Identification of vitamin K-dependent carboxylase activity in lung type II cells but not in lung macrophages

Reidar WALLIN and Stephen R. RANNELS

Department of Physiology, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, PA 17033, U.S.A.

Fluorography of ^{14}C -labelled glutamic acid residues in vitamin K-dependent protein precursors in lung microsomes (microsomal fractions) shows that the lung has several substrates that are not found in the liver. These precursor proteins unique to the lung have apparent molecular masses of 65, 53, 50, 36, 31 and 13 kDa. Type II epithelial cells appear to synthesize most of the vitamin K-dependent proteins in the lung. The 36 and the 31 kDa precursors also found in Type-II-cell microsomes have a similar molecular mass to those of surfactant-associated proteins, and we have previously shown [Rannels, Gallaher, Wallin & Rannels (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5952–5956] that the 36 kDa protein is one of the precursors for these proteins. Immunoblotting of membrane fragments of Type-II-cell microsomes with plasma prothrombin antibodies identified two prothrombin-like antigens of apparent molecular masses 68 and 65 kDa. This raises the question as to whether Type II cells are also a potential site for synthesis of prothrombin and possibly other vitamin K-dependent clotting factors. Pulmonary macrophages appear to be devoid of vitamin K-dependent carboxylase activity. However, Type II epithelial cells have significant activity, and this activity was unaltered when these cells were maintained in primary culture for 3 days, suggesting that carboxylase activity is expressed in lung alveolar epithelium independently of culture-induced changes in cellular differentiation. Carboxylase activity in Type II cells was enhanced 2-fold when cells were cultured for 24 h in the presence of 50 μM -warfarin. Type II cells, therefore, resemble hepatocytes with regard to their response to coumarin anticoagulant drugs.

INTRODUCTION

Vitamin K-dependent carboxylation is a post-ribosomal modification reaction that converts glutamic acid residues in protein precursors into γ -carboxylated proteins, and this reaction requires the reduced form of the vitamin (Suttie 1978, 1980). The reaction is catalysed by vitamin K-dependent carboxylase, an integral membrane protein of the endoplasmic reticulum (Suttie, 1978, 1980; Vermeer & De Boer-Van Den Berg, 1985).

Several coagulation factors, namely factors II, VII, IX, X, protein Z, protein C and protein S, are vitamin K-dependent proteins. Although their primary site of synthesis is the hepatocyte (Olson *et al.*, 1966; Graves *et al.*, 1982; Fair & Bahnak, 1984; Fair & Malar, 1986), other cell types have been shown to produce vitamin K-dependent coagulation factors. Endothelial cells synthesize protein S (Fair *et al.*, 1986), and various tumour-cell lines produce vitamin K-dependent procoagulant activity, which may contribute to tumour metastasis (Donati & Semeraro, 1984). Factors II, VII, IX and X have also been measured in 24 h cultures of murine peritoneal macrophages (Osterud *et al.*, 1980), but this observation remains controversial.

In the present studies we have focused our attention on the lung and isolated lung cells in order to investigate synthesis of vitamin K-dependent proteins in this tissue and its cells. We show that the lung has several precursors

of vitamin K-dependent proteins, many of which are not present in the liver. By using an assay system which measures vitamin K-dependent carboxylase activity in isolated cells, macrophages and Type II epithelial cells isolated from rat lung were tested for this activity. No carboxylation could be measured in macrophages, an observation which questions the ability of these cells to produce vitamin K-dependent procoagulant activity (Lyberg, 1984). However, Type II cells, the lung cells which synthesize, store and secrete surfactant material (Katyal & Sing, 1984), revealed significant activity.

EXPERIMENTAL

Materials

Vitamin K_1 and the zwitterionic detergent Chaps were purchased from Sigma Chemical Co. The vitamin was reduced to vitamin K_1H_2 with dithionite as described by Sadowski *et al.* (1976). Pansorbin was from Calbiochem, La Jolla, CA, U.S.A. En³Hance was from NEN, Boston, MA, U.S.A. The pentapeptide Phe-Leu-Glu-Glu-Leu was from Vega Fox Biochemicals Division (Tucson, AZ, U.S.A.). $\text{NaH}^{14}\text{CO}_3$ (60 mCi/mmol) and ^{125}I -Protein A (> 30 mCi/mg) were from Amersham Corp., Arlington Heights, IL, U.S.A.). The insecticide VAPONA was a gift from the Shell Oil Co. (Houston, TX, U.S.A.). Protein was measured by using the Bio-Rad (Richmond,

Abbreviations used: Chaps, 3-[(3-cholamidopropyl)dimethylammonio]propanesulphonic acid; VAPONA (Shell), 2,6-dichlororinyl dimethyl phosphate; Pansorbin (Calbiochem), *Staphylococcus aureus* Protein A; TMEM, Joklik's modified minimal essential medium; DCS, donor-calf serum; DMEM, Dulbecco's modified minimal essential medium; DTT, dithiothreitol; PAGE, polyacrylamide-gel electrophoresis; $\text{iPr}_2\text{P-F}$, di-isopropyl phosphorofluoridate.

