No evidence for vitamin K-dependent carboxylation of canine surfactant apoproteins, 28–36 kDa

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Recent research has shown that rat surfactant apoproteins (26–38 kDa) are vitamin K-dependent [Rannels, Gallaher, Wallin & Rannels (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5952–5956]. We have investigated the effect of the vitamin K antagonist warfarin on this family of apoproteins in surfactant from dog lung. Our data suggest that warfarin does not interfere with synthesis and secretion of these proteins into dog lung surfactant. Abnormal surfactant apoproteins, produced in response to warfarin treatment of the dog, were also not found in lung surfactant. 4-Carboxyglutamic acid analysis of purified dog apoproteins also failed to detect the vitamin K-modification. When vitamin K-dependent ^{14}C labelling of precursors of vitamin K-dependent proteins was carried out, fluorography of these precursors, when electrophoresed into SDS/polyacrylamide gels, revealed ^{14}C -labelled proteins of apparent molecular mass 74, 46, 42, 34, 31 and 23 kDa. Antibodies produced against purified dog surfactant apoproteins recognized precursors of the surfactant apoproteins in lung microsomes but did not recognize any ^{14}C -labelled carboxylase substrates. These precursors appeared on immunoblots with apparent molecular mass 29, 32, 33 and 50 kDa. Our data suggest that there are significant differences between this class of surfactant apoproteins in the rat and the dog.

INTRODUCTION

Pulmonary surfactant is a distinctive mixture of phospholipids and specific apoproteins which lower the surface tension at the air/water interface of the lung (King, 1982; Claypool *et al.*, 1984). These lipids and apoproteins are synthesized by type II epithelial cells (Kikkawa *et al.*, 1975; Claypool *et al.*, 1984; Balis *et al.*, 1984; Walker *et al.*, 1986). Prior to their secretion into the alveolar space the phospholipids, and possibly the apoproteins, are assembled and stored as lamellar bodies in type II cells (Walker *et al.*, 1986). Once in the alveolar space the lamellar bodies form tubular myelin, a unique bilayer structure found only in the lung (Williams, 1977). Formation of this structure requires Ca^{2+} (Sanders *et al.*, 1980; Benson *et al.*, 1984a,b). It is currently believed that the surfactant apoproteins are necessary for the structure as well (Benson *et al.*, 1984a).

Two families of surfactant apoproteins have been described. One family of proteins isolated from dog lung have molecular masses of 28, 32 and 36 kDa (Hawgood *et al.*, 1985). These proteins appear to differ only in their carbohydrate content (Benson *et al.*, 1985). The other family comprise some very hydrophobic polypeptides, and these polypeptides from dog lung have molecular masses of 5, 8 and 18 kDa (Hawgood *et al.*, 1987).

Rannels *et al.* (1987) have recently presented data which show that the higher-molecular-mass family of surfactant apoproteins present in rat lung lavage are vitamin K-dependent Gla-containing proteins. This observation promoted an investigation to determine whether or not coumarin anticoagulant drug treatment results in production of surfactant apoproteins that lack

the vitamin K modification. These drugs cause the vitamin K-dependent carboxylation system in liver to produce inactive forms of the vitamin K-dependent clotting factors which appear in blood (Suttie, 1978). These abnormal clotting factors have lost their ability to bind to phospholipids in the presence of Ca^{2+} , which prevents formation of the prothrombinase complex (Mammen, 1986). We have tested the effect of warfarin on the 28–36 kDa family of apoproteins isolated from dog lung surfactant. The dog was chosen as the experimental animal because most of the available biochemical data are on dog surfactant apoproteins. No difference was found between this family of apoproteins isolated from lavage of normal dogs and from a warfarin-treated dog. In fact, Gla analysis revealed that these apoproteins purified from dog surfactant are not vitamin K-dependent proteins.

MATERIALS AND METHODS

Animals

Dogs fed on a standard laboratory diet were used for these experiments. All dogs were inspected by a veterinary surgeon at The Milton S. Hershey Medical Center Animal Farm and found to be in good health.

Warfarin treatment

One female beagle dog (15 kg) was treated with warfarin as follows: 10 mg/kg was given orally on two consecutive days; on the third day no drug was given; on the fourth day 30 mg of warfarin/kg was given intravenously. After the last treatment (24 h later), 9 parts of

Abbreviations used: Gla, 4-carboxyglutamic acid; PAGE, polyacrylamide-gel electrophoresis.

