Phospholipid-transfer activities in cytosols from lung, isolated alveolar type II cells and alveolar type II cell-derived adenomas

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We have examined phospholipid-transfer activities in cytosols from rat and mouse whole lung, isolated rat alveolar type II cells and alveolar type II cell-derived mouse pulmonary adenomas. We report an enrichment in phosphatidylcholine and phosphatidylglycerol (but not phosphatidylinositol) protein-catalysed transfer in the type II cell and adenoma cytosols compared with the whole-lung cytosols. The activities from these cytosols were resolved using column chromatofocusing, which clearly demonstrated the presence of a phosphatidylcholine-specific transfer protein in each of the four tissues. In addition, two proteins (rat) or three proteins (mouse) catalysing both phosphatidylcholine and phosphatidylglycerol transfer were resolved from whole lung, whereas in both the rat isolated alveolar type II cells and the mouse type II cell-derived adenomas one of these less specific proteins is not present.

A body of evidence is accumulating that suggests that phospholipid-transfer proteins may have a role in the mammalian pulmonary surfactant system. It has been suggested that these proteins may catalyse the transfer of surfactant phospholipid from its site of synthesis on the alveolar type II cell endoplasmic reticulum to the developing lamellar bodies (Robinson *et al.*, 1978; Engle *et al.*, 1978; Spaulding & Hook, 1979). PtdCho-transfer activity has been demonstrated in lung soluble fractions from sheep (Robinson *et al.*, 1978), mice (Engle *et al.*, 1978), rabbit (Spaulding & Hook, 1979) and rat (van Golde *et al.*, 1980) and has been shown to correlate with foetal maturation of the surfactant system of the mouse (Engle *et al.*, 1978) and with the specific alveolar surface area in a series of six vertebrate species (Lumb *et al.*, 1980). PtdGro-transfer activity has been observed in rat (van Golde *et al.*, 1980) and sheep (Whitlow *et al.*, 1980) lung cytosols.

Because of the complexity of the mammalian lung with its many different cell types (Sorokin, 1970) it is important to determine whether the observed activities are intra- or extra-cellular, and to determine the cell type in which specific phospholipid-transfer proteins occur. Isolation of alveolar type II cells in primary culture has proven to be a powerful tool in clarifying mechanisms of surfactant phospholipid

synthesis (Mason & Dobbs, 1980) and its control (Batenburg, 1980; Post *et al.*, 1980b; King & Martin, 1981). Phospholipid-transfer activities have been observed in rat lung isolated alveolar type II cells (Post *et al.*, 1980a) but no separation of activities was achieved. Urethane-induced pulmonary adenomas of BALB/c mice (Snyder *et al.*, 1973; Wykle *et al.*, 1977) are derived from alveolar type II cells and have proven to be a useful model for studying surfactant synthesis (Snyder & Malone, 1975; Voelker *et al.*, 1976; Stoner *et al.*, 1978; Voelker & Snyder, 1979; Wykle *et al.*, 1980). We report in the present paper the distributions of phospholipid-transfer proteins in whole rat liver and mouse lung cytosols determined by chromatofocusing and compare that with the distributions of transfer proteins in cytosols derived from isolated rat alveolar type II cells and from urethane-induced mouse pulmonary adenomas.

Materials and methods

Preparation of whole-lung cytosol

Adult Sprague–Dawley rats and adult BALB/c mice were killed by cervical dislocation without anaesthesia and the lungs removed, dissected free of bronchi, rinsed with 10 mM-Tris/HCl buffer, pH 7.4, containing 0.25 M-sucrose and 1 mM-EDTA (SET buffer). A 20% homogenate was prepared in SET buffer using five strokes of a Potter–Elvehjem tissue homogenizer (clearance 0.125 mm). The homogenate was centrifuged at 15000 g for 20 min, the

Abbreviations used: PtdCho, phosphatidylcholine; PtdGro, phosphatidylglycerol; PtdIns, phosphatidylinositol.

supernatant decanted and re-centrifuged at 105 000 *g* for 60 min. The decanted lung supernatant was termed whole-lung cytosol. For one experiment the rat was anaesthetized with sodium pentobarbital (50 mg/kg body wt.), the lung gently lavaged with SET buffer via a tracheal cannula and the lung perfused via the right atrium with SET buffer until completely blanched. Cytosol from this lung was prepared as described above.

