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Evidence against lung galaptin being important to the synthesis or organization of the elastic fibril

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Previously it has been suggested that galaptin, an endogenous β-galactoside-binding lectin, may function in the organization of lung elastic fibres. Galaptin was not present in preparations of rat or porcine lung elastic fibrils, neither did it bind to any of the fibril-associated proteins when these were separated by SDS/polyacrylamide-gel electrophoresis. Elastin and galaptin synthesis and secretion were investigated in lung fibroblast cultures and in anatomically preserved slices from developing rat lung. In both systems the synthesis and secretion of elastin was unmodified by the presence of β-galactosides or antigalaptin in the culture medium. The synthesis of galaptin was unmodified by the presence of anti-elastin or β-aminoproprionitrile in the culture medium. Cultured fibroblasts secreted elastin but only trivial amounts of galaptin. When cultures were treated with iodoacetamide (10^{-5} M) galaptin synthesis was maintained but elastin synthesis ceased. These results argue against galaptin having an important role in the synthesis, secretion or organization of the elastic fibril.

INTRODUCTION

A protein which is highly conserved between species and has a widespread distribution would appear to have an important biological function. These attributes apply to the small soluble, β-galactoside-specific mammalian lectins, here termed galaptins. These galaptins also appear to be developmentally regulated and it has been proposed that they may function in the organization of extracellular glycoconjugates [1]. However, whilst this is an area of active research, their function remains unknown.

Originally a galaptin with a subunit molecular mass of 13–14 kDa was described in different species and organs [2–5] but now it is obvious that a family of galaptins, with overlapping saccharide specificity, exist [6–8]. Immunocytochemical studies have suggested that the smallest galaptin (13–14 kDa) is localized predominantly extracellularly [1,9]. In rat lung, immunocytochemical studies with electron microscopy have suggested that the galaptin is localized along elastic fibres [10]. This framework of elastic fibres is rapidly synthesized during the alveolarization of the lung [11] and in the rat peak elastin synthesis probably occurs between days 7 and 12 postnatally [12]. Crosslinking of elastin appears to occur more slowly and the desmosine plus isodesmosine content of lung increases steadily from day 7 to day 21 [13]. The time period of most rapid cross-linking coincides with peak concentrations and synthesis of galaptin [13,14]. It has also been observed that galaptin appears to be a differentiation marker for elastic chondrocytes from bovine ligamentum nuchae in culture: galaptin anti-sera bind strongly to the chondrocyte surface when the cells become competent to secrete elastin (R. Mecham, personal communication).

With this information it seemed reasonable to postulate that the predominant β-galactoside-binding protein or galaptin (13 kDa) in lung interacted with glycoconjugates associated with elastic fibres. Elastin itself is not glycosylated but many of the microfibrillar glycoproteins are candidate ligands for galaptin. The experiments reported here do not suggest that galaptin has an important extracellular function in the organization of elastic fibrils.

MATERIALS AND METHODS

All materials for tissue culture were obtained from Gibco–BRL (Paisley, Scotland), radiochemicals were from Amersham U.K. and other chemicals were of the best available grade. Second antibodies were from Dakopatts (High Wycombe, U.K.). Gel electrophoresis was performed with the Pharmacia PHAST system.

Porcine lung elastic fibrils were prepared by the method of Richmond [15]. Rat lung elastic fibrils were prepared by the method of Ross & Bornstein [16]. Galaptin was prepared from porcine and rat lungs by affinity chromatography on lactosyl-Sepharose and the products reacted with iodoacetamide to maintain their activity [17]. Galaptin was iodinated by using Na[^{125}I] and Iodogen surface catalyst [18]. The most efficient labelling was obtained after denaturation of carboxyamidomethylated galaptin. Galaptin (100 μg) was dissolved in 4 M deionized urea containing 20 mM-sodium phosphate, pH 7.4 (400 μl), and allowed to stand at room temperature for 2 h before iodination. After iodination the labelled galaptin was separated from excess Na[^{125}I] and urea by chromatography on Sepharose G-25 (1 cm × 25 cm). The labelled protein was then dialysed overnight against 20 mM-sodium phosphate, pH 7.4, to complete renaturation. Elastin was prepared from rat aorta using cyanogen bromide extractions [19].

