Biochem. J. (1984) **223**, 769–774
Printed in Great Britain

Endogenous ligands of rat lung β-galactoside-binding protein (galaptin) isolated by affinity chromatography on carboxyamidomethylated-galaptin–Sepharose

Janet T. POWELL*‡ and Philip L. WHITNEY†
**Department of Biochemistry, Charing Cross Hospital Medical School, Fulham Palace Road, London W6 8RF, U.K., and †Division of Pulmonary Medicine (R120), University of Miami School of Medicine, P.O. Box 016960, Miami, FL33101, U.S.A.*

(*Received 10 May 1984/Accepted 13 July 1984*)

Rat lung β-galactoside-binding protein (galaptin) is developmentally regulated during postnatal lung development. In common with other vertebrate galaptins, it is very labile when purified and dependent on the presence of exogenous thiol reagents. Reaction of rat lung galaptin with iodoacetamide resulted in a stable active carboxyamidomethylated galaptin that could be coupled to Sepharose. The resultant affinity matrix bound asialoglycoproteins, and these could be quantitatively eluted with disaccharide haptens. The carboxyamidomethylated-galaptin–Sepharose affinity matrix was used to search for endogenous ligands in 13-day-rat lung. Cytosolic fractions of developing rat lung contained no moieties that could be specifically eluted with disaccharide hapten. Only when membranous fractions were extracted with 1% Triton were glycoproteins solubilized that bound to the affinity matrix and could be specifically eluted with disaccharide hapten. The eluted glycoproteins were potent inhibitors of galaptin binding to asialo-orosomucoid. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis identified these glycoproteins as being of high M_r, with three components of M_r 160000–200000 and a smaller component of M_r 75000. This is the first evidence for specific membrane-associated glycoproteins being the ligands of rat lung galaptin.

The lung, in common with many other tissues, shows marked developmental regulation of a β-galactoside-binding protein, the maximum concentration coinciding with the peak of alveolarization of the lung (Powell & Whitney, 1980). Similar β-galactoside-binding proteins (also termed 'galaptins') that are developmentally regulated have been described in diverse species and in many tissues (Barondes, 1981). These galaptins are characterized by their lectin activity in haemagglutination. The molecular properties of these vertebrate galaptins are strikingly similar from chicken to cow to man (Barondes, 1981). Immunocytochemistry has been used to demonstrate their presence within a large number of cell types, and extracellularly (Podleski & Greenberg, 1980; Beyer & Barondes, 1980; Cerra *et al.*, 1984). Although it has been postulated that these galaptins may function in cellular-recognition and -adhesion phenomena, there is scant evidence to support this. Indeed there is little evidence, other than circumstantial, that these galaptins have any biochemical or biological function.

To what endogenous ligands do these galaptins bind and why? Here we present an approach to defining the endogenous ligands of rat lung galaptin during postnatal lung development. We describe a simple chemical-modification procedure to obtain a stable galaptin derivative. We then describe use of this derivative as an affinity-chromatography ligand to isolate endogenous galaptin-binding moieties from developing rat lung.

Abbreviations used: CAM, carboxyamidomethyl(ated); SDS, sodium dodecyl sulphate.

‡ To whom correspondence and requests for reprints should be addressed.

Experimental procedures

Rat lung galaptin was prepared essentially as previously described (Powell, 1980), but we used 13-day-rat lung as the source material to optimize yields. The galaptin was concentrated on a column of lactosyl-Sepharose (Levi & Teichberg, 1981).

The galaptin migrated as a single band when subjected to polyacrylamide-gel electrophoresis by the method of Laemmli (1970). Routinely the galaptin was assayed by haemagglutination with unfixed trypsin-treated rabbit erythrocytes (Simpson *et al.*, 1977; Powell, 1980). In chemical-modification experiments the galaptin was also assayed by the binding of ^{125}I-asialo-orosomucoid fragments (25μg, 10μCi) as previously described (Powell, 1980). Briefly, galaptin was incubated with 25μg of labelled glycosylated asialo-orosomucoid fragment and inhibitor (where appropriate) at 25°C for 30min in a total volume of 0.2ml. Controls were incubated with thiodigalactoside (5mM). Galaptin–asialo-orosomucoid-fragment complexes were collected by precipitation with poly(ethylene glycol), washed, and analysed for ^{125}I radioactivity in a Packard Autogamma counter. The assays were performed in triplicate and were linear with 0–5μg of both galaptin and carboxyamidomethylated galaptin.