Preparation of alveolar type II cell cytosol

Freshly isolated alveolar type II cells were obtained after enzymic dissociation of adult rat lungs with elastase and after purification by centrifuging on metrizamide density gradients as described previously (Dobbs *et al.*, 1980). Cytosol from these cells was obtained by homogenizing 4×10^8 alveolar type II cells in 1 ml of SET buffer. Three different cell preparations of type II cells, with a calculated purity of $88 \pm 5\%$ type II cells, were pooled in order to obtain sufficient material for column chromatofocusing. After five strokes of a Potter–Elvehjem homogenizer the homogenate was centrifuged at 105 000 *g* for 60 min and the decanted supernatant used as the cytosolic fraction.

Preparation of adenoma cytosols

Urethane-induced pulmonary adenomas were obtained as a generous gift from Dr. Fred Snyder (Medical and Health Science Division, Oak Ridge Associated Universities, Oak Ridge, TN, U.S.A.). Adenomas were induced in BALB/c mice by urethane injection as described previously (Snyder *et al.*, 1973; Wykle *et al.*, 1977), dissected from the lungs and frozen at -70°C until used. Cytosolic fractions were prepared from the thawed adenomas using the same procedures as for whole lung.

Column chromatofocusing

Column chromatofocusing was performed using PBE 94 chromatofocusing gel (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) equilibrated with 25 mM-imidazole/HCl at pH 7.4 (rat) or pH 6.4 (mouse). Cytosolic fractions were applied directly to the columns and eluted with Polybuffer 74 (Pharmacia Fine Chemicals), pH 4.0. Details are provided in the legend to Fig. 1.

Assay of phospholipid transfer

Protein-catalysed phospholipid transfer was assayed using sonicated phospholipid vesicles (Whitlow *et al.*, 1980) as donors (0.0125 μmol of phospholipid phosphorus per assay tube) and hand-shaken acceptor multilamellar vesicles (5 μmol of phospholipid phosphorus per assay tube). Acceptor multilamellar vesicles consisted of 90 mol% PtdCho (Sigma; fraction V) and 10 mol% dicetyl phosphate and were prepared by gentle swirling in 50 mM-

Tris/HCl, pH 7.4, containing 5 mM-EDTA. Transfer of PtdCho and PtdGro was assayed using sonicated donor phospholipid vesicles consisting of 80 mol% egg phosphatidylethanolamine (Sigma type III), 10 mol% egg PtdCho (Sigma type V E) and 10 mol% PtdGro (from egg yolk lecithin; Sigma Chemical Co.). Tracer quantities of (di[^3H]palmitoyl)PtdCho, (2-[^{14}C]linoleoyl-1-palmitoyl)PtdCho or (di[^{14}C]palmitoyl)PtdGro and either [^3H]- or [^{14}C]hexadecylcholesteryl ether (as a non-exchangeable marker) were included. All radiochemicals were purchased from New England Nuclear Corp., Boston, MA, U.S.A. Transfer of PtdIns was determined by using the same donor vesicle composition except that [^3H]PtdIns and unlabelled PtdIns were substituted for the PtdGro. Donor and acceptor vesicles were incubated with the appropriate protein fractions in 50 mM-Tris/HCl, pH 7.4, containing 5 mM-EDTA at 37°C in a total volume of 0.5 ml. The assays were terminated by addition of 1.5 ml of cold buffer and centrifuging at 12 000 *g* for 30 min. The acceptor multilamellar vesicle pellets were resuspended in buffer, re-centrifuged and the radioactivity in the pellets determined. All transfer values are corrected for co-sedimentation of donor vesicles as reported by the non-exchangeable marker ($<10\%$) and for transfer in the absence of protein ($<5\%$). To determine the specific activities shown in the Tables, several different volumes of each fraction appropriate to give up to 15% transfer were assayed and the linear regions of transfer were used. Protein concentrations were determined by the dye-binding procedure of Bradford (1976) with bovine serum albumin as standard. Phospholipid phosphorus was determined by the method of Rouser *et al.* (1970).