Anti-sera to galaptin and α-elastin were raised in rabbits and assayed by double immunodiffusion and

Abbreviation used: ELISA, enzyme-linked immunosorbent assay.

enzyme-linked immunosorbent assay (ELISA). For the production of anti-serum to galaptin, rabbits (New Zealand cross-lops) were initially immunized with 150 μg of carboxyamidomethylated galaptin [17] in complete Freund's adjuvant distributed between three intradermal sites. Rabbits were boosted at weekly intervals for a further 3 weeks with 100 μg of carboxyamidomethylated galaptin in complete Freund's adjuvant distributed between two intradermal sites along the back. Peak titre anti-serum was obtained 5–8 weeks after initial inoculation. For the production of anti-serum to α-elastin, rabbits were initially immunized with 400 μg of α-elastin in complete Freund's adjuvant distributed between three intradermal sites. Rabbits were then boosted at weekly intervals for 4 weeks with 150 μg of α-elastin in complete Freund's adjuvant intradermally. The titre of anti-serum was maintained by continued boosts of 100 μg of α-elastin given intradermally at 3-weekly intervals. For galaptin ELISA the plates were coated with porcine galaptin (10 μg/ml) and for elastin ELISA the plates were coated with α-elastin (10 μg/ml). Rabbits immunized with galaptin produced precipitating antibodies, which were further purified by ion-exchange and affinity chromatography. The serum was fractionated with Na_2SO_4 and the 9–14% precipitate (25 °C) was resuspended and dialysed against 50 mM-sodium phosphate, pH 7.7, overnight. The IgG-enriched fraction was then applied to a column (2 cm × 35 cm) of DE-52 equilibrated with 50 mM-sodium phosphate, pH 7.7. The fractions passing directly through the column were pooled and applied, in batches, to a column (8 ml) of carboxyamidomethylated galaptin-Sepharose (0.2 mg of galaptin/ml of gel). Affinity-purified anti-serum was eluted with 0.1 M-glycine, pH 2.5, containing 25% polyethylene glycol (PEG 4000). One rabbit (out of three) immunized with α-elastin also produced an anti-serum which formed immunoprecipitates and this anti-serum was used without further purification, but its specificity was tested in Western blots. These Western blots used the culture media of rat aortic smooth-muscle cells and rat lung fibroblasts cultured in the presence of 25 μg of β-aminoproprionitrile/ml. The anti-sera reacted with major components at 77 and 71 kDa and minor components at 68 kDa and 60 kDa suggesting that it reacted with tropoelastin and its degradation products [20].

Rat lung fibroblasts were cultured in Dulbecco's Modified Eagles Medium supplemented with 10% fetal-calf serum and were obtained from explant cultures of lungs from rats aged 4, 7, 10, 16 and 22 days. Porcine lung fibroblasts were obtained from explant cultures of porcine lung (30 kg pig). Rat lung slices were prepared from agar-inflated lungs of different age rats. The lungs were inflated with 1% agar (Sigma type VII) at 38 °C and 20 cm H_2O (196 Pa) pressure. The inflated lungs were rapidly chilled to 4 °C to allow the agar to set. The lungs were then sectioned into 50 μm-thick slices and these slices were allowed to adhere to 35 mm dishes whilst being just covered with CMRL 1066 medium (complex tissue culture medium) supplemented with 10% fetal-calf serum. The slices were allowed to adhere and equilibrate overnight at 37 °C. The medium and non-adherent slices were aspirated and adherent slices treated with 1% agarase (Sigma A6162) in CMRL for 2 h. The agarase was removed and the slices just covered in CMRL 1066 containing 10% fetal-calf serum and radiolabelled amino acids or anti-sera or other supplements as necessary. The following concentrations of supplements were used in both lung slices and fibroblast cultures: β-aminoproprionitrile (25 μg/ml), lactose (5 mM), anti-galaptin (1:100), anti-elastin (1:50), [^{14}C]phenylalanine (0.6 mM, 5 μCi/μM). [^{14}C]valine (1.0 mM, 5 μCi/μM). The slices (10–12/dish) were incubated overnight under the different conditions; the medium was removed for the determination of secreted proteins and the slices washed 3 times with ice-cold phosphate-buffer saline (containing 7.2 g of NaCl, 1.48 g of NaH_2PO_4 and 0.43 g of KH_2PO_4 per litre, pH 7.2) and fixed in ice-cold 5% trichloroacetic acid. Proteins were extracted into 0.2 M-NaOH and DNA and then extracted by using 2 M-NaOH. The protein extract was rapidly neutralized with 6 M-HCl. Residual insoluble elastin was extracted by the Lansing method [21] and estimated by scintillation counting. DNA was measured by using a fluorimetric determination method [22], protein, by the Coomassie dye-binding assay [23] and total protein synthesis, by scintillation counting. In the aspirated medium galaptin and elastin were determined by ELISA and by immunoprecipitation using Protein A with determination of radioactivity in the immunoprecipitate. The specificity of the immunoprecipitation was checked by Western blotting. Cellular proteins were estimated in a similar manner.

