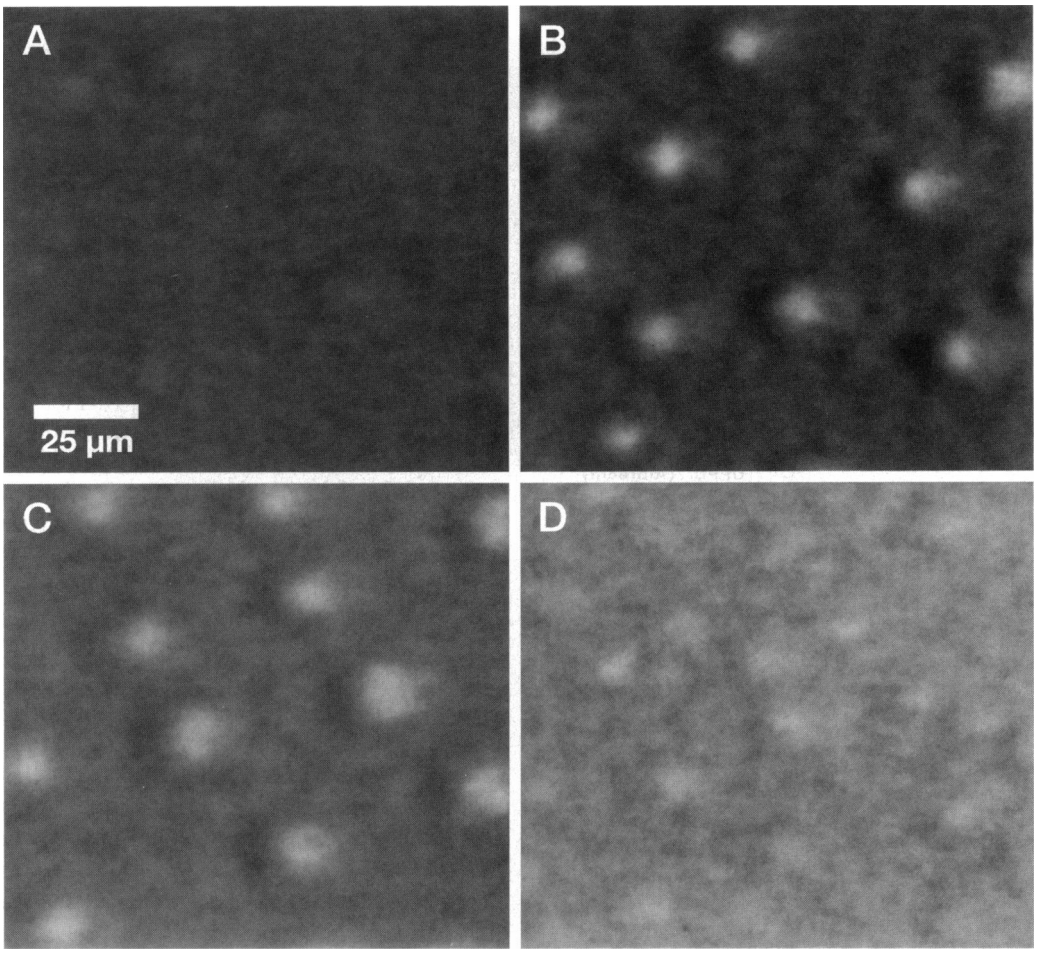

FIGURE 3 Brewster angle micrographs of CLSE monolayers. Chloroform solutions of CLSE were spread on HSC buffer at room temperature and compressed to the desired surface pressure. Scale bar represents 25 μm. Images were recorded at surface pressures of (*A*) 10 mN/m; (*B*) 20 mN/m; (*C*) 30 mN/m; (*D*) 41 mN/m. The relative grayscale has been preserved for this sequence of images. The dark and light shadows to the left and right, respectively, of each domain are due to interference of the reflected coherent light at the domain edges.

fluorescent lipid probe (Lösche et al., 1983; McConnell et al., 1984; Peters and Beck, 1983). Tightly packed lipids in the LC phase exclude the large fluorescent chromophore on the probe, which remains soluble in more disorganized LE regions of the film. The nonfluorescent domains appear in monolayers containing a single component at the onset of the LE-LC plateau and grow during further compression until they coallesce. (Knobler, 1990). In the BAM images, reflectivity varies because of differences in refractive index and thickness of the film (Hénon and Meunier, 1991; Hönig and Möbius, 1991). Bright domains in single-component films again indicate regions of condensed phase lipid. We are unaware of studies that demonstrate phase coexistence in films with the compositional complexity of pulmonary surfactant, let alone any that prove the domains represent condensed phase. But the previous results with much simpler monolayers certainly support the hypothesis that the domains in the surfactant films represent highly organized condensed lipids.