Results and discussion

Cytosolic fractions from whole rat and mouse lung, lavaged and perfused rat lung, rat isolated alveolar type II cells and pulmonary adenomas all catalyse the transfer of PtdCho, PtdGro and PtdIns between phospholipid bilayers (Table 1). The specific activities of transfer for all three phospholipids are higher in the lavaged and perfused lung cytosol than in the whole-lung cytosol; this supports an intracellular localization of the observed activities. In isolated alveolar type II cell cytosol the specific activity of PtdCho transfer is enriched more than 3-fold and PtdGro transfer almost 6-fold compared with the values for whole lung; the specific activity of PtdIns transfer in the type II cell cytosol, however, is only about half that in whole lung cytosol. A similar pattern of specific activities is seen in the mouse tissues, with PtdCho- and PtdGro-transfer activities increased and PtdIns-transfer activity decreased in the adenoma compared with the whole lung. These data support the hypothesis

Table 1. *Phospholipid-transfer activities of whole rat lung, lavaged and perfused rat lung and alveolar type II cell cytosols*

Phospholipid transfer from sonicated phospholipid vesicles to multilamellar vesicles was determined as described in the Materials and methods section using three concentrations of cytosol protein. Incubations were carried out at 37°C for 120 min. Activity is expressed as units/mg of protein per h; 1 unit = transfer of 1% of radiolabelled phospholipid pool in donor single bilayer liposomes.

	Phospholipid-transfer activity		
	PtdCho	PtdGro	PtdIns
Rat lung	131	44	487
Lavaged and perfused lung	195	79	695
Alveolar type II cells	440	259	265
(Type II/rat lung)*	(3.4)	(5.9)	(0.6)
Mouse lung	297	89	95
Pulmonary adenoma	449	159	82
(Adenoma/mouse lung)†	(1.5)	(1.8)	(0.9)

* Ratio of transfer activity in Type II cell cytosol to that in rat whole-lung cytosol.

† Ratio of transfer activity in adenoma cytosol to that in mouse whole-lung cytosol.

that PtdCho- and PtdGro-transferring proteins play a role in the functions of the type II pneumocyte.

When cytosol derived from whole lungs was chromatographed on Sephadex G-75 (results not shown) it was apparent that the transfer activities of the three different phospholipid species did not co-chromatograph when assayed with our system, but little separation of activities was achieved. However, when whole-rat lung cytosol was subjected to column chromatofocusing (pH gradient of 7.5 to 4.8) three clear regions of transfer activity were detected (Fig. 1a). A peak eluting before the beginning of the pH gradient (pH > 7.5) was specific for PtdCho transfer, and peaks eluting at pH 6.6 and at pH 5.1 appeared to be less specific, catalysing the transfer of both PtdCho and PtdGro. An essentially identical pattern of activities and specificities was observed when we chromatographed cytosol derived from lavaged and perfused lung on a chromatofocusing column, further supporting the intracellular location of all three of the observed activities. When whole-lung soluble fraction was eluted from a chromatofocusing column using a pH gradient from 9.0 to 6.0 the PtdCho-specific protein eluted at a pH of 8.0 (results not shown), which is similar to the isoelectric point of a PtdCho-specific transfer protein isolated from rat liver (Lumb *et al.*, 1976).