Similar experiments were conducted using fibroblasts at passage three, cultured in 35 mm dishes. In these experiments acid phosphatase [24] and lactate dehydrogenase [25] were also determined both in the medium and in lysed cells as an index of cell number. After removal of the medium, cells were washed in phosphate-buffered saline containing 5 mM-lactose to remove cell-surface galaptin and the cells were lysed with 10 mM-Tris/Cl^-, pH 7.5, containing 0.25% Triton X-100 and 1 mM-dithiothreitol for the determination of intracellular proteins. The dishes were then extracted with 4 M-urea to extract galaptin from remaining extracellular matrix. After treatment with 4 M-urea the culture dishes did not stain with peroxidase-conjugated anti-galaptin. Galaptin, elastin and DNA were quantified in the same way as tissue slices.

Fibroblasts from explants of 6–7-day-old rat lungs were seeded at passage three into 35 mm dishes for stress experiments with thiol-reactive agents. Confluent cultures were rinsed 3 times with phosphate-buffered saline and exposed to iodoacetamide (10^{-5} or 10^{-6} M) in Dulbecco's Modified Eagle's Medium containing 2% fetal-calf serum. After 4 h the cells were radiolabelled with 100 μCi of [^{35}S]methionine/ml or 1 μCi of [^{14}C]phenylalanine/ml for a further 4–6 h.

RESULTS

Elastic fibrils

The proteins and glycoproteins of pig lung elastic fibrils were separated by dialysis (at pH 8.0 and 4.5) and washing with urea and SDS [15]. These were then analysed in an 8–25%-gradient polyacrylamide gel in the presence of SDS (Fig. 1) and blotted onto nitrocellulose for 4 h to ensure transfer of high molecular mass proteins. The blot was not stained by sequential incubation with anti-galaptin, peroxidase-conjugated goat anti-(rabbit IgG) serum and diaminobenzidine. The blots were also probed with porcine lung ^{125}I-galaptin and again no

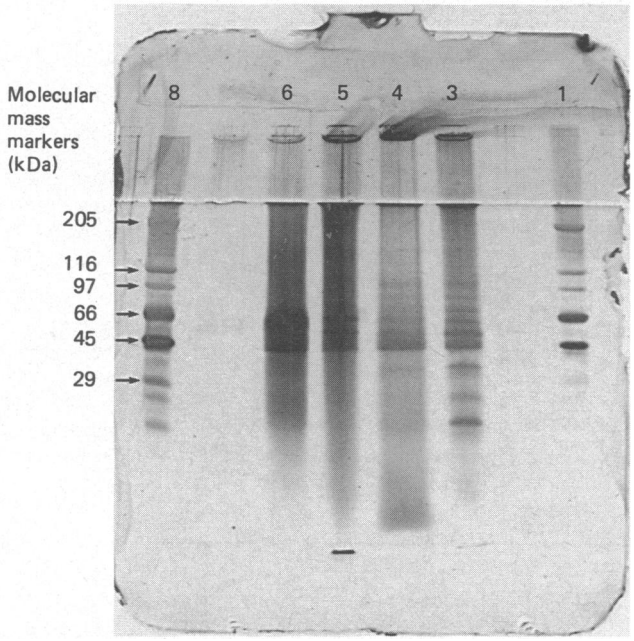

Fig. 1. Elastic fibril components separated by SDS/polyacrylamide-gel electrophoresis

The different fractions were prepared from elastic fibrils according to Richmond [15] and run in an 8–25 % gradient gel followed by silver staining. Lanes 1 and 8 carry molecular mass markers, lane 3 external acidic glycoproteins, lane 4 mesoacidic glycoproteins, lane 5 elastic fibres and lane 6 elastin-rich fractions. The nomenclature is taken from Richmond [15]. The prominent band at ~ 45000 Da present in all lanes bound ^{125}I-concanavalin A. The external acidic glycoproteins and mesoacidic glycoproteins, obtained by extracting fibrils, appear to be very similar.