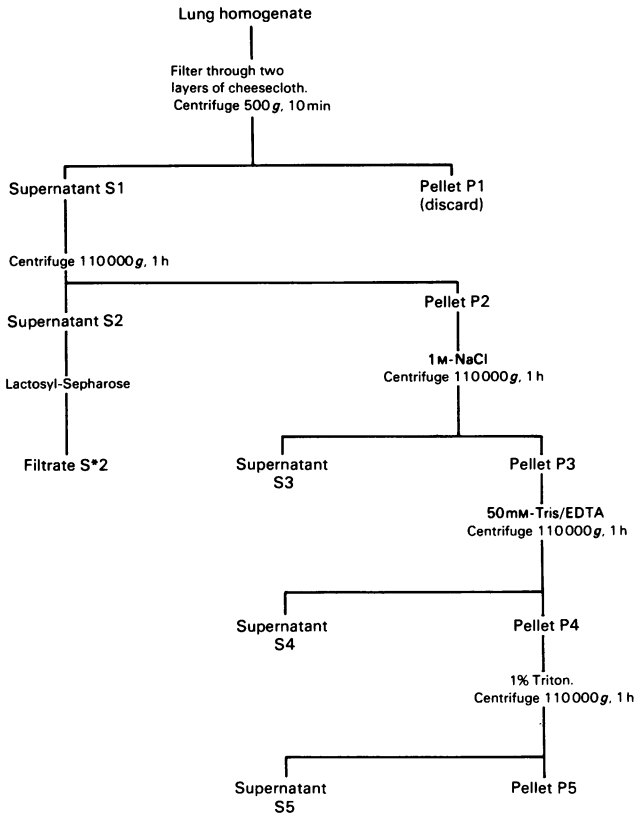
Carboxyamidomethylation was performed with iodoacetamide during elution from an affinity column. Rat lung galaptin was applied to a column of lactosyl-Sepharose (1ml) in 20mM-sodium phosphate, pH7.4, containing 0.14M-NaCl and 20mM-2-mercaptoethanol. The loaded column was washed with 10vol. of the same buffer and then with a further 10vol. of the same buffer devoid of 2-mercaptoethanol. The galaptin was eluted sharply with 50mM-Tris, pH8.6, containing 0.1M-lactose and 0.1M-iodoacetamide. Lactose and unchanged iodoacetamide were removed by dialysis against phosphate-buffered saline (7.2g of NaCl, 1.48g of Na_2HPO_4 and 0.43g of KH_2PO_4/litre). Carboxyamidomethylated galaptins (CAM-galaptin) can be prepared directly from the final concentrating column of lactosyl-Sepharose; when it was prepared in this manner the yield was 160μg of CAM-galaptin/g of lung.

The modified galaptin was analysed by molecular-sieve chromatography on a column (1cm × 50cm) of Bio-Gel P100 equilibrated with phosphate-buffered saline, gel electrophoresis and amino acid analysis.

The CAM-galaptin was coupled to Sepharose at pH8.5 by the method of March *et al.* (1974). The capacity of the matrix was determined by the binding of ^{125}I-asialo-orosomucoid. The affinity matrix bound 125μg of asialo-orosomucoid/ml of packed gel, and this could be quantitatively eluted with buffers containing 0.1M-lactose or 10mM-thiodigalactoside. The affinity matrix also bound asialofetuin. Since the matrix was to be used with crude lung homogenates, the risk of ligand degradation was high, even in the presence of proteinase inhibitors. After each cycle of use the binding capacity of the matrix was assessed with ^{125}I-asialo-orosomucoid; after three cycles the affinity matrix bound 78μg of asialo-orosomucoid/ml, and after six cycles the matrix bound only 33μg of asialo-orosomucoid/ml of packed gel. This diminution in binding activity of the matrix could result from ligand degradation or from tightly bound moieties. However, when the columns were used with crude homogenates in the absence of proteinase inhibitors, only 35% of the binding activity remained after three cycles. Inactive columns were washed with urea (4M). They could not be regenerated and no specific components were eluted. Columns were only used when they had a binding capacity of more than 75μg of asialo-orosomucoid/ml.