When alveolar type II cell cytosol was eluted from a chromatofocusing column (pH gradient of 7.5 to 4.8) peaks of transfer activity were observed in the same three pH regions as in whole lung but the

Fig. 1. *Chromatofocusing elutions of rat cytosols loaded on PBE 94 gel columns and developed with Polybuffer 74 (pH 4.0)*

(a) Rat whole-lung cytosol (10 ml) was loaded on a column (1 cm × 25 cm) and developed with 200 ml of buffer at 15 ml/h. Fractions (2 ml) were collected. The results presented are representative of several runs, which confirmed the profile. (b) Rat alveolar type II cell cytosol (1.2 ml) was loaded on a column (0.6 cm × 9 cm) and developed with 100 ml of buffer at 5 ml/h. Fractions (1 ml) were collected. The results presented are representative and were confirmed by duplicate elution. PtdCho-transfer (○) and PtdGro-transfer (●) was determined as described in the Materials and methods section; 1 unit = transfer of 1% of the radiolabelled phospholipid pool in the sonicated donor liposomes. The horizontal lines above the peaks of transfer show the fractions that were pooled and used to determine the specific activities shown in Table 2.

region at pH 6.6 catalysing the transfer of both PtdCho and PtdGro appeared to be substantially reduced (Fig. 1b). Table 2 shows that whereas this pH region represented 20% of the PtdCho-transfer activity (and 31% of the PtdGro-transfer activity) recovery from whole lung, it represented only 4% of the PtdCho-transfer activity (and 12% of the PtdGro-transfer activity) recovered from type II cell cytosol. The total amount of PtdCho- and PtdGro-transfer activity per g of tissue is increased in the

Table 2. *Specific activities of phospholipid transfer by fractions derived from chromatofocusing of rat whole lung and alveolar type II cell cytosols*

Regions of phospholipid transfer activity from chromatofocusing elutions were pooled and phospholipid-transfer activity was determined using three different protein concentrations. Transfer was determined as described in the Materials and methods section; incubations were carried out at 37°C for 120 min. Activity is expressed as units/h per g of wet tissue, where 1 unit represents the transfer of 1% of radiolabelled phospholipid pool in donor single bilayer liposomes.

		Phospholipid transfer			
		PtdCho		PtdGro	
	pH of elution	(%)*	(units · h ⁻¹ · g ⁻¹)	(%)*	(units · h ⁻¹ · g ⁻¹)
Rat lung	7.5	36	857	—	—
	6.6	20	438	31	292
	5.1	44	764	69	424
Alveolar type II cells	7.5	36	1932	—	—
	6.6	4	205	12	137
	5.1	60	3174	88	974

* % of total recovered activity.

Table 3. *Specific activities of phospholipid transfer by fractions derived from chromatofocusing of mouse whole lung and pulmonary adenomas*

Regions of phospholipid transfer activity from chromatofocusing elutions were pooled and phospholipid-transfer activity was determined using three different protein concentrations. Transfer was determined as described in the Materials and methods section; incubations were carried out at 37°C for 120 min. Activity is expressed as units/h per g of wet tissue, where 1 unit represents the transfer of 1% of radiolabelled phospholipid pool in donor single bilayer liposomes.

		Phospholipid transfer			
		PtdCho		PtdGro	
	pH of elution	(%)*	(units · h ⁻¹ · g ⁻¹)	(%)*	(units · h ⁻¹ · g ⁻¹)
Mouse lung	5.4	10	609	—	—
	5.1	60	3618	57	615
	4.7	21	1271	27	289
	4.5	9	541	16	176
Adenoma	5.4	12	586	—	—
	5.1	8	399	7	49
	4.7	37	1796	48	318
	4.5	43	2095	45	304

* % of total recovered activity

type II cell cytosol compared with that for whole lung for both the pH > 7.5 PtdCho-specific protein and the pH 5.1 protein; however, the total amount of activity of the pH 6.6 non-specific protein is reduced in the type II cell cytosol (Table 2).