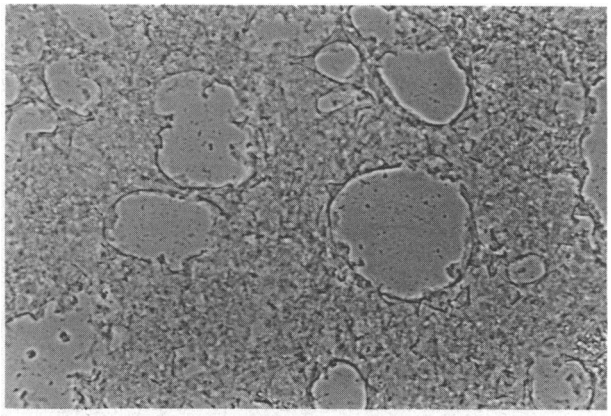

Fig. 2. Lung slice from immature rat

The lung of a 7-day-old rat was inflated with 1 % agar at a pressure of 20 cmH_2O (196 Pa), chilled at 4 °C and sectioned into 5 μm slices. Final magnification is 58 times.

bands were revealed by autoradiography. Similarly rat lung ^{125}I-galaptin did not bind to components of rat lung elastic fibrils. ^{125}I-Concanavalin A (0.9 μCi/μg) was used as a positive control and did label some bands. Galaptin could only be labelled to low specific activity (0.2 μCi/μg) unless it was denatured in 4 M-urea when labelling to

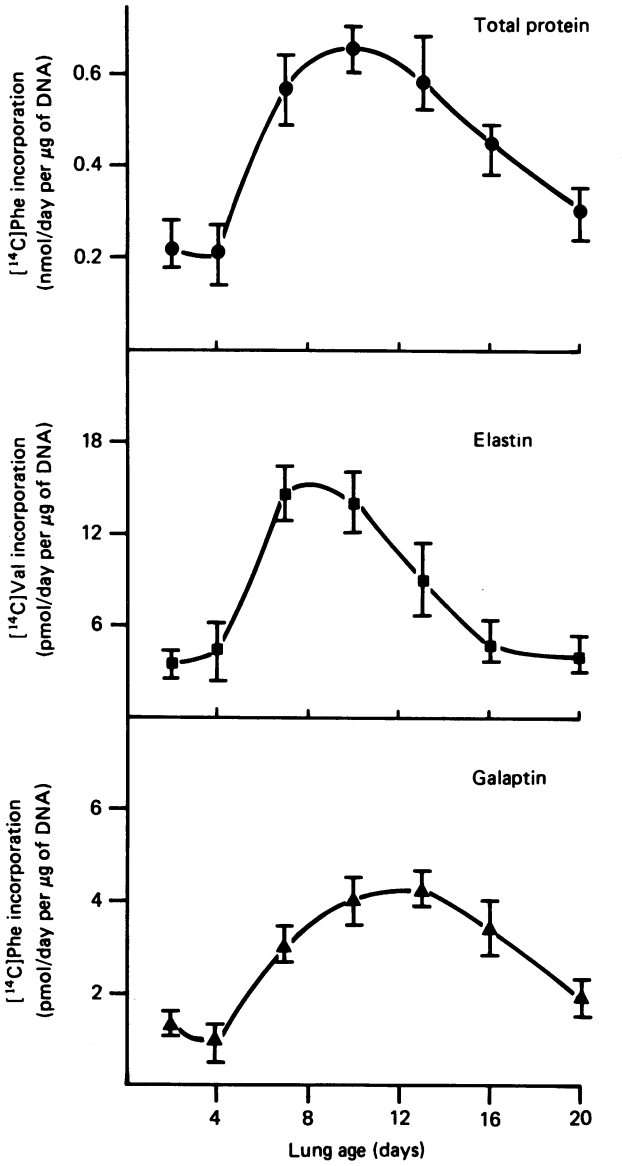

Fig. 3. Protein, elastin and galaptin synthesis in thin slices of developing rat lung

The incorporation of ^{14}C-labelled amino acid is shown at different time points in the postnatal development of rat lung. The saccular phase remains until day 4 or 5, after which alveolarization commences and is complete by day 21 (see reference [11]). Each point is the result of three or more experiments and the error bars show S.D.

high specific activity (1 μCi/μg) occurred. The galaptins could be renatured by dialysis and regained full haem-agglutination and lactosyl-bovine serum albumin-binding activity. The high specific activity ^{125}I-galaptins did not bind either directly to the gel or to nitrocellulose blots. The different preparations of elastic fibrils from porcine lung [15] and rat lung [16], therefore, neither contain galaptin nor bind ^{125}I-galaptin.