CA, U.S.A.) protein assay kit. 4-Chloro-1-naphthol was also from Bio-Rad. All other chemicals were reagent grade or better.

Animals

Male Sprague-Dawley rats weighing 200–250 g (Charles River Laboratories, Wilmington, MA, U.S.A.) used for these studies were fed a standard laboratory diet. Rats receiving sodium warfarin were given 30 mg of the drug/kg in saline as intraperitoneal injections. The rats were fasted 24 h and killed by decapitation 24 h after warfarin administration.

Preparation of microsomes

(a) **From tissue.** All adult rat livers were perfused through the portal vein with ice-cold saline (0.9% NaCl). The livers were homogenized as described by Suttie *et al.* (1976) in 250 mM-sucrose/25 mM-imidazole, pH 7.2 (SI-buffer), containing 1 mM-VAPONA (SI-VAPONA buffer) (Wallin & Martin, 1987), 2 ml of SI-VAPONA buffer being used per g of wet tissue. The homogenate was centrifuged twice at 10000 g and then at 100000 g for 60 min to obtain a microsomal pellet. The pellets from the 100000 g spin were resuspended in the SI-VAPONA buffer and repelleted a second time by centrifugation at 100000 g for 60 min. The supernatant was discarded and the pellet stored in liquid N₂ with no detectable loss of carboxylase activity. Adult rat lungs were perfused through the pulmonary artery with ice-cold saline. Lung microsomes were obtained as described for rat liver.

(b) **From cells.** Cells were washed three times in the SI-VAPONA buffer. Cells [(50–100) × 10⁶] in 2 ml of SI-VAPONA buffer were sonicated on ice in the presence of 2 mM-iPr₂P-F for three 10 s intervals. The sonicated cells were diluted to 5 ml with the SI-VAPONA-iPr₂P-F-buffer and subsequently homogenized with six strokes in a Potter-Elvehjem tissue homogenizer. The homogenate was centrifuged at 10000 g for 10 min. The supernatant from the 10000 g spin was centrifuged at 100000 g for 60 min. The pellet was stored in liquid N₂.

Isolation of Type II pneumocytes and pulmonary macrophages

Type II pulmonary epithelial cells were prepared from lungs of male Sprague-Dawley rats (200–250 g) with modifications of the method of Fabisiak *et al.* (1986). Lungs were cleared of blood by saline infusion via the pulmonary artery, followed by intratracheal lavage with 3 × 10 ml of saline to remove macrophages. Macrophages obtained at this step were cleared of contaminating red blood cells by differential adherence to plastic surfaces in the presence of 10% (v/v) foetal-bovine serum. Macrophages which attached to the surface were removed from the surface by gentle agitation and with a rubber policeman. Cells were dispersed from intact tissue by intratracheal instillation of 0.1% elastase in Joklik's modified minimal essential medium (JMEM) containing 0.05% BaSO₄. After incubation at 37 °C for 30 min, proteolytic digestion was terminated by instillation of 0.08% (w/v) soybean trypsin inhibitor and DNAase (80 µg/ml) in JMEM with 50% (v/v) donor-calf serum (DCS). The lung parenchyma was then minced finely and vortex-mixed; dispersed cells were collected by filtration through HCl60 Nitex bolting cloth (Tetco) and were pelleted by centrifugation at 400 g for 10 min. Pellets

were resuspended in 50–100 ml of JMEM containing DNAase (80 µg/ml) and incubated at 37 °C for 20 min, then at room temperature for 10 min. The cell suspension from one or two rats was layered on a discontinuous density gradient of Percoll (8 ml, 1.08 g of Percoll/ml; 8 ml, 1.04 g of Percoll/ml, 20 ml of cell suspension). Gradients were centrifuged for 30 min at 400 g (4 °C) and cells were collected from the 1.08/1.04-relative-density interface. Cells were washed, resuspended in Dulbecco's modified minimal essential medium (DMEM) containing 10% foetal-bovine serum and purified further by differential adherence to plastic culture dishes as described above. Macrophages were also obtained at this step and were found to be free of contaminating erythrocytes and Type II cells. Non-adherent cells were collected, and the viability of the final cell preparations was > 95% as assessed by the exclusion of erythrocin B or Trypan Blue. In most preparations, Type II cell purity was approx. 90% on the basis of staining of lamellar bodies by haematoxylin/Li₂CO₃ or by tannic acid as described by Mason *et al.* (1985). Type II cells were plated and maintained in primary culture in DMEM containing 10% foetal-bovine serum for 3 days as described by Fabisiak *et al.* (1986) and Rannels & Rannels (1987).