Chromatofocusing of mouse lung cytosol (Fig. 2a) and pulmonary adenoma cytosol (Fig. 2b) on a pH gradient from 6.4 to 4.0 results in the separation of four regions that catalyse the transfer of phospholipids. A region at pH 5.4 appears to be quite specific for PtdCho transfer, whereas regions eluting at pH 5.1, 4.7 and 4.5 transfer both PtdCho and PtdGro. The primary difference between the whole lung and the type II-derived adenoma is again a reduction in one of the regions catalysing the transfer of both PtdCho and PtdGro; in the

adenoma model, the total amount of the PtdCho-specific protein per g of tissue is about the same, whereas the two lower-pH non-specific proteins are increased (Table 3).

In both type II pneumocyte model systems there is a substantial reduction in a non-specific transfer protein while the low-pI activity transferring both PtdCho and PtdGro is increased. It is clear that a PtdCho-specific protein is present in both the alveolar type II cell and in the alveolar type II cell-derived urethane-induced pulmonary adenoma.

These results differ from those obtained by Post *et al.* (1980a,b), who reported three proteins in whole-rat lung (PtdCho-specific, PtdGro-specific and non-specific transfer proteins). In their isolated type II cell preparations they were only able to resolve a

Fig. 2. Chromatofocusing elution of mouse cytosols loaded on PBE 94 gel columns and developed with Polybuffer 74 (pH 4.0)

Cytosol (3 ml) was loaded on a column (0.6 cm × 36 cm) and developed with 140 ml of buffer at 7.5 ml/h. Fractions (1 ml) were collected. The results presented are representative of several runs. (a) Mouse whole-lung cytosol, concentrated by dialysis against 30% sucrose. (b) Mouse pulmonary adenoma cytosol. PtdCho-transfer (O) and PtdGro-transfer (●) were determined as described in the Materials and methods section; 1 unit = transfer of 1% of the radiolabelled phospholipid pool in the sonicated donor liposomes. The horizontal lines above the peaks of transfer show the fractions that were pooled and used to determine the specific activities shown in Table 3.

non-specific region of phospholipid transfer activity. Our ability to resolve multiple regions of transfer activities in the type II cells may be the result of our comparatively minimal treatment of the cytosolic fractions, the use of a different assay system or the different resolving power of column chromatofocusing as applied to cytosolic fractions. In addition our type II cell preparations were isolated using elastase instead of trypsin; trypsin used in such isolations has been shown to cause alterations in some cellular proteins (Finkelstein & Mavis, 1979).

Phospholipid-transfer proteins may transfer newly synthesized surfactant phospholipid from its site of synthesis on the endoplasmic reticulum (Baranska &

van Golde, 1977; Voelker & Snyder, 1979) or mitochondrion (Mavis & Vang, 1981) to the developing lamellar bodies. This role would require a net transfer of phospholipid; such net transfer has recently been demonstrated using both specific (Wilson *et al.*, 1980; Wirtz *et al.*, 1980) and non-specific (Crain & Zilversmit, 1980) transfer proteins. Experiments with rabbit lung slices incubated with liposomal PtdCho species suggest that transfer proteins may have a role in determining the degree of unsaturation of surfactant PtdCho (Tsao, 1980). Experimental data of Hallman *et al.* (1981) also raise the possibility that phospholipid-transfer proteins may be involved in a bidirectional flux of surfactant phospholipid between the alveolar subphase and intracellular compartments of the alveolar type II cell. Phospholipid-transfer proteins may be involved in sorting phospholipids in the various compartments to determine the specific composition of pulmonary surfactant phospholipid.

In the present paper we have demonstrated increased surfactant phospholipid-transfer activity in surfactant-producing cells compared with whole lung; the increases are in both specific activity and total activity per g of tissue. In the surfactant-producing cells there are proteins that are highly specific for PtdCho transfer and proteins that catalyse the transfer of both PtdCho and PtdGro. Further investigations ought now to be directed specifically at these proteins.

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