The subcellular localization of the β -galactoside-binding protein of rat lung

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The subcellular localization of the β -galactoside-binding protein, or lectin, from rat lung was investigated by the specific binding of anti-lectin immunoglobulin G to subcellular fractions. We used both adult and immature (12-day-old) rats; the immature rat lungs have an 8–10-fold greater concentration than adult rat lungs [Powell & Whitney (1980) *Biochem. J.* **188**, 1–8]. In both groups of animals we observed greater specific binding of anti-lectin immunoglobulin G to intracellular membranes (mitochondrial and microsomal fractions) than to plasma membranes. Pre-incubation of membrane fractions with lactose resulted in a marked diminution of anti-lectin immunoglobulin G binding. In the adult rat lung most (approx. 80%) of the lectin activity was membrane-associated. In the immature rat lung only approx. 30% of the lectin activity was membrane associated and most of the β -galactoside-binding protein appeared to be a soluble cytoplasmic component. The rat lung β -galactoside-binding protein appeared to have a broad but predominantly intracellular location, being associated with membranes through one of its galactoside-binding sites.

The lung is one of several organs in the rat that have an abundance of β -galactoside-binding protein. The newborn rat lung had negligible levels of this β -galactoside-binding protein. Maximum levels were observed at 10–14 days of age followed by a 10-fold decline to adult levels (Powell & Whitney, 1980). This period (postnatal days 10–14) is the period of maximum alveolarization of rat lung. The β -galactoside-binding protein of rat lung has properties that have led to the operational terminology of lung lectin for this protein. The rat lung lectin bound two or more molecules having a terminal β -galactoside moiety, agglutinated erythrocytes with exposed β -galactoside termini but was devoid of any glycosidase activity (Powell, 1980). In the adult rat lung the lectin was membrane-associated; lectin solubility from whole lung or membranous fractions was dependent on the presence of lactose, or another hapten, in the homogenizing buffer (Powell, 1980).

These properties of lung lectin had suggested that the lectin might function in a cell-surface process, perhaps cell–cell interaction, cell–substratum interaction or as a surface receptor for a regulatory

ligand. We had expected, therefore, to be able to isolate lung lectin from plasma membranes and reciprocally we had expected to locate anti-lectin immunoglobulin-binding sites in plasma-membrane fractions isolated from lung. However, the present experiments suggest that lung lectin is located predominantly intracellularly.

Experimental and results

Materials

[³H]Formaldehyde (sp. radioactivity 85 Ci/mol) and Aquasol-2 were purchased from New England Nuclear, North Billerica, MA, U.S.A. Sodium cyanoborohydride was obtained from Aldrich, Milwaukee, WI, U.S.A., and calf thymus DNA from Sigma, St. Louis, MO, U.S.A. Whatman GF/F filters were from a local supplier.

Immunological procedures

β -Galactoside-binding protein, or lectin, was prepared from rat lung as previously described (Powell, 1980). A goat was injected four times, at weekly intervals, with 0.2–0.3 mg of rat lung lectin in physiological saline (0.9% NaCl; 0.5 ml) suspended in an equal volume of Freund's complete adjuvant. The goat was then subjected to plasmaphoresis twice a week for 3 weeks to yield serum (2 litres) having

Abbreviation used: IgG, immunoglobulin G.

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an anti-lectin titre of 1:12 on Ouchterlony immunodiffusion plates. The serum was precipitated at an $(\text{NH}_4)_2\text{SO}_4$ concentration of 35% and the precipitate further purified by ion-exchange chromatography on DE-52 DEAE-cellulose (Stanworth, 1960). The anti-lectin titre was found to be 1:32 on Ouchterlony immunodiffusion plates. A single precipitin line was observed against lung lectin prepared from adult or immature (12-day-old) rats. Both anti-lectin IgG and pre-immune serum IgG were labelled with [^3H]formaldehyde by reductive methylation performed as described by Jentoft & Dearborn (1979). The specific radioactivity of the labelled IgG varied with the preparation [6405 ± 685 d.p.m./mg of protein (mean $\pm$ s.d.)]. The labelled anti-lectin IgG retained its immunological properties as determined by Ouchterlony immunodiffusion. The anti-lectin IgG-lectin reaction was followed by immunoprecipitation using a nephelometer; we used these data to calculate the amount of specific anti-lectin IgG in the IgG fraction. Unfortunately neither lactose (10 mM) nor thiodigalactoside (1 mM) inhibited immunocomplex formation. The antibody was therefore not directed against the active site of the lectin.