Carboxylase activity measurements

Liver and lung microsomes were resuspended in 25 mM-imidazole, 0.5% Chaps, 1 mM-VAPONA, pH 7.2 (imidazole/Chaps/VAPONA buffer), containing 1 mM-MnCl₂ and 1 mM-DTT by using a tight-fitting Dounce homogenizer. Cells were washed in 25 mM-imidazole, 1 mM-VAPONA, pH 7.2, and sonicated on ice for 3 × 10 s in the same buffer. Chaps (0.5%), MnCl₂ (1 mM) and DTT (1 mM) were added from concentrated stock solution to the sonicated samples to bring the cell suspension into the imidazole/Chaps/VAPONA buffer. Vitamin K-dependent peptide carboxylase activity was measured in the microsomal and cell suspensions as ¹⁴CO₂ incorporation into the synthetic pentapeptide Phe-Leu-Glu-Glu-Leu as described by Esmon & Suttie (1976). Incubations contained 2 mM-pentapeptide, 5 mM-DTT and chemically reduced vitamin K₁H₂ (100 µg/ml).

Protein carboxylation

Vitamin K-dependent [¹⁴C]carboxylation of endogenous protein in microsomes and cells suspended in the imidazole/Chaps/VAPONA buffer was triggered with vitamin K₁H₂ (100 µg/ml) in the presence of H¹⁴CO₃⁻ (40 µCi/ml). Incubation was carried out for 30 min at 25 °C. Protein carboxylation activity was measured as described by Esmon & Suttie (1976) as vitamin K-dependent ¹⁴CO₂ fixation of endogenous proteins. To recover ¹⁴C-labelled proteins from the reaction mixture, the mixture was subjected to gel filtration on Sephadex G-25 in 0.1 M-NH₄HCO₃. The void-volume fraction was collected and freeze-dried in 0.5 ml portions. Control samples were prepared as described above from incubations in the absence of vitamin K₁H₂.

Antiserum

Rat prothrombin was purified from the plasma of Sprague-Dawley rats by using the method described by Grant & Suttie (1976). The purified protein appeared as a single band on SDS/PAGE in 10%-(w/v)-polyacrylamide reducing gels. The protein was used for anti-

Fig. 1. SDS/PAGE of ^{14}C -labelled carboxylase substrate protein in lung and rat liver microsomes

Microsomes from warfarin-injected rats were suspended in imidazole/Chaps/VAPONA buffer and incubated for 30 min in the presence of $40\ \mu\text{Ci}$ of $\text{NaH}^{14}\text{CO}_3/\text{ml}$ and $100\ \mu\text{g}$ of vitamin $\text{K}_1\text{H}_2/\text{ml}$. Control incubations were prepared in the absence of vitamin K_1H_2 . The incubation mixtures were desalted on Sephadex G-25 in $100\ \text{mM-NH}_4\text{HCO}_3$. Freeze-dried microsomal protein was dissolved in the SDS/PAGE buffer and subjected to electrophoresis in 10%-(w/v)-polyacrylamide reducing gels. Lane A, fluorogram of $150\ \mu\text{g}$ of liver microsomal proteins from incubations with vitamin K_1H_2 ; lane B, fluorogram of $80\ \mu\text{g}$ of lung microsomal proteins from incubations with vitamin K_1H_2 . The SDS/PAGE gels of liver and lung proteins were exposed to X-ray film for $2\frac{1}{2}$ and 4 weeks respectively. Arrows identify protein bands shown on the fluorograms. Values on the right are apparent molecular masses in kDa determined with ^{14}C -methylated standard proteins. 'P' identifies the prothrombin substrate for the vitamin K-dependent carboxylase.

prothrombin antibody production in 10-week-old New Zealand White rabbits. The antigen ($0.5\ \text{mg}$ in Freund's complete adjuvant) was given intradermally at multiple sites over the entire back of the animal. The rabbits were boosted with $0.5\ \text{mg}$ of antigen in saline, which was given subcutaneously to the animals. Blood was drawn by ear-vein puncture and the serum stored at -20°C before use. Ouchterlony (1948) immunodiffusion of antiserum and rat plasma resulted in a single precipitin arch between the two wells, and the antiserum neutralized prothrombin activity in rat plasma.

Immunoabsorption

Freeze-dried proteins from incubations with $\text{H}^{14}\text{CO}_3^-$ containing either vitamin K_1H_2 or ethanol (control) were dissolved in $25\ \text{mM}$ -imidazole/ $0.5\ \text{M}$ -KCl/ 1.5% Triton X-100/ $1\ \text{mM}$ -VAPONA, pH 7.2 (SIK/VAPONA buffer) and allowed to react with prothrombin antiserum or preimmune serum. The reaction mixtures were left overnight at 4°C before Pansorbin was added to the

mixtures ($200\ \mu\text{l}/50\ \mu\text{l}$ of antiserum). Incubation with Pansorbin was carried out on ice for 10 min with occasional shaking. The *Staphylococcus* particles were pelleted by using an Eppendorf table microcentrifuge. The particles were washed twice with $25\ \text{mM}$ -imidazole/ $0.5\ \text{M}$ -NaCl/ 0.05% Triton X-100, pH 7.2, before the immunocomplexes and IgG were released from the particles by boiling them for 2 min in the SDS/PAGE buffer containing 2% (w/v) SDS and 5% (v/v) mercaptoethanol. The released proteins were subjected directly to SDS/PAGE.