Information on the chemical composition of the domains can be obtained by comparing the fractional composition of the CLSE constituents with the fractional area covered by the domains. The fraction of the interface ϕ_s occupied by domains with a molecular area $\bar{A}_s$ and containing the fraction f of the molecules present is given by

$$\phi_s = f \cdot \frac{\bar{A}_s}{\bar{A}},$$

where $\bar{A}$ is the average molecular area for the entire film (Knobler, 1990). DPPC represents the constituent of calf surfactant most likely to exist in condensed phase under the conditions of our experiments. For hypothetical domains containing only DPPC, this compound constitutes 30% (mol/mol) of the lipid in CLSE (Kahn et al., 1995). The domains achieved their maximum area when $\bar{A}$ was 60 Å^2/molecule. This figure for $\bar{A}$ included lipid molecules but ignored the 1% (w/w) content of protein (Hall et al., 1994). The calculated value of ϕ_s then depends on the value of $\bar{A}_s$ assigned from studies of pure DPPC. Compression isotherms indicate that condensed DPPC has a molecular area of 51 Å^2/molecule (Albrecht et al., 1978, and data not

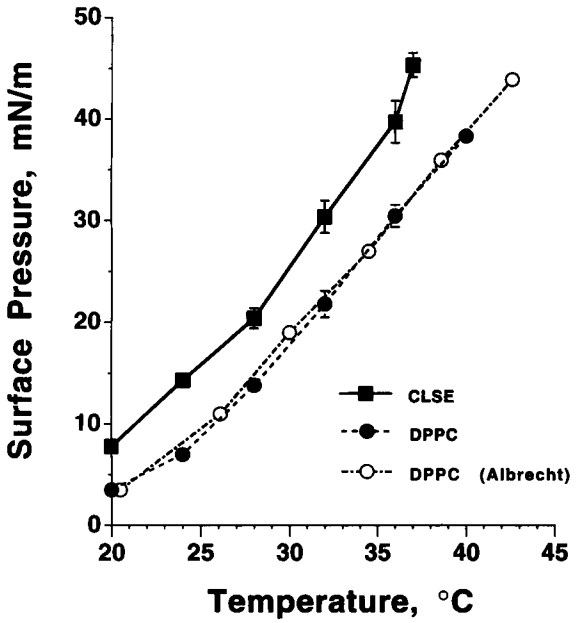

FIGURE 4 Temperature dependence of surface pressure at which epifluorescence microscopy first detected domains. Isothermal compressions were conducted at the temperatures indicated to determine the surface pressure at which domains first occurred in epifluorescent images of CLSE. Chloroform solutions were spread to an initial area of 150 Å^2/phospholipid molecule and compressed at 2.8 Å^2/phospholipid molecule/min. Data are mean ± SD for three experiments. Open symbols represent the onset of the LE-LC coexistence plateau in previously published isotherms for DPPC (Albrecht et al., 1978).

shown), which would predict that DPPC in CLSE would occupy 26% of the interface. The value of 43 Å^2/molecule obtained by Weis and McConnell (1985) from their epifluorescence studies would predict 22%. Both calculated values of ϕ_s agree with the measured value of 25 ± 5% for the maximum area covered by domains.

The domains, however, may well contain components other than DPPC. Since both phases are in thermodynamic equilibrium, the domains are saturated with the other constituents, and the surrounding film is saturated with DPPC. The surface pressure at which the domains first emerge in CLSE exceeds the value for DPPC by 8–10 mN/m. This difference may reflect the nonideality of the mixed film, resulting from the presence of components other than DPPC in the domains. The close proximity over the broad range of temperatures of the pressures at which the domains emerge for DPPC and CLSE does provide further evidence that DPPC is the most important component of the domains. The LE-LC phase transition for all of the other phospholipids is absent over this range of temperatures or occurs at pressures well above the values observed here (Kahn et al., 1995). Our data, however, do not exclude the possibility that the domains contain significant levels of other components in addition to the DPPC.