Subcellular fractionation

The fractionation was followed with biochemical markers: ouabain-sensitive ATPase for plasma membranes, cytochrome *c* oxidase for mitochondria and glucose 6-phosphatase for microsomes (Basu *et al.*, 1979), DNA for nuclei (Brunk *et al.*, 1979) and acid phosphatase for lysosomes (Barrett, 1972). Protein was measured by the dye-binding assay described by Bradford (1976). Lectin activity was monitored by haemagglutination (Simpson *et al.*, 1977).

The method of Ryan & Smith (1971) for the isolation of plasma membranes from rat lung was used as the basis of the subcellular fractionation (Scheme 1). Long Evans hooded rats (50–75 g body wt.) were given intraperitoneal injections of sodium pentobarbital (30 mg/kg body wt.) and killed by exsanguination from the abdominal aorta, the lungs removed, trimmed and weighed. The tissue from either group of animals was minced and homogenized in 0.25 M-sucrose containing 100 mM-KCl and 50 mM-Tris/maleate, pH 7.5, (buffer A) using 6 ml/g of tissue. Three strokes in a loose-fitting homogenizer were followed by three strokes in a Potter-Elvehjem homogenizer, with a 0.1 mm clearance. This fractionation scheme yielded reproducible results when immature rat lungs were used; fractionation of adult lungs yielded a more variable preparation, probably because of the large amount of connective tissue in these lungs. The relative enrichment and contamination of the different fractions is shown in Table 1.

The immature rat lungs yielded fractions appro-

priately enriched (4–7-fold) in the markers. Unfortunately all fractions except the nuclei were contaminated with acid phosphatase, which could find its origin in either lysosomes or lamellar bodies.

The fractions obtained from adult lungs presented more variability. The plasma membranes were difficult to obtain free of other membrane contaminants. The main contaminating enzyme of all fractions was again acid phosphatase. The microsomal fractions were too contaminated to be used for decisive experiments. However, excellent mitochondrial and nuclear fractions could be obtained from time to time and were used for binding experiments. The marker characteristics of these fractions are shown in Table 1.

Extraction of lung lectin

It has been observed previously that only approx. 20% of the lectin activity could be solubilized, in the absence of lactose, from adult rat lung (Powell, 1980). Very similar results were observed with Tris/maleate buffer (buffer A) used in the subcellular fractionation scheme. However, in the immature rat lung a different situation prevailed and approx. 70% of the total lactose-inhibitable lectin activity was extracted into the 100 000 g supernatant solution in the absence of lactose, the remainder (approx. 30%) being found in the precipitable fractions (Table 2). The lectin activity was assayed as previously described (Powell, 1980) but serial dilutions from 1:3 and 1:5 were used in addition to 1:4.

The presence of anti-lectin IgG did not result in the loss of lectin from the membrane fractions of adult lung; a minimal loss of lectin (<15%) was observed after the membrane fractions of immature rat lungs were incubated with anti-lectin IgG.

Anti-lectin immunoglobulin binding

Lectin associated with rat lung subcellular fraction was detected by measuring the specific binding of [^3H]anti-lectin IgG. Binding assays were done as follows. The concentration of subcellular fraction was varied in 0.38 ml total volume, containing 1.25 mg of bovine serum albumin/ml and 2.5% poly(ethylene glycol) 6000 in phosphate-buffered saline, pH 7.5. The mixture was incubated with 0.37 mg of [^3H]IgG (20 μl containing 11 μg of specific anti-lectin IgG) for 1 h at 25°C. Bound anti-lectin IgG was separated from unbound product by filtration using GF/F glass-fibre filters. The filters were rinsed with 5 ml of Aquasol-2 added and the radioactivity on each filter determined by scintillation counting. The radioactivity due to non-specific binding was subtracted from experimental values. Control experiments were performed using labelled pre-immune serum and pre-incubation with unlabelled anti-lectin IgG. The apparent association cons-

Scheme 1. *Subcellular fractionation scheme*

tants (K_d) for anti-lectin IgG binding were estimated from the initial slopes of the graph [^3H]-anti-lectin IgG bound/total anti-lectin IgG against the membrane protein concentration. All such plots were biphasic in accord with the bivalency of IgG. The immunoglobulin bound/protein concentration ratio was estimated from the same graphs. The

binding assays were performed on at least three different membrane preparations from each age group of rats.