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blood was drawn into 1 part of citrate (2.85%) for prothrombin-time determinations (Denson & Biggs, 1976). The dog was killed for preparation of lung surfactant and lung microsomes. Five other dogs that had not received warfarin were also killed and used to prepare samples to be used as controls.

Purification of surfactant and surfactant apoproteins

Surfactant was isolated from dog lungs as described by King & Clements (1972), with modifications described by Hawgood *et al.* (1985). The 28–36 kDa family of surfactant apoproteins was isolated from surfactant as described by Hawgood *et al.* (1987). These proteins were stored frozen at -25°C in 5 mM-Tris/HCl, pH 7.4.

Vitamin K-dependent carboxylase

Lavaged lung tissue was weighed, minced and suspended in 2 vol. (v/w) of the homogenization buffer (80 mM-KCl/200 mM-sucrose/20 mM-Tris/5 mM-benzamidine, pH 7.8). The suspension was dispersed by using a Polytron homogenizer for 10 s at low setting. The slurry was then homogenized in a Potter–Elvehjem homogenizer with a tight-fitting pestle. The homogenate was centrifuged at 10000 *g* for 10 min, and the resulting supernatant was centrifuged for 60 min at 100000 *g*. The pelleted microsomes obtained after the 100000 *g* spin were stored in liquid N_2 .

Carboxylase activity was measured in lung microsomes suspended in 25 mM-imidazole/0.5% 3-[3-cholamidopropyl]dimethylammonio]propanesulphonic acid (CHAPS)/5 mM-benzamidine/1 mM- MnCl_2 , pH 7.2 (buffer A), as described previously (Wallin & Martin, 1987). Activity was measured as $^{14}\text{CO}_2$ incorporation into the synthetic peptide Phe-Leu-Glu-Glu-Leu (peptide carboxylation) and also as $^{14}\text{CO}_2$ incorporation into endogenous microsomal proteins (protein carboxylation) (Esmon & Suttie, 1976). Incubations contained 5 mM-dithiothreitol (DTT) and 100 μg of chemically reduced vitamin K_1H_2 /ml. Controls had no added vitamin K_1H_2 .

For isolation of ^{14}C -labelled carboxylase substrates in lung microsomes, vitamin K-dependent ^{14}C -carboxylation of endogenous microsomal proteins suspended in buffer A was triggered with 100 μg of vitamin K_1H_2 /ml in the presence of 40 μCi of $\text{H}^{14}\text{CO}_3^-$ /ml. Incubation was carried out for 15 min at 25°C . ^{14}C -labelled proteins in the incubation mixture are recovered in the void-volume fraction resulting from gel filtration of the carboxylation system *in vitro* on Sephadex G-25 in 0.1 M- NH_4HCO_3 . Control samples were prepared as described above from microsomes incubated in the absence of vitamin K_1H_2 . Recovered proteins were freeze-dried and stored at -20°C .

Electrophoresis

SDS/polyacrylamide-gel electrophoresis (SDS/PAGE) was carried out in 10% reducing gels by the method of Laemmli (1970). Before electrophoresis samples were boiled for 2 min in the presence of 5% (v/v) 2-mercaptoethanol and 1% (w/v) SDS. Electrophoresed proteins were fixed and stained as described previously (Wallin *et al.*, 1987). Gels selected for fluorography were fixed overnight in 10% (w/v) trichloroacetic acid/10% (v/v) acetic acid/25% (v/v) methanol. The fixed gels were treated with the auto-

radiography enhancer EN³HANCE (New England Nuclear, Boston, MA, U.S.A.) for 1 h and subjected to two washes in 20% (v/v) glycerol (15 min and 30 min) before they were dried. Fluorograms of gels were obtained by exposing the dried gels to Kodak X-Omat XAR-5 film at -70°C . Molecular masses of the electrophoresed proteins were estimated from the migration of [^{14}C]methylated standard proteins purchased from Amersham (Arlington Heights, IL, U.S.A.).