Rat lung homogenates were prepared from 13-day-old rats and fractionated as shown in Scheme 1. Briefly, rats were given an intraperitoneal injection of sodium pentobarbital and killed by exsanguination from the abdominal aorta with simultaneous saline cardiac perfusion; this procedure yielded essentially blood-free lungs. The lungs (total wt. 2.5–3g) were removed and minced with scissors before disruption with a Polytron homogenizer in 20mM-sodium phosphate, pH7.2 (25ml), containing 0.14M-NaCl, 10mM-2-mercaptoethanol, *N*-ethylmaleimide (10μg/ml) and phenylmethanesulphonyl fluoride (10μg/ml). The pellet obtained by centrifugation at 500g for 10min was discarded and the supernatant (S1) centrifuged at 25000rev./min ($g_{max.}$ 110000) for 1h. (All further extraction buffers contained *N*-ethylmaleimide and phenylmethanesulphonyl fluoride; extractions were made with the Polytron homogenizer and all homogenates were centrifuged at 110000$g_{max.}$ for 1h). Galaptin was removed from the supernatant (S2) by passage over lactosyl-Sepharose to yield the filtrate (S*2). The pellet (P2) was re-extracted first with high-ionic-strength buffer (20ml of 20mM-sodium phosphate, pH7.2, containing 1M-NaCl) and then with the same volume of 50mM-Tris, pH7.8, containing 1mM-EDTA; this yielded the supernatant solutions S3 and S4 and a pellet, P4 (see Scheme 1). Finally the pellet P4 was extracted with 20ml of 50mM-Tris, pH7.8, containing 1mM-EDTA and 1% Triton X-100 to yield the supernatant solution S5 and a precipitate P5.

The supernatant fractions (S*2, S3, S4 and S5) were subjected to chromatography on CAM-galaptin–Sepharose (2ml columns binding 250μg of asialo-orosomucoid). Columns were washed with the appropriate extration buffers (10–20vol.) before elution by the same extraction buffer to which thiodigalactoside (10mM) was added. Protein was determined by the method of Bradford (1976), the procedure described by Hudgin *et al.* (1974) being used to remove Triton X-100 where necessary.



Scheme 1. *Lung fractionation*

Isolated glycoproteins were analysed by molecular-sieve chromatography on a column (1 cm × 45 cm) of Sephacryl 300 equilibrated with 50 mM-Tris, pH 7.8, containing 1 mM-EDTA and 0.2% Triton X-100, and by electrophoresis in 0.1%-SDS/6.5%-acrylamide gels using cross-linked bovine serum albumin as an M_r marker. Acrylamide gels were stained with Coomassie Brilliant Blue for protein and periodate/Schiff stain for carbohydrate (Glossman & Neville, 1971).

Results

Properties of CAM-galaptin

The activities of CAM-galaptin and pure rat lung galaptin were identical in the haemagglutination assay. CAM-galaptin and pure rat lung galaptin bound, per μg of protein, 0.82 μg and 0.78 μg of asialo-orosomucoid fragment respectively. The hapten inhibition of CAM-galaptin and pure rat lung galaptin was very similar (see Table 1). CAM-galaptin was eluted coincidentally with native rat lung lectin on Bio-Gel P100 molecular-sieve chromatography to give an estimated M_r of 30000. CAM-galaptin migrated as a single band (M_r 14000) on SDS/polyacrylamide-gel electrophoresis. Amino acid analysis showed that CAM-galaptin contained four to five modified cysteine residues per subunit (P. L. Whitney & J. T. Powell, unpublished work). CAM-galaptin was stable as determined by haemagglutination over a pH range of 4–9 for a period of 2 h at 4°C. CAM-galaptin retained 95% of its activity after 24 h at

Table 1. *Hapten inhibition of rat lung galaptin and CAM-galaptin*

C_{50} is the concentration of hapten required to inhibit the binding (by 50%) of asialo-orosomucoid fragment (25 μg) to galaptin (2 μg).