All the subcellular fractions from immature rat lung showed some specific binding of [^3H]anti-lectin IgG. The plasma membranes, mitochondrial and microsomal fractions showed a similar capacity to

Table 1. *The enrichment of markers in the subcellular fractions of rat lung*

The enrichment was calculated relative to the original tissue homogenate; specific activities were used for enzyme markers and quantification for DNA. For ATPase, the ouabain-sensitive activity was measured; glucose 6-phosphatase was assayed as soon as the fractionation was complete. Abbreviation: n.d., not determined. The diagonal lines emphasize the recognized marker for the particular subcellular fraction.

		Enrichment (relative to original tissue homogenate)				
		ATPase	Cytochrome c oxidase	Glucose 6-phosphatase	DNA	Acid phosphatase
Plasma membrane	Immature	6.0	0.7	0.6	0.6	3.1
	Adult	5.0	3	4	n.d.	n.d.
Mitochondria	Immature	2	6.3	1.2	0.3	2.4
	Adult	1.1	14.8	0	n.d.	n.d.
Microsomes	Immature	1.9	1.4	6.5	0.5	4.4
	Adult	0	1.7	1.8	n.d.	n.d.
Nuclei	Immature	1.7	0	0	3.7	0.5
	Adult	n.d.	2.5	n.d.	3.1	1.4

Table 2. *Lectin extraction from rat lung*

Rat lung (1 g) was disrupted in 6 ml of homogenizing medium using a Polytron homogenizer. Soluble lectin was estimated from the lactose-inhibitable haemagglutination activity of the first 100000g supernatant fraction prepared from 1 g of rat lung. Total lectin was estimated from the cumulative haemagglutination activity yielded from repeated extraction of rat lung (1 g) with 20 mM-sodium phosphate, pH 7.2, containing 0.14 M-NaCl and 100 mM-lactose. Buffer A was the homogenizing medium used for the subcellular fractionation. All buffers contained 20 mM-2-mercaptoethanol.

	Immature rats		Adult rats	
	Soluble lectin (units)	Soluble lectin/ total lectin (%)	Soluble lectin (units)	Soluble lectin/ total lectin (%)
Homogenizing medium				
0.14 M-NaCl/20 mM-sodium phosphate (pH 7.2)	145 ± 15	66	5.6 ± 1	19
0.14 M-NaCl/20 mM-sodium phosphate (pH 7.2) + 20 mM-lactose	184 ± 25	83	24.0 ± 2	80
Buffer A	159 ± 20	74	7.2 ± 1	22
Buffer A + 20 mM-lactose	178 ± 25	82	26.3 ± 2	82

Table 3. *The binding of [³H]anti-lectin IgG to subcellular fractions of rat lung*

The apparent K_a was calculated as the slope of (bound anti-lectin/total anti-lectin) against membrane protein concentration. The amount of anti-lectin IgG bound was calculated from the linear region of the same graph. The results shown above are from a single experiment but similar results were obtained on three or more occasions.

Subcellular fraction	Age of rat	$10^{-3} \times$ Apparent K_a	Anti-lectin IgG bound (μ g/mg of protein)
Plasma membrane	Immature	2	33
	Adult	2	33
Mitochondria	Immature	3	65
	Adult	8	141
Microsomes	Immature	2	52
	Adult	—	—
Nuclei	Immature	<1	<5
	Adult	<1	<5

bind anti-lectin IgG (Fig. 1, Table 3). The nuclear fraction had a very low binding capacity for anti-lectin IgG. The capacity to bind anti-lectin

IgG/mg of protein decreased in the following order: mitochondria > microsomes > plasma membranes > nuclei (Table 3). The apparent binding affinity of

Fig. 1. *The binding of anti-lectin IgG to subcellular fractions prepared from immature rat lung*

The fractional binding of $[^3\text{H}]$ anti-lectin IgG (bound/total) is shown as a function of protein concentration in different fractions: O, mitochondrial enriched fraction; □, plasma-membrane-enriched fraction; Δ, nuclear fraction. Results of a single experiment are shown; separate experiments gave very similar results.

Fig. 2. *The binding of anti-lectin IgG to subcellular fractions prepared from adult rat lung*

The fractional binding of $[^3\text{H}]$ anti-lectin IgG (bound/total) is shown as a function of protein concentration in different fractions: O, mitochondrial enriched fraction; □, plasma-membrane-enriched fraction; Δ, nuclear fraction. Results of a single experiment are shown; separate experiments gave very similar results.

anti-lectin IgG for the nuclear fraction was less than for the other fractions, which had very similar binding affinities (Table 3).

The plasma-membrane fraction was resuspended in a buffer containing 0.1M-lactose, collected by centrifugation and then resuspended in the original buffer at the original concentration. This lactose-treated plasma-membrane fraction had a much diminished capacity to bind anti-lectin IgG; only 30% of the specific anti-lectin IgG binding was observed, although background binding of the pre-immune serum was unchanged.