The formation of the domains occurs by a process that differs from the typical first-order phase transition of pure DPPC. The isotherm is smooth without any evidence of a plateau. The domains in CLSE enlarge over a broad range of pressures, extending at room temperature from 9 to 35 mN/m rather than over the narrow LE-LC coexistence region required of a first-order transition and observed for DPPC. The results of Dluhy and co-workers provide further supporting evidence for phase coexistence (Dluhy et al., 1989). They observed ordering of acyl chains in films of pulmonary surfactant continually over the range of surface pressures from 0 to 45 mN/m, in marked contrast to the rapid change seen for DPPC between 0 and 10 mN/m. CLSE and DPPC reflect this difference in the behavior of the condensed phase.

The appearance, growth, and disappearance of the domains implies that the miscibility of the constituents of CLSE depends on surface pressure. In this model, enlargement of the domains during compression reflects the progressive separation of DPPC into a condensed phase. The subsequent decrease in total area of the domains observed at lower temperatures with further compression represents remixing of the constituents. Such a gap in miscibility in the phase diagram, with a homogeneous system at low and high pressures separated by a region in which immiscible phases coexist has been seen previously for binary mixtures of DPPC with both cholesterol (Rice and McConnell, 1989; Slotte, 1995; Subramaniam and McConnell, 1987) and dioleoyl phosphatidylcholine (DOPC) (Nag and Keough, 1993). The decrease in the total area of the domains for DPPC:DOPC was suggested previously to result not from a true decrease in the condensed regions but from a change in the partitioning of the fluorescent probe. Our results with BAM, however, show that the domains also decrease sharply in films that contain no probe and indicate that the fall in condensed area for CLSE is real.

The remixing of the constituents at high pressures may result from a narrowing of the difference in density of the two phases. Calculations of the molecular area of the film surrounding the domains support this possibility. The condensed domains should be relatively incompressible. If their molecular area changes little during compression, then our results predict that the density of the expanded phase increases significantly. The area per molecule $\bar{A}_f$ for the expanded phase is given by

$$\bar{A}_f = \bar{A} \cdot \frac{(1 - \phi_s)}{(1 - \phi_s(\bar{A}/\bar{A}_s))}$$

(Weis and McConnell, 1985). Values of $\bar{A}_f$ calculated from our measurements of $\bar{A}$ and ϕ_s decreased throughout compression. For $\bar{A}_s$ = 51 Å^2/molecule, for instance, obtained from DPPC isotherms (Albrecht et al., 1978), $\bar{A}_f$ decreased from 101 to 57 Å^2/molecule during compression from 5 to 40 mN/m. Our BAM images provide some additional support for this progressive increase in molecular density of the phase that surrounds the domains. The grayscale, and hence the optical thickness, of the domains remained relatively constant during compression while it increased in the surrounding film (Fig. 3). The steady increase in the optical