Isoelectric focusing of surfactant apoproteins was carried out in a pH 4–7 reducing gradient under denaturing conditions using a Bio-Rad Mini-Protein II dual-slab-gel apparatus. To prepare the gradient gel, the following solution was prepared: 5.7 g of urea was dissolved in 4.0 ml of water, 1.2 ml of glycerol, 2.2 ml of an acrylamide stock solution (29.2 g of acrylamide/0.8 g of bisacrylamide in 100 ml of water), 100 μl of ampholyte 3.5/10, 800 μl of ampholyte 4/6, 100 μl of ampholyte 5/7, 16 μl of $\text{NNN}'\text{N}'$ -tetramethylethylenediamine and 45 μl of ammonium sulphate (0.5 g in 5 ml of water). The gel was allowed to polymerize for 50 min. The anolyte and catholyte solutions were 0.02 M-acetic acid and 0.02 M-NaOH respectively. Before protein focusing, the gel was prerun for 30 min at 400 V. Protein samples were dissolved in the sample buffer; 0.24 g of urea and 16 mg of dithiothreitol dissolved in 200 μl of water, 75 μl of glycerol, 5 μl of ampholyte 3.5/10, 25 μl of ampholyte 4/6. Focusing was carried out for 2 h at 400 V using a cooling device in the electrophoresis cell. Standard proteins for reference pI values were purchased from Bio-Rad. Gels were fixed for 10 min in 20% trichloroacetic acid, then for 10 min in fixative II (40% ethanol/10% acetic acid/0.25% SDS). Before staining with 0.125% Coomassie Blue R-250 dissolved in 40% ethanol/10% acetic acid, the gel was washed twice in the destaining solution (40% ethanol/10% acetic acid).

Phospholipid and protein determination

Phospholipid concentration of isolated surfactant was determined based on the phosphorus content [$\text{Pi} \times 25$ (Sueishi & Benson, 1981)]. Protein was determined by the method of Schaffner & Weissmann (1973).

Immunological methods

Antiserum against purified surfactant apoproteins was raised in New Zealand White rabbits, and IgG was isolated as described by Fahey (1967). The IgG fraction was subjected to immunoaffinity chromatography on a column of dog serum proteins as recently described (Rannels *et al.*, 1987). The antibodies were used for quantitative determination of apoproteins in purified surfactant using a dot-blot assay (Riederer *et al.*, 1987).

Immunoadsorption of ^{14}C -labelled freeze-dried lung proteins was carried out as follows: proteins from incubations with vitamin K_1H_2 or controls were dissolved in 25 mM-imidazole/0.5 M-KCl/1.5% Triton X-100/5 mM-benzamidine, pH 7.2, and reacted with the antibodies overnight at 4°C . Immunocomplexes were adsorbed on to *Staphylococcus aureus* Protein A (Pansorbin; Calbiochem, La Jolla, CA, U.S.A.), washed twice with 25 mM-imidazole/0.5 M-NaCl/0.05% Triton X-100 and released from the *Staphylococcus* particles by boiling them for 2 min in the SDS/polyacrylamide-gel electrophoresis buffer containing 2% SDS and 5% mercaptoethanol. The released proteins were applied

Table 1. Vitamin K-dependent carboxylase activity in dog lung microsomes

Carboxylase activity was measured in microsomes from normal dog lung and lung from a dog treated with warfarin (see the Materials and methods section). Activity was determined with reduced vitamin K₁H₂ in the test system and was measured as ¹⁴CO₂ incorporation into a synthetic carboxylase substrate ('Peptide') and into endogenous microsomal protein ('Protein'). The values are means for three parallel incubations; individual values differed by less than 5%.

Lung microsomes	Carboxylase activity (c.p.m./mg)	
	Peptide	Protein
Normal dog	23890	6185
Warfarin-treated dog	72724	14397

directly to SDS/PAGE gels for development of fluorograms as described above.

Immunoblotting of lung microsomal proteins was carried out in the Mini-Protein II dual-slab-gel apparatus as described previously (Wallin & Rannels, 1988). The nitrocellulose membrane was allowed to react with blotting buffer containing the antibody preparation in a 1:1000 dilution. Horseradish-conjugated antibodies were used as second antibodies. These antibodies were used in a 1:3000 dilution with the blotting buffer (Wallin & Rannels, 1988). Immunoreactive protein bands were revealed after horseradish-peroxidase reduction of 4-chloro-1-naphthol.

4-Carboxyglutamic acid (Gla) analysis

Determination of Gla in surfactant apoproteins was carried out after vacuum hydrolysis of purified proteins in 2.5 M-KOH (Hauschka, 1977). H.p.l.c. analysis of Gla was performed as described by Kuwada & Katayama (1981) using a Nucleosil SB 5 µm column. [¹⁴C]Norleucine was used as internal standard.