In the adult rat lung the binding of anti-lectin IgG to membranes was similar to that observed in the immature rat lungs. Again the mitochondria appeared to bind more anti-lectin IgG/mg of protein than the plasma membrane (Fig. 2), whereas a low level of binding was observed to the nuclear fraction. (The 'microsomal' fractions also bound large amounts of anti-lectin IgG.) The apparent binding affinity of anti-lectin IgG to the adult lung membranes was very similar to that observed in immature rat lung membranes (Table 2). Again lactose-treated plasma membranes lost 70% of their binding sites for anti-lectin IgG.

Discussion

The lung is a complex organ composed of more than forty different cell types. Perhaps this provides an explanation for the scant literature on subcellular fractionation of the lung. A procedure has been published for the purification of plasma membranes from rat lung (Ryan & Smith, 1971) and we used this procedure as the basis of our fractionation scheme. By using both immature and adult rat lungs we were able reproducibly to obtain subcellular fractions enriched 5–15-fold in specific biochemical markers. The enzyme profiles permitted discrimination between plasma-membrane-enriched fractions and intracellular fractions, although we provide no morphological evidence.

Previous experiments with β -galactoside-binding protein from adult rat lungs have shown that most of the lectin activity remained associated with the membranous fractions of lung homogenates if lactose was not included in the homogenizing medium (Powell, 1980). Little lectin activity could be recovered from the low-speed precipitates enriched in connective tissue elements. We, therefore, inferred that the lung lectin was associated with a membranous structure through one of its galactoside-binding sites. This hypothesis would be strengthened if we could demonstrate the specific binding of anti-lectin IgG to lung membrane fractions and its abolition in the presence of lactose.

We did observe specific binding of anti-lectin IgG to plasma-membrane-enriched fractions of both

immature and adult rat lungs (Figs. 1 and 2). The dose-response curve was non-linear, which is in accordance with the bivalency of immunoglobulin G. Association constants were estimated by using data in the linear region of the binding curves; they were closely similar for both groups of animals. Pre-incubation of the plasma-membrane fractions with lactose caused a marked reduction (>70%) in the specific binding of anti-lectin IgG, but little reduction in the background non-specific binding.

Subcellular fractions enriched in nuclei bound anti-lectin IgG very poorly but other intracellular membrane fractions, mitochondria and microsomes, consistently bound more anti-lectin IgG/mg of protein than did the plasma-membrane fractions (Table 3). The estimated association constants of the anti-lectin IgG for these intracellular membranes were very similar to those for binding to plasma membranes. Therefore it seemed probable that the anti-lectin IgG was binding to the same protein moiety in the different membrane fractions rather than binding to degraded fragments of the β -galactoside-binding protein. This evidence suggests that lung lectin may have a broad but predominantly intracellular distribution.

Immature rat lung has an 8–10-fold greater concentration of lectin activity than adult rat lung (Powell & Whitney, 1980). The β -galactoside-binding proteins purified from these two groups of animals appear to be biochemically and immunologically identical. Why, therefore, was there not a much greater amount of specific antibody binding to the membranes from immature rat lungs? The loss of antibody-binding sites after incubation with lactose argued against the binding of a non-specific immunoglobulin. The answer proved simple. Most of the lectin activity (approx. 70%) in immature rat lung homogenates was obtained in supernatant fractions, even in the absence of lactose (Table 2). The lectin activity was still dependent on the presence of exogenous thiol reducing agents, so we suppose that in the immature rat lung most of the lectin is a cytoplasmic protein rather than a soluble component of the interstitium.

These data strongly suggest that β -galactoside-binding protein, or lectin, has a predominantly intracellular location. The adult lung lectin appeared

to be associated, by a galactoside-binding site, with the membranes of microsomes, mitochondria and plasma membranes. In the immature rat lung much of the lectin was not membrane-associated and was either a free cytoplasmic protein or included within vesicles.

Immunofluorescence experiments with cultured chick embryo fibroblasts (Briles *et al.*, 1979) and with chick myoblasts (Nowak *et al.*, 1977; Podleski & Greenberg, 1980) have also suggested that the β -galactosyl lectins associated with these cells are predominantly intracellular. Although a small proportion of the lectin appears to be associated with the plasma membrane it seems unlikely that the β -galactoside-binding protein functions in extracellular processes unless it mediates the internalization of a ligand. We should perhaps seek an intracellular role for the β -galactoside-binding protein, possibly in glycoprotein transport.

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