Gel electrophoresis

SDS/PAGE was performed as described by Laemmli (1970) in 10%-(w/v)-polyacrylamide gels. Before electrophoresis, all samples were boiled for 2 min in the presence of 5% mercaptoethanol and 1% SDS. Standard proteins used for M_r determinations on fluorograms were the low- M_r ^{14}C -methylated standard proteins purchased from Amersham Corp. Gels selected for fluorography were fixed overnight in 10% (w/v) trichloroacetic acid, 10% (v/v) acetic acid and 25% (v/v) methanol (all final concns.). The fixed gels were treated with En^3Hance for 1 h and subjected to two washes (15 min and 30 min) in 20% (v/v) glycerol before they were dried. Fluorograms of gels were obtained by exposing the dried gels to Kodak X-Omat XAR-5 film at -70°C .

Immunoblotting

Transfer of proteins from SDS/polyacrylamide gels to nitrocellulose was carried out in a Bio-Rad Trans-Blot cell using an SDS/PAGE buffer (Reiderer *et al.*, 1987) containing 20% methanol. Electrophoresis was carried out for 3 h at a constant voltage of 60 V. The nitrocellulose membrane was treated with Blotto and allowed to react with antibodies as described by Reiderer *et al.* (1987). Prothrombin antiserum or preimmune serum was added to Blotto in a ratio of $1:2000$ (v/v) respectively. For detection of immunoreactive protein bands, either ^{125}I -Protein A or horseradish-peroxidase-conjugated anti-rabbit IgG (affinity-purified, from Bio-Rad) were used. When ^{125}I -Protein A was used, the nitrocellulose membranes were incubated for 2 h with $1\ \mu\text{Ci}$ of ^{125}I -Protein A in $100\ \text{ml}$ of Blotto. After incubation the membranes were washed extensively with $50\ \text{mM}$ -Tris/HCl/ $150\ \text{mM}$ -NaCl, pH 7.4. The membranes were dried and submitted to autoradiography. When horseradish-peroxidase-conjugated antibodies were used, the antibody solution was added to Blotto in a ratio of $1:3000$ (v/v). With this method, immunoreactive protein bands were revealed after horseradish peroxidase reduction of 4-chloro-1-naphthol.

RESULTS

The liver is the primary site of synthesis of vitamin K-dependent coagulation factors. However, before the present studies neither the identity nor the biological function of any of the γ -carboxylated proteins produced by the lung was known. A main objective of the present work was to establish the identity of such proteins. To approach this goal, we first radiolabelled microsomal precursors of vitamin K-dependent proteins as described in the Experimental section.

Since all proteins present in our carboxylation system could be analysed by SDS/PAGE, it was possible to

Table 1. Carboxylase activity in purified pulmonary macrophages and Type II cells

Peptide carboxylase activity was measured in cells that were sonicated and suspended in the imidazole/Chaps/VAPONA buffer as described in the Experimental section. Activity was determined in freshly isolated pulmonary macrophages and Type II cells, and in Type II cells after 3 days in primary culture (see the Experimental section). Peptide carboxylase activity was measured in the presence ('+Peptide') and absence ('-Peptide') of exogenously added pentapeptide substrate. Carboxylation of endogenous proteins was also measured ('Protein'). Values are means $\pm$ S.D. for three parallel incubations. All data are corrected for background radioactivity measured in incubations with no vitamin K₁H₂. Background radioactivities for peptide carboxylation in macrophages, Type II cells and Type II cells after 3 days in culture were 920, 880 and 860 c.p.m./ml per 10⁷ cells respectively. Background radioactivities for protein carboxylation in macrophages and Type II cells were 104 and 162 c.p.m./ml per 10⁷ cells.

Cell type	Carboxylase activity (c.p.m./10 ⁷ cells)		
	+ Peptide	- Peptide	Protein
Macrophages	*	*	*
Type II cells	2080 $\pm$ 95	82 $\pm$ 28	420 $\pm$ 18
Type II cells after 3 days in culture	2120 $\pm$ 12	78 $\pm$ 24	430 $\pm$ 20

* Not significant over background.

establish the apparent M_r values of ¹⁴C-labelled γ -carboxylated proteins that were synthesized in microsomes from lung and liver. For these studies, microsomes from warfarin-injected rats were used. These microsomes have a higher carboxylase activity and yield quantitatively more ¹⁴C-labelled γ -carboxylated proteins on carboxylation *in vitro* (Suttie, 1978). Fluorograms of SDS/10%-PAGE gels with proteins from the lung and the liver carboxylation systems are shown in Fig. 1. On the fluorogram of liver protein, four or five bands can be seen (Fig. 1, lane A). Of these bands, the one labelled 'P' was identified as the prothrombin substrate for the liver vitamin K-dependent carboxylase (see below). A longer exposure time (4 weeks) was required to obtain a fluorogram of the ¹⁴C-labelled proteins synthesized in the lung carboxylation system (Fig. 1, lane B). The fluorogram, however, revealed a heavily ¹⁴C-labelled protein migrating with the same M_r (70000) as the heavy band appearing on the fluorogram of liver proteins. In addition γ -carboxylated proteins with apparent M_r values of 65000, 53000, 50000, 36000, 31000 and 13000 were found among the lung proteins (Fig. 1, lane B). These proteins were not found on the fluorogram of liver proteins (Fig. 1, lane A). Therefore it appears that several vitamin K-dependent proteins produced by the lung are not produced by the liver.