Materials

Vitamin K₁ was purchased from Sigma Chemical Co. The vitamin was reduced to vitamin K₁H₂ with dithionite as described by Sadowski *et al.* (1970). NaH¹⁴CO₃ (60 mCi/mmol) was from Amersham. The pentapeptide Phe-Leu-Glu-Glu-Leu was from Vega Fox (Tucson, AZ, U.S.A.). All other chemicals were reagent grade or better.

RESULTS

Administration of warfarin according to our protocol had a significant effect on the dog's coagulation system. On the fifth day after initiating warfarin administration, prothrombin time had increased from 7.4 to 53 s. This increase in prothrombin time was equivalent to the prothrombin time measured in 5%-diluted normal dog plasma. Warfarin had also clearly affected the vitamin K-dependent carboxylation system in the dog lung. The specific activity for peptide and protein carboxylation measured in lung microsomes from the warfarin-treated dog was 3.0- and 2.3-fold higher than these

Fig. 1. SDS/PAGE of dog surfactant apoproteins

Surfactant apoproteins were isolated from normal dog lungs and lungs from a dog treated with warfarin (see the Materials and methods section). Lane A, apoproteins from normal lungs (7 µg); lane B, apoproteins from lungs of a warfarin-treated dog (5 µg); lanes ST, standard proteins.

activities measured in microsomes from normal dog lung (Table 1).

Coumarin anticoagulant drugs result in the liver synthesizing abnormal undercarboxylated vitamin K-dependent clotting factors which are secreted into blood (Suttie, 1978). To establish whether warfarin caused secretion of abnormal surfactant apoproteins into lung surfactant, surfactant apoproteins were isolated from the warfarin-treated dog. Warfarin had no apparent effect on the ratio of phospholipid to total protein or the ratio of phospholipid to apoprotein in lung surfactant. Also, similar amounts of surfactant and surfactant apoproteins were isolated from lungs of normal and warfarin-treated dogs (results not shown). In addition the apoprotein content of surfactant isolated from normal dogs and the warfarin-treated dog was determined to be 37% (±2%, n = 3) and 40% (±2%, n = 3) of total surfactant protein respectively. These results suggest that warfarin had not interfered with synthesis and secretion of surfactant lipids and apoproteins into the alveolar lumen.

SDS/PAGE of surfactant apoproteins isolated by the method of Hawgood *et al.* (1987) is shown in Fig. 1. Lanes A and B show the proteins isolated from normal and the warfarin-treated dog respectively. The electrophoretic picture of the two preparations of apoproteins were identical, and both showed the quantitative difference between the 28, 32 and 36 kDa proteins that is characteristic for the apoprotein preparation obtained from dog surfactant (Benson *et al.*, 1985). From the

Fig. 2. Isoelectric focusing for surfactant apoproteins

Surfactant apoproteins isolated from normal dog lungs (lane A, 7 μ g) and lungs from a warfarin-treated dogs (lane B, 7 μ g) were subjected to isoelectric focusing in 8 M-urea (see the Materials and methods section for details). Arrows identify two proteins in lane B that appear to be less stained than the equivalent proteins in lane A.

migration of standard proteins was estimated the molecular mass of the various proteins appearing in the gel to be 31.5, 34.5 and 41 kDa. These values are somewhat higher than those reported (28, 32 and 36 kDa) by Benson *et al.* (1985). The discrepancy most likely results from the use of different SDS/PAGE systems. Previous work (results not shown) has shown that rat liver DT-diaphorase (EC 1.6.99.2) (molecular mass 30 kDa) also exhibits a higher apparent molecular mass (34 kDa) when estimated from SDS/PAGE in the Mini Portran II dual-slab-gel apparatus. However, a higher molecular mass for the apoproteins has also been reported by others (Sueishi & Benson, 1981). No difference in the migration of these proteins in a pH gradient was seen when we subjected the proteins to isoelectric focusing under denaturing conditions (Fig. 2). However, the amount of protein in the bands designated by arrows in Fig. 2 seems to be somewhat lower in the preparation from the warfarin-treated dog. The difference in relative intensity of these bands could be due to biological variation. Because the presence of undercarboxylated abnormal apoproteins was expected to change the isoelectric-focusing pattern, this experiment suggested to us that no such vitamin K-dependent proteins were present in the apoprotein preparation from the warfarin-treated dog. Agarose electrophoresis of the two preparations in the presence of 2 mM- Ca^{2+} and 2 mM-EDTA