Hapten	C_{50} (mM) Lectin	CAM-lectin
Galactose	30	30
Lactose	2.8	3.0
Thiodigalactoside	0.45	0.4
Asialofetuin	0.28	0.32
CAM-galaptin-binding protein (reducing termini)	0.02	0.04

16°C. The CAM-galaptin could be stored at 4°C after freeze-drying.

Affinity chromatography on CAM-galaptin–Sepharose

The fractionation provided four supernatant fractions (S*2, S3, S4 and S5) for analysis, the yields being given in Table 2. The supernatant fractions S*2, S3 and S4 contained soluble components extracted with 0.14M-NaCl high-ionic-strength and low-ionic-strength buffers respectively. These extraction procedures were designed to separate galaptin from soluble components and loosely associated membrane components. At each stage, membranous components were precipitated. Only fraction S5 was obtained by detergent extraction and therefore only fraction S5 is likely to contain integral membrane components.

Fractions S*2, S3 and S4 did not contain any glycoconjugates binding to CAM-galaptin–Sepharose that could be specifically eluted with thiodigalactoside. In contrast, supernatant S5 (the only supernatant to contain detergent) contained glycoproteins that bound to CAM-galaptin–Sepharose and could be specifically eluted with thiodigalactoside. From a starting pooled lung weight of 2.5g, a fraction containing 210μg of protein was eluted from a column with a maximal binding capacity of 195μg of asialo-orosomucoid.

Five separate experiments confirmed that S*2, S3 and S4 contained no components that could be eluted from CAM-galaptin–Sepharose with thiodigalactoside. In contrast, supernatant solution S5 always contained glycoprotein(s) that could be isolated on CAM-galaptin–Sepharose; the yields were 210, 160, 185, 90 and 105μg of protein in five separate experiments. After dialysis to remove thiodigalactoside, the glycoprotein(s) were re-applied to CAM-galaptin–Sepharose (1ml) and could again be eluted with thiodigalactoside, but in much reduced yield (90, 75, 30, 25 and 40μg respectively).

Table 2. *Yields of the supernatant fractions (described in Scheme 1) from 2.6g of lung*

Fraction	Volume (ml)	[Protein] (mg/ml)	Haemagglutination titre
S2	12.2	22.6	1024
S*2	25.6	11.3	4
S3	18.2	2.4	32
S4	17.6	3.5	8
S5	17.5	9.8	—

Properties of glycoproteins binding to CAM-galaptin–Sepharose

The glycoprotein fraction isolated by affinity chromatography on CAM-galaptin–Sepharose was a potent inhibitor of native rat lung galaptin as determined by asialo-orosomucoid-fragment-binding assay (Table 1). The glycoprotein fraction consisted of four main components that could be separated by molecular-sieve chromatography (on Sephacryl 300) or by polyacrylamide-gel electrophoresis. Molecular-sieve chromatography revealed two peaks of galaptin-inhibitory activity (Fig. 1): a broad inhibition peak was observed (M_r 190000$\pm$30000) and a sharper peak (M_r 80000) (the amount of protein was too small for reliable assay in the presence of Triton). In each experiment the profile obtained on SDS/polyacrylamide-gel electrophoresis was very similar. Three high-M_r (200000–160000) components were observed, along with a smaller component (M_r 75000); each component stained for both protein and carbohydrate. Comparative densitometric traces from three experiments are shown in Fig. 2. In one of the five separate experiments a discrete small protein component was observed (Fig. 2*a*); this was perhaps a result of degradation (see below).

Initial experiments were performed in the absence of proteinase inhibitors. Under these conditions, glycoproteins from supernatant S5 could be specifically eluted from CAM-galaptin–

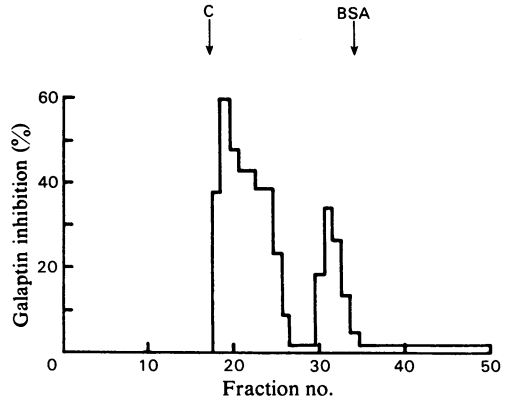


Fig. 1. *Molecular-sieve chromatography of glycoproteins isolated on CAM-galaptin–Sepharose*