No radioactivity was detected by the photographic film when proteins from control incubations with H¹⁴CO₃⁻ but no added vitamin K₁H₂ were analysed by SDS/PAGE (results not shown). Therefore the bands shown in Fig. 1 correspond to ¹⁴C-labelled proteins

Table 2. Effect of warfarin on carboxylase activity in cultured Type II cells

Type II cells were maintained in culture for 48 h as described in the Experimental section. Warfarin (50 μ M) and saline were then added to test cultures and control cultures respectively, and the cells were maintained for an additional 24 h before they were harvested from the plastic culture surface for peptide carboxylase activity measurements. Values for peptide carboxylase activity are means $\pm$ S.D. for three parallel incubations.

Addition to Type II cells	Carboxylase activity in Type II cells (c.p.m./ml per 10 ⁷ cells)	Activity (% of control)
Saline	2110 $\pm$ 86	100
Warfarin	4093 $\pm$ 159	194

synthesized in response to vitamin K₁H₂. These proteins are indeed vitamin K-dependent proteins.

Identification of the liver prothrombin substrate for the vitamin K-dependent carboxylase was carried out as shown in Fig. 2. When the complete '*in vitro*'-carboxylation system was subjected to SDS/PAGE and transferred to nitrocellulose, specific prothrombin antibodies and subsequent staining with peroxidase-conjugated secondary antibody identified a 78 kDa protein as the prothrombin antigen present in our liver system [Fig. 2a, both lanes (i) identical]. Fluorography of the proteins in the complete '*in vitro*' system is shown in Fig. 2(b), lane (ii). Among the ¹⁴C-labelled proteins present, the prothrombin antibodies recognized a single protein with a molecular mass of 78 kDa as the prothrombin antigen, as shown by electrophoresis of the immunoabsorbed material (Fig. 2b, lane iii).

In order to trace the production of vitamin K-dependent proteins in the lung to certain cell types, we isolated Type II pulmonary epithelial cells and pulmonary macrophages for additional studies. Preliminary experiments using Type II cells isolated from rat lung showed that carboxylase activity could be measured in an '*in vitro*' system prepared from unfractionated disrupted cells. When sonication of cells was followed by addition of the zwitterionic detergent Chaps, a test system was produced in which the synthetic pentapeptide could be used as substrate for the carboxylase. Freezing and thawing of the cells before this treatment was found to destroy the activity.

We prepared the '*in vitro*' system from purified pulmonary macrophages and Type II cells starting with a suspension of (50–75) $\times$ 10⁶ cells/ml. Carboxylase activity was measured in Type II cells immediately after their isolation and also in Type II cells after 3 days in culture. Activity was measured in the absence or presence of the synthetic pentapeptide to evaluate the requirement for the carboxylase substrate in the assay. In the absence of peptide ('-Peptide', Table 1) we measured in Type II cells, but not in macrophages, a small increase in radioactivity (50–90 c.p.m./ml per 10⁷ cells) over a background radioactivity in the absence of vitamin K₁H₂. When the pentapeptide substrate was added to the test system, Type II cells revealed significant peptide carboxylase activity, but no activity could be measured

in macrophages ('+Peptide', Table 1). Significant endogenous protein carboxylation was also measured in Type II cells, but again, no activity could be measured in macrophages (Table 1). These data strongly suggest that the macrophages we studied were devoid of vitamin K-dependent carboxylase activity.

Type II cells harvested after 3 days in culture had approximately the same carboxylase activity per 10^7 cells as freshly isolated Type II cells (Table 1). Therefore the ability of these cells to synthesize vitamin K-dependent proteins does not appear to change during a time in primary culture when these cells are known to undergo significant morphological and functional alterations (Rannels & Rannels, 1987). These changes do not significantly alter the vitamin K function of the cells. However, cells which did not adhere, or those which were released from the plastic surface during culture, had no carboxylase activity (results not shown). Because many of these cells were viable, as assessed by Trypan Blue exclusion, expression of carboxylase activity after several days in culture may depend on the maintenance of some degree of cell-substratum contact. This conclusion is consistent with an important role for the extracellular matrix in the maintenance of differentiated-Type-II-cell function, particularly with respect to surfactant homoeostasis (Rannels & Rannels, 1987).