Fig. 3. Fluorography of carboxylase substrates in dog lung microsomes

Protein substrates for the vitamin K-dependent carboxylase in dog lung microsomes were ^{14}C -labelled in a vitamin K-dependent γ -carboxylation reaction as described in the Materials and methods section. After γ -carboxylation, the microsomal proteins were subjected to SDS/PAGE in 10% reducing gels and prepared for fluorography as described in the Materials and methods section. Lane B, fluorogram of total microsomal protein after γ -carboxylation *in vitro*; lane C, same as lane B, except that γ -carboxylation was carried out in the absence of vitamin K_1H_2 (control); lane A, fluorogram of immunocomplexes obtained by allowing the ^{14}C -labelled proteins in lane B to react with antibodies against purified surfactant apoproteins (for details see the Materials and methods section).

respectively was carried out by the method of Stenflo & Ganrot (1972). Again, no difference was found between the two preparations, and the electrophoretic mobility of the apoproteins was not significantly different with EDTA or Ca^{2+} in the electrophoresis buffer.

As shown in Table 1, vitamin K-dependent proteins are indeed synthesized in the dog lung. Therefore, in a second set of experiments, we decided to investigate the protein substrates for the vitamin K-dependent carboxylase in lung microsomes from the warfarin-treated dog and their potential immunological relationship to the surfactant apoproteins. A fluorogram of an SDS/PAGE gel with these substrates is shown in Fig. 3, lane B. Before SDS/PAGE the substrates were ^{14}C -labelled *in vitro* in a vitamin K-dependent carboxylation reaction (see the Materials and methods section). The fluorogram revealed a heavily ^{14}C -labelled protein of apparent molecular mass 74 kDa. Additionally, ^{14}C -labelled pro-

Fig. 4. Immunoblotting of microsomal precursors of surfactant apoproteins

Dog lung microsomal proteins were subjected to SDS/PAGE in 10% reducing gels and transferred to nitrocellulose for immunoblotting with antibodies raised against purified surfactant apoproteins (for details see the Materials and methods section). Lane A, dog microsomal proteins; lane B; purified surfactant apoproteins.

teins of apparent molecular masses 46, 42, 34, 31 and 23 and one smaller protein were also detectable (Fig. 3, lane B). A fluorogram of proteins from a control incubation where ^{14}C -labelling had been carried out in the absence of vitamin K_1H_2 is shown in Fig. 3, lane C. No ^{14}C -labelled proteins were detectable on the fluorogram, which demonstrates that the bands seen in Fig. 3, lane B, indeed represent vitamin K-dependent proteins. Recently, we have used antibodies against the vitamin K-dependent coagulation factors II and X to successfully immunoprecipitate ^{14}C -labelled precursors of these factors from a liver *in vitro* vitamin K-dependent carboxylation system (Wallin & Martin, 1986; Wallin & Rannels, 1988). When a similar approach was employed using the vitamin K-dependent carboxylation system prepared from dog lung microsomes, we did not isolate a ^{14}C -labelled antigen that was detectable on fluorography (Fig. 3, lane A). The antibodies did, however, recognize precursors of surfactant apoproteins in lung microsomes. An immunoblot is shown in Fig. 4, lane A. Four immunoreactive bands with apparent molecular masses 29, 32, 33 and 50 kDa were found. An immunoblot of the purified surfactant apoproteins (Fig. 3, lane B) produced a band of apparent molecular mass 62 kDa (Fig. 3, lane C) in addition to the three bands seen in a Coomassie Blue-stained SDS/polyacrylamide

gel (Fig. 1). Whether this protein was a contaminant in the surfactant apoprotein preparation or a protein immunologically related to the apoproteins was not investigated.

These results led us to investigate whether or not the purified surfactant apoproteins were vitamin K-dependent Gla-containing proteins. Gla analysis failed to detect the vitamin K modification. A 100 μg portion of apoproteins from normal and warfarin-treated dogs was hydrolysed for the analysis, but Gla was not detectable in the eluate, even when the h.p.l.c. column was overloaded.