The column (1cm x 45cm) of Sephacryl 300 was monitored by determining the inhibitory effect of aliquots (50μl) of each fraction on the binding of rat lung galaptin (2μg) to labelled asialo-orosomucoid fragment (25μg). The amount of protein was too small to enable reliable determination in the presence of Triton X-100. Reference points mark the elution of catalase (C; M_r 260000) and bovine serum albumin (BSA; M_r 68000).

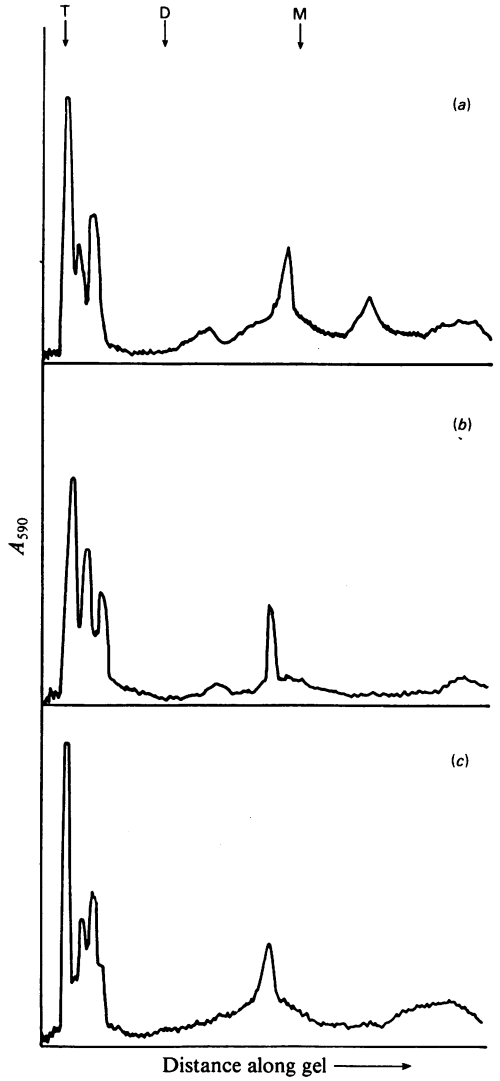


Fig. 2. *Densitometer tracings of polyacrylamide gels loaded with the glycoprotein fraction eluted from CAM-galaptin–Sepharose*

(*a*), (*b*) and (*c*) are the results of experiments with three separate lung homogenates. The gels (6.5% acrylamide/0.1% SDS) were stained for protein with Coomassie Brilliant Blue, and a calibration scale taken from the comparative migration of cross-linked bovine serum albumin [monomer (M) M_r 68000; dimer (D) M_r 136000; trimer (T) M_r 204000)] is shown at the top.

Sepharose with thiodigalactoside, and SDS/polyacrylamide-gel electrophoresis revealed the presence of seven or more bands (M_r 90000–15000), with a band of M_r 75000 being prominent.

Discusssion

The galaptins are a group of developmentally regulated, β-galactosyl-specific, vertebrate lectins. These properties might argue for the galaptins playing a role in recognition processes at a time of rapid cell proliferation and differentiation. However, currently there is scant evidence as to the endogenous ligands or biological function of these galaptins. This lack of information can, in part, be attributed to the lability of the isolated galaptins. The purified galaptins require the presence of high concentrations of exogenous thiol reagents, dithiothreitol or mercaptoethanol, to maintain their lectin activity. This lability of the galaptins has precluded the identification of endogenous ligands and the raising of antibodies to the saccharide-binding site of galaptins.

No galaptin is present in newborn-rat lung, and a surge of galaptin expression is coincident with the peak of alveolarization of the lung. At this time (10–13 days after birth), the galaptin is readily soluble when lung is homogenized in the absence of saccharide haptens (Sanford *et al.*, 1982*a*). In the adult-rat lung the levels decline and the presence of a competing hapten (lactose) in homogenization buffers is essential for galaptin extraction from rat lung. Immunocytochemical techniques have demonstrated galaptin both intracellularly (smooth muscle and alveolar cells), and extracellularly associated with elastic fibres in adult rat lung (Cerra *et al.*, 1984).