The effect of the anticoagulant drug warfarin on carboxylase activity in Type II cells was also investigated. Culturing Type II cells for 24 h in the presence of $50 \mu\text{M}$ warfarin enhanced their carboxylase activity 94% over the activity measured in control cultures where no drug was added (Table 2). Type II pneumocytes, therefore, respond to warfarin in a manner similar to that reported for hepatoma cells (Fair & Bahnak, 1984) and rat liver (Suttie, 1978). This new important observation suggests that the vitamin K-dependent carboxylase enzyme complex is similar in these diverse cell types.

For identification of vitamin K-dependent precursor proteins in Type II cells, we first prepared microsomes from these cells, using the vitamin K-dependent carboxylase as a marker enzyme to identify the microsomal pellet. Microsomes from alveolar macrophages were prepared by the same procedure. Cell microsomes were subjected to vitamin K-dependent carboxylation by the same procedure as described for crude lung and liver microsomes. Previously we have shown that Type II cell microsomes have ^{14}C -labelled vitamin K-dependent precursor proteins of molecular masses 87, 65, 36, 31 and 13 kDa (Rannels *et al.*, 1987). However, ^{14}C -labelled proteins could not be detected in microsomes from alveolar macrophages. Applying more microsomal protein from the macrophage preparation to the SDS/polyacrylamide gel also did not result in any detectable radiolabelled bands (results not shown). This observation was consistent with the carboxylase activity measurements in macrophages and gave additional support for our data, which suggest that alveolar macrophages are devoid of this activity. The vitamin K-dependent carboxylase is a microsomal enzyme (Suttie, 1985). Therefore all the carboxylase activity measured in our test system prepared from whole cells is related to the microsomal particles present in the test system. The activity is certainly an expression of the cell's ability to synthesize vitamin K-dependent proteins. Only in those samples exhibiting activity could ^{14}C -labelled proteins be detected by fluorography.

Fig. 2. Identification of the prothrombin substrate for the vitamin K-dependent carboxylase in liver

Vitamin K-dependent proteins in microsomes from livers of warfarin-injected rats were ^{14}C -labelled and freeze-dried as described in the legend to Fig. 1. A sample (11 mg) of freeze-dried protein was dissolved in 1 ml of the SIK-VAPONA buffer and allowed to react with $50 \mu\text{l}$ of prothrombin antiserum for isolation of the prothrombin liver precursor. Immunoabsorbed material was electrophoresed into SDS/10%-polyacrylamide gels (b, lane iii) simultaneously with crude freeze-dried proteins dissolved in the SDS/PAGE buffer (b, lane ii). (b) Shows a fluorogram of the SDS/PAGE gel. (a) (lanes i) shows immunoblotting of two identical samples of crude freeze-dried proteins in the SDS/10%-PAGE gel. Horseradish-peroxidase-conjugated IgG was used for the detection of immunoreactive material.

We have recently shown that Type II cells produce γ -carboxylated surfactant-associated proteins (Rannels *et al.*, 1987). Do Type II cells also produce the coagulation factor prothrombin? The following experiment has triggered this question. Microsomes isolated from Type II cells were washed twice in 25 mM-imidazole/HCl/0.5% Chaps/1 mM-VAPONA/1 mM- $\text{iPr}_2\text{P-F}$, pH 7.2. When these washed membrane vesicles were subjected to SDS/PAGE and transferred to nitrocellulose for immunoblotting with antibodies against plasma prothrombin, immunoreactive materials were found. As shown in Fig. 3(a), lane (ii), the antibodies identified two prothrombin-like precursors of apparent molecular masses 65 and 68 kDa. The 65 kDa antigen had the same molecular mass as one of the ^{14}C -labelled precursors previously found in Type II cell microsomes (Rannels *et al.*, 1987). However, both of these proteins migrated with a lower molecular mass than that (78 kDa) of the liver prothrombin precursor (Fig. 3a, lane i). Fig. 3b shows Type-II-cell membrane vesicles as in Fig. 3(a), lane (ii), which were allowed to react with preimmune serum.

DISCUSSION

Fluorography of ^{14}C -labelled proteins synthesized in a vitamin K-dependent reaction in lung and liver micro-

Fig. 3. Identification of prothrombin-like antigens in Type II cell microsomes

Microsomes from livers of warfarin-treated rats and microsomes from Type II cells isolated from untreated rats were isolated as described in the Experimental section. The microsomes were washed twice in 25 mM-imidazole/0.5% Chaps/1 mM-VAPONA/1 μ M- i Pr₂P-F, and the insoluble membrane vesicles were recovered after each wash by centrifugation at 100 000 *g* for 60 min. The washed membrane vesicles (50 μ g of protein) were dissolved in 2% SDS and 5% mercaptoethanol, boiled for 2 min, and then subjected to SDS/PAGE in 10%-polyacrylamide gels. Panel (a), lanes (i) and (ii), shows immunoblots of electrophoresed proteins from rat liver and Type-II-cell microsomes respectively, using prothrombin antibodies and ¹²⁵I-Protein A for the detection of immunoreactive protein bands. The electrophoresed proteins in panel (b) are identical with those in panel (a), lane (ii), but immunoblotting was carried out with preimmune serum.