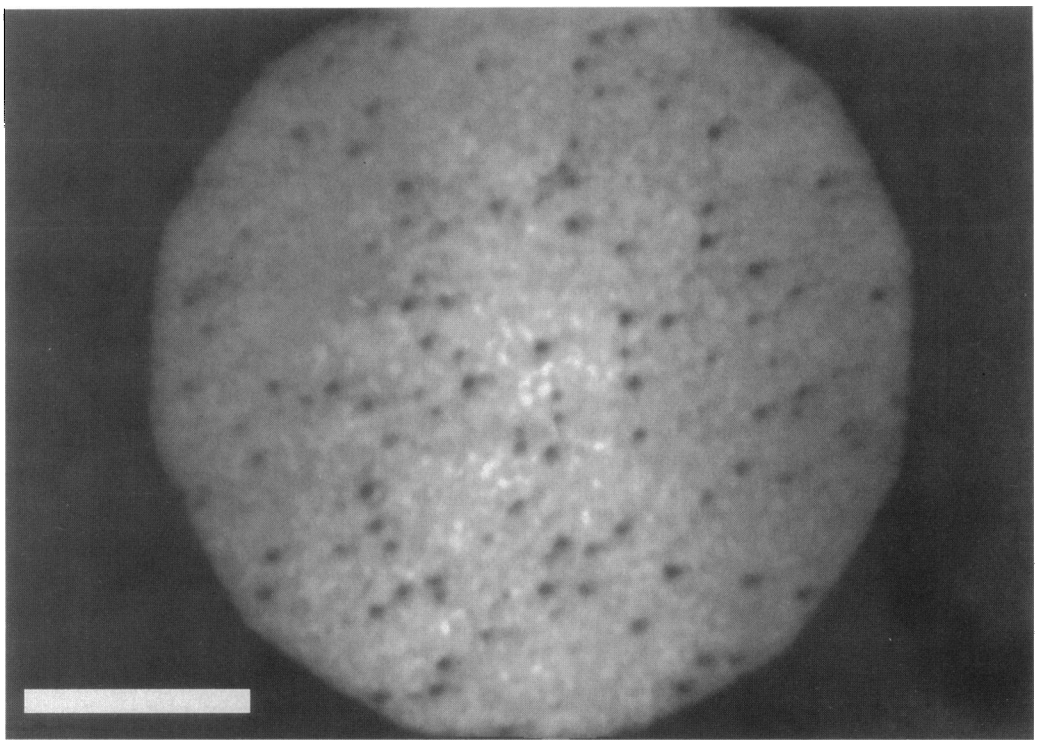

FIGURE 5 Epifluorescence micrographs of surfactant preparations at 37°C. Chloroform solutions were spread to 150 Å^2/phospholipid molecule on HSC buffer and compressed at 2.8 Å^2/phospholipid molecule/min to 45 mN/m. Scale bar is 50 μm.

thickness of the surrounding film also agrees with the increase in ordering of acyl chains observed by Dluhy and co-workers using vibrational spectroscopy (Dluhy et al., 1989). All of these results suggest that the density of the surrounding film approaches that of the domains. Such a decrease in the difference between the density of the two phases may lead to remixing of the constituents.

Interpretation of the physiological importance of our findings is complicated by the difficulty of extrapolation to experimental conditions not accessible in these studies. Surface pressures in the lung are well above the maximum levels of 45 mN/m that we could study here. Our results, however, do have significant implications for surfactant function, particularly at the upper end of our range of pressures.

One major issue concerns the role of phase separation in the refinement of the surfactant films. The characteristics of monolayers that reach the high surface pressures seen in the lungs suggest that they contain predominantly condensed phase DPPC, which implies a substantial change in composition from the material originally secreted by the alveolar type II cells. In vitro, the compressibility of the surfactant films at high surface pressures approaches that of LC DPPC (Putz et al., 1994). In excised lungs, the temperature dependence of pulmonary mechanics suggests that alveolar films contain LC DPPC (Clements, 1977). The interfacial component of the pulmonary elastic recoil increases abruptly between 40 and 44°C, which correlates well with the melting transition of gel phase DPPC at 41°C. These observations have led to the widely held belief that pulmonary surfactant must undergo substantial refinement of its composition before it can achieve high surface pressures during compression. Refinement could occur during formation of the interfacial film, a process that we have not studied here. The more widely held view, however, has been that compression to surface pressures above equilibrium values selectively eliminates surfactant constituents from the interface. Refinement by this process might proceed via two mechanisms. Constituents might depart from a homogeneous film, which consists of a single phase, according to characteristics of the individual molecules such as their shape. Alternatively, separation of a film into two phases with different collapse pressures could result in refinement. The more disordered LE phase, for instance, might fold from the surface at lower pressures than the condensed phase. This second mechanism, but not the first, requires a separation of two phases. Our studies confirm that phase separation can occur in surfactant films despite the absence of any discontinuity in the compression isotherms. Phase separation, however, occurs only in a narrow regime of temperature and surface pressure. Distinction of these two mechanisms will require studies of phase behavior at the high pressures and dynamic conditions present in the lung.

In summary, compression of films of extracted calf surfactant leads to a partial demixing of the constituents through the formation of condensed domains that are surrounded by a less densely packed film. The temperature dependence and area of the domains suggest that they

contain DPPC predominantly but not exclusively. The surface pressure at which the domains emerge rises with temperature. Most interesting, however, is our finding at 20°C that the constituents remix at sufficiently high surface pressures to form a largely homogeneous monolayer. This observation implies the existence of a miscibility gap in the surface films of extracted calf surfactant.

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