DISCUSSION

The data provide evidence that the 28–36 kDa family of dog surfactant apoproteins are not vitamin K-dependent proteins. Gla analysis by h.p.l.c. failed to detect this vitamin K modification in an alkaline hydrolysate of purified apoproteins. The vitamin K-antagonist warfarin also had no effect on the appearance of these apoproteins in lung surfactant. The ratio between phospholipid and protein in surfactant from control dogs and the warfarin-treated dog was found to be similar and the numbers are in close agreement with those reported by Sueishi & Benson (1981). Only one dog was used for warfarin treatment. As judged by the prothrombin time, the dog responded normally to the drug, and we therefore feel the data are representative for a normal dog population.

In contrast with the association between the vitamin K-dependent clotting factors and phospholipids (Mammen, 1986), the association between the apoproteins and surfactant lipids appears to be independent of Ca^{2+} (King *et al.*, 1983). Therefore it was expected that undercarboxylated abnormal apoproteins would be present in surfactant from the warfarin-treated dog. The half-life of the apoproteins in lung has been estimated at 10 h (King *et al.*, 1977). Therefore, if the lung carboxylation system is similar to the liver system (Wallin *et al.*, 1987), a significant decline in the concentration of normal apoproteins was also expected in surfactant from the warfarin-treated dog. The data suggest the abnormal apoproteins were not present in surfactant from this dog.

Rannels *et al.* (1987) have reported that, in the rat, these apoproteins are vitamin K-dependent Gla-containing proteins. Their Gla analysis was carried out on apoproteins excised from SDS/polyacrylamide gels after electrophoresis of rat surfactant. Rat surfactant contains a significant amount of serum proteins (Katyal & Singh, 1984). Thus contamination of the apoprotein preparation by vitamin K-dependent serum proteins of similar molecular mass is possible. Such proteins could be the Gla-containing activation fragments of vitamin K-dependent rat coagulation factors (Esmon *et al.*, 1975). The preparation of surfactant apoproteins used in this study is less likely to contain contaminating serum proteins. The purification protocol used in the present paper (Hawgood *et al.*, 1987) removes serum proteins by selective extraction into 20 mM-octyl glucoside before isolation of surfactant proteins.

All known vitamin K-dependent proteins are synthesized with a pro-piece that is believed to be the recognition site for the vitamin K-dependent carboxylase (Pan & Price, 1985). The vitamin K-dependent clotting factors and also the unrelated bone Gla protein (Pan

et al., 1985) have this pro-piece extending from the N-terminal end of the native proteins, starting with arginine in position -1 and ending with phenylalanine in position -16. The amino acid sequence for the dog apoproteins has been deduced from their cDNA sequence (Benson *et al.*, 1985). This pro-piece found on precursors of vitamin K-dependent proteins cannot be found in the extended sequence of the surfactant apoproteins, an observation that raises questions about whether or not these proteins can serve as substrates for the vitamin K-dependent carboxylase.

Since antibodies against surfactant apoproteins did recognize antigens, but no ^{14}C -labelled carboxylase substrates, in dog lung microsomes, the data suggest that other vitamin K-dependent proteins, immunologically related to the apoproteins, are also not produced in the dog lung. However, two of the ^{14}C -labelled carboxylase substrates in the dog lung (31 and 34 kDa; see Fig. 3) have molecular masses that are quite similar to those of the surfactant apoproteins.

The fluorogram of carboxylase substrates in dog lung microsomes is quite similar to a fluorogram of such substrates detected in rat lung microsomes (Wallin & Rannels, 1988). This suggests that similar vitamin K-dependent proteins are produced in the two species. Since we failed to prove that the canine 28–36 kDa family of surfactant apoproteins are vitamin K-dependent proteins, the vitamin K-dependent proteins produced in the dog lung are still unknown. These proteins may play an important role in the lung, a possibility that warrants further investigation. If, as has been suggested by Rannels *et al.* (1987), the rat apoproteins are vitamin K-dependent proteins, the data presented here suggest that there is a significant difference between the rat and dog surfactant systems. As shown here, vitamin K-dependent apoproteins are not secreted by the dog lung into the alveolar space to be part of the 28–36 kDa family of surfactant apoproteins that are known to have a Ca^{2+} -dependent effect on spreading of surfactant lipids on the water/air interface (Hawgood *et al.*, 1985).

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