some demonstrates that the lung has precursors of vitamin K-dependent proteins, many of which are not found in the liver. It appears that Type II epithelial cells can synthesize most of the vitamin K-dependent proteins found in the lung. Of the ¹⁴C-labelled vitamin K-dependent precursors found in microsomes from lung tissue, 70% had the same molecular mass as ¹⁴C-labelled precursors found in microsomes from Type II cells. We also found that antibodies against the coagulation factor prothrombin recognized antigens in alveolar Type II epithelial cells. Immunoblotting of extensively washed microsomal vesicles from these cells identified two prothrombin-like antigens of apparent molecular masses 65 and 68 kDa. This finding raises the question whether the Type II cell is also a potential site for prothrombin synthesis. If we can exclude that proteolysis did not occur in our system in the presence of i Pr₂P-F and VAPONA, our data would suggest that the carboxylase in Type II cells uses a lower-*M_r* prothrombin precursor as substrate for γ -carboxylation. Type II cells may, however, synthesize a prothrombin molecule that is different from the prothrombin molecule produced by

hepatocytes. Fibrinogen, another coagulation protein produced in the liver, is also synthesized in megakaryocytes and stored in the α -granules of blood platelets (Leven *et al.*, 1985). Immunological (Plow & Edington, 1975) and structural (Ganguly, 1972) differences have been shown to exist between plasma and platelet fibrinogen. Whether or not Type II cells produce an altered prothrombin molecule needs further investigation.

Evidence for vitamin K-dependent carboxylase activity in Type II cells was obtained in an '*in vitro*' carboxylation system prepared from freshly isolated whole cells. In this system the synthetic pentapeptide was accepted as substrate for the enzyme. Type II cells were also shown to resemble hepatocytes with regard to their response to the anticoagulant drug warfarin, and also retained similar carboxylase activity after 3 days in culture. In these experiments (Table 2), Type II cells were cultured under conditions in which differentiated morphology and function diminished with time. These changes include loss of lamellar inclusion bodies and a decline in the synthesis and secretion of surfactant materials (Rannels & Rannels, 1987). Thus cells which are known to 'de-differentiate' during culture, possibly into a 'Type-I-like' epithelial cell, still retain the same per-cell carboxylase activity. These results suggest that this biological activity may be characteristic of lung alveolar epithelium in any stage of cellular differentiation. This observation may reflect the potential of the lung to maintain synthesis of vitamin K-dependent proteins after lung injury, when Type I cells are irreversibly damaged and the Type II epithelium divides to re-establish the normal Type I/Type II ratio (Crapo *et al.*, 1982).

Vitamin K-dependent carboxylase activity measured in isolated cells should coincide with production of vitamin K-dependent procoagulant activity by these cells. It is well established that macrophages produce factor V (Rothberger & McGee, 1984) and tissue factor, the latter being the potent trigger of the extrinsic coagulation system (Lyberg, 1984; Rothberger *et al.*, 1984). However, reported data showing production of vitamin K-dependent coagulation proteins by these cells are conflicting (Shands, 1986). Osterud *et al.* (1980) have proposed that murine peritoneal macrophages produce the entire set of the classical vitamin K-dependent coagulation factors. Shands (1986) reported that these cells produce a vitamin K-dependent factor-VII-like activity, but question their ability to synthesize factor X. In addition, Chapman *et al.* (1985) also reported that [³⁵S]methionine-labelled factor VII could be isolated from cultured human alveolar macrophages with anti-(factor VII) antibodies. On the other hand, Rothberger & McGee (1984) found no evidence that pulmonary macrophages can produce factor II, VII and X, which suggest that their cells were unable to produce vitamin K-dependent coagulation factors. This finding is consistent with our results, as we were unable to measure carboxylase activity in pulmonary macrophages.

Mononuclear phagocytes, after leaving the marrow, reside in various tissues as macrophages of differing degrees of maturation (Adams & Hamilton, 1984). Our preparations of pulmonary macrophages represent a mixture of such cells. Since our cell preparations were devoid of carboxylase activity, it appears that activity is not expressed in macrophages that have progressed through specific stages of maturation. In addition, we

have failed to measure carboxylase activity both in macrophages derived from extensive alveolar lavage fluids and in a separate population derived from elastase-disrupted tissue after differential adherence on plastic surfaces. These latter cells are predominantly of interstitial origin and may be acutely involved in injury-repair mechanisms. We have used our '*in vitro*' carboxylation system to measure vitamin K-dependent carboxylase activity in several cell lines (Wallin & Al-Mondhiry, 1987) and, so far, macrophages are the only cells we have studied that appear to be devoid of carboxylase activity. Our data do not exclude the possibility that the genes for the vitamin K-dependent clotting factors are expressed in macrophages, but the data suggest that functional vitamin K-dependent clotting factors are not produced by these cells.