We have circumvented the problem of galaptin lability by a simple chemical-modification procedure. Carboxyamidomethylation, under non-denaturing conditions, provides a stable, active, rat lung galaptin (Sanford *et al.*, 1982*b*). This derivative of rat lung galaptin, CAM-galaptin, has the same molecular characteristics as the native galaptin, having a dimeric structure of M_r 30000. Further, the chemical modification has little effect on the saccharide specificity (Table 1). When coupled to Sepharose the galaptin retained at least one accessible saccharide-binding site as determined by its ability to bind asialoglycoproteins, which could be specifically eluted with lactose or thiodigalactoside.

The fractionation procedure for 12-day-rat lungs (Scheme 1) was designed to separate galaptin from other soluble lung components before removing components loosely associated with membranes or connective tissue. No soluble cytoplasmic or loosely associated membranous proteins were isolated on CAM-galaptin–Sepharose.

When the CAM-galaptin–Sepharose was used, specific glycoproteins were only isolated from detergent extracts. These endogenous glycoprotein ligands are therefore membrane-bound components. The stoichiometry of the binding of these

glycoproteins to CAM-galaptin–Sepharose suggests that they are larger than CAM-galaptin. These glycoproteins isolated on CAM-galaptin–Sepharose were more potent inhibitors of galaptin–asialo-orosomucoid binding than other haptens tested by an order of magnitude (Table 1). Analysis of these glycoproteins by polyacrylamide-gel electrophoresis in the presence of SDS showed the principal component to have M_r 200000, with three less prominent components of M_r 190000, 160000 and 75000. The smallest component could be resolved by chromatography on Sephacryl 300; both large and small components inhibited galaptin–asialo-orosomucoid binding, but the quantities available were insufficient to determine separate inhibition constants. It seems possible that the three smallest components result from degradation of the principal component, since, when experiments were performed in the absence of proteinase inhibitors, only low-M_r components were isolated, the 75000-M_r component being prominent.

CAM-galaptin–Sepharose appears to be a useful probe in determining the endogenous ligands of lung galaptin, despite its limitation to components that can be solubilized. Specific membrane-bound glycoproteins have been identified as potent inhibitors of rat lung galaptin and probable endogenous ligands. The characterization and localization of these endogenous galaptin inhibitors should provide insight as to the biological function of lung galaptins.

Part of this work was supported by a grant (HL 24261) from the National Institutes of Health.

References

Barondes, S. H. (1981) *Annu. Rev. Biochem.* **50**, 207–231

Beyer, E. C. & Barondes, S. H. (1980) *J. Supramol. Struct.* **13**, 219–227.

Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254

Cerra, R. F., Haywood-Reid, P. L. & Barondes, S. H. (1984) *J. Cell Biol.* **98**, 1580–1589

Glossman, H. & Neville, D. M. (1971) *J. Biol. Chem.* **246**, 6339–6346

Hudgin, R. L., Pricer, W. E., Ashwell, G., Stockert, R. J. & Morrell, A. G. (1974) *J. Biol. Chem.* **249**, 5536–5543

Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

Levi, A. & Teichberg, V. I. (1981) *J. Biol. Chem.* **256**, 5737–5740

March, S. C., Parikh, I. & Cuatrecasas, P. (1974) *Anal. Biochem.* **60**, 149–152

Podleski, T. R. & Greenberg, I. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1054–1058

Powell, J. T. (1980) *Biochem. J.* **187**, 123–129

Powell, J. T. & Whitney, P. L. (1980) *Biochem. J.* **188**, 1–8

Sanford, G. L., Davis, D. L. & Powell, J. T. (1982*a*) *Biochem. J.* **204**, 97–102

Sanford, G. L., Whitney, P. L. & Powell, J. T. (1982*b*) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41**, 898

Simpson, D. L., Thorne, D. R. & Loh, H. H. (1977) *Nature (London)* **226**, 367–369