We have not measured carboxylase activity in murine peritoneal macrophages and can, therefore, not argue whether or not this tissue population of macrophages can produce vitamin K-dependent procoagulant activity. However, there is no convincing evidence in the literature that suggests these cells should be distinctly different from the other cells of the mononuclear-phagocyte system (Walker, 1976).

This work was supported by grants (32070-02 and HL-31560) from the National Institutes of Health and a grant (851337) from the American Heart Association. S.R.R. is an Established Investigator of the American Heart Association. We thank Ms. Susan D. Patrick and Ms. Carol Stinson Fisher for technical assistance.

REFERENCES

- Adams, D. O. & Hamilton, T. A. (1984) *Ann. Rev. Immunol.* **2**, 283–318
- Chapman, H. A., Allen, C. L., Stone, O. L. & Fair, D. S. (1985) *J. Clin. Invest.* **75**, 2030–2037
- Crapo, J. D., Barry, B. E., Gehr, P., Bachofen, M. & Weibel, E. R. (1982) *Am. Rev. Respir. Dis.* **125**, 740–745
- Donati, M. B. & Semeraro, N. (1984) *Haemostasis* **14**, 422–429
- Esmon, C. T. & Suttie, J. W. (1976) *J. Biol. Chem.* **251**, 6238–6243
- Fabisiak, J. P., Rannels, S. R., Vessel, E. S. & Rannels, D. E. (1986) *Am. J. Physiol.* **250**, C871–C879
- Fair, D. S. & Bahnak, B. R. (1984) *Blood* **64**, 194–204
- Fair, D. S. & Marlar, R. A. (1986) *Blood* **67**, 64–70
- Fair, D. S., Marlar, R. A. & Levin, E. C. (1986) *Blood* **67**, 1168–1171
- Ganguly, P. J. (1972) *J. Biol. Chem.* **247**, 154–156
- Grant, G. A. & Suttie, J. W. (1976) *Arch. Biochem. Biophys.* **176**, 650–662
- Graves, C. B., Munns, T. W., Willingham, A. K. & Strauss, A. W. (1982) *J. Biol. Chem.* **257**, 13108–13113
- Katyal, S. L. & Sing, G. (1984) *Exp. Lung Res.* **6**, 175–189
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Leven, R. M., Schicu, P. U. & Budzinski, A. Z. (1985) *Blood* **65**, 501–504
- Lyberg, T. (1984) *Haemostasis* **14**, 430–439
- Mason, R. J., Walker, S. R., Shields, B. A., Henson, J. E. & Williams, M. E. (1985) *Am. Rev. Respir. Dis.* **131**, 786–788
- Olson, J. R., Miller, L. L. & Troup, S. B. (1966) *J. Clin. Invest.* **45**, 690–701
- Osterud, B., Lindahl, U. & Seljelid, R. (1980) *FEBS Lett.* **120**, 41–43
- Ouchterlony, O. (1948) *Acta Pathol. Microbiol. Scand.* **25**, 186–191
- Plow, E. F. & Edington, T. S. (1975) *Thromb. Res.* **7**, 729–742
- Rannels, S. R. & Rannels, D. E. (1987) in *Toxicology of the Lung* (Gardner, D. E., Crapo, J. D. & Massaro, E. J., eds.), Raven Press, New York, in the press
- Rannels, S. R., Gallaher, K. J., Wallin, R. & Rannels, D. E. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5952–5956
- Reiderer, B. M., Zagon, I. S. & Goodman, S. R. (1987) *J. Neurosci.* **7**, 804–874
- Rothberger, H. & McGee, M. P. (1984) *J. Exp. Med.* **160**, 1880–1890
- Rothberger, H., McGee, M. P. & Lee, T.-K. (1984) *J. Clin. Invest.* **73**, 1524–1531
- Sadowski, J. A., Esmon, C. T. & Suttie, J. W. (1976) *J. Biol. Chem.* **251**, 2770–2776
- Shands, J. W. (1986) *Blood* **65**, 169–175
- Suttie, J. W. (1978) in *The Fat-Soluble Vitamins [Handbook of Lipid Research (De Luca, H. F., ed.), vol. 2]*, pp. 211–277, Plenum Press, New York
- Suttie, J. W. (1980) *CRC Crit. Rev. Biochem.* **8**, 191–223
- Suttie, J. W. (1985) *Annu. Rev. Biochem.* **54**, 459–477
- Suttie, J. W., Hageman, J. M., Lehrman, S. R. & Rich, (1976) *J. Biol. Chem.* **251**, 5827–5830
- Vermeer, C. & De Boer-Van Den Berg, M. A. G. (1985) *Haematologia* **18**, 71–97
- Walker, W. S. (1976) in *Immunobiology of the Macrophage* (Nelson, D. S., ed.), pp. 91–110, Academic Press, New York
- Wallin, R. & Al-Mondhiry, H. (1987) *Clin. Res.* **35**, 603A
- Wallin, R. & Martin, L. F. (1987) *Biochem. J.* **241**, 389–396

Received 18 May 1987/18 September 1987; accepted 4 November 1987