Elastin, galaptin and protein synthesis in lung slices

The agar-inflated lung slices were used to approach a culture system with preserved architecture and adequate diffusion of nutrients; a typical slice is shown in Fig. 2. Trypan Blue staining of such slices suggested that

Table 1. Elastin and galaptin synthesis in thin lung slices

Results (tissue+secreted), given as mean±S.D., were from three or more experiments. β-Aminoproprionitrile (β-APN) was present at 25 μg/ml and lactose at 5 mM.

Age of rat (days)	[^{14}C]Valine incorporation into elastin (pmol/day per μg of DNA)			[^{14}C]Phenylalanine incorporation into galaptin (pmol/day per μg of DNA)		
	β-APN	β-APN+lactose	β-APN+anti-galaptin	Control	β-APN	Anti-elastin
2	3.2±0.9	3.9±0.5	2.9±0.8	1.4±0.2	1.4±0.2	1.3±0.3
4	4.3±1.3	3.7±1.0	4.0±1.0	0.9±0.5	1.1±0.4	0.7±0.3
7	14.5±1.4	13.6±1.6	13.1±1.8	3.1±0.4	3.2±0.5	2.9±0.6
10	13.7±1.4	11.9±1.3	12.6±1.4	4.0±0.5	4.1±0.5	4.3±0.6
13	8.9±1.1	9.2±0.9	8.2±0.7	4.3±0.5	4.6±0.6	4.8±0.6
16	4.8±1.1	5.0±0.9	5.5±0.4	3.4±0.6	3.2±0.5	3.1±0.6
20	4.2±0.8	3.6±0.8	4.0±0.7	1.9±0.4	1.8±0.4	2.2±0.6
Adult (100 g)	3.6±0.8	3.4±0.8	4.0±0.9	1.2±0.5	1.4±0.3	1.4±0.3

Table 2. Elastin, galaptin and protein synthesis in rat lung fibroblasts

Confluent fibroblasts at third passage were used for these experiments. Results are given as mean±S.D.

Age of rat (days)	Protein [^{14}C]Phe (nmol/day per μg of DNA)	Elastin [^{14}C]Val (pmol/day per μg of DNA)	Galaptin [^{14}C]Phe (pmol/day per μg of DNA)
4	0.59±0.08	2.3±0.3	2.6±0.3
7	0.64±0.08	9.2±0.4	4.2±0.6
10	0.72±0.10	6.5±0.5	4.6±0.5
13	0.73±0.10	4.3±0.4	3.9±0.5
20	0.61±0.09	3.1±0.5	3.3±0.6
Adult	0.58±0.08	2.6±0.3	2.6±0.4

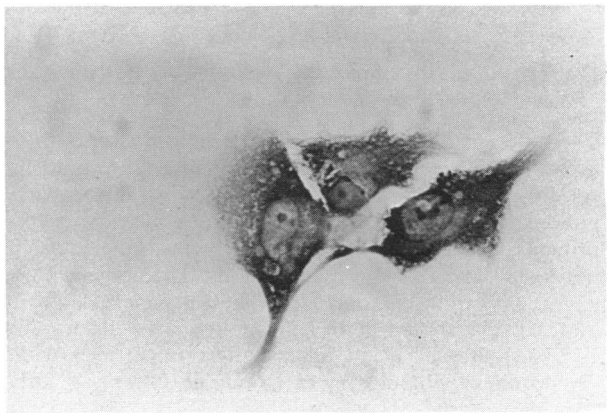

Fig. 4. Localization of galaptin in lung fibroblasts

Fibroblasts from 7-day-old rat lungs were fixed with acetone and incubated with peroxidase-conjugated anti-galaptin, washed and reacted with diaminobenzidine to provide permanent coloration. Final magnification is 525 times.

>85% of the cells were still viable. Total protein synthesis reached a maximum in slices of 10-day-old rat lung (Fig. 3). In the presence or absence of β-aminoproprionitrile elastin synthesis reached a maximum in slices from 7-day-old rat lung (Fig. 3 and Table 1). Galaptin synthesis reached a maximum in 10–13-day-old rat lung (Fig. 3 and Table 1). Galaptin secretion was low at all ages. The effects of lactose, anti-galaptin and anti-elastin are shown in Table 1: lactose and anti-galaptin had minimal effect on elastin synthesis or secretion, anti-elastin or β-aminoproprionitrile had no effect on galaptin synthesis or secretion.

Elastin, galaptin and protein synthesis in lung fibroblasts

Fibroblasts cultured from 6–7-day-old rat lung synthesized more elastin than fibroblasts cultured from 10, 16 or 22-day-old rat lung (Table 2). The synthesis of galaptin by fibroblasts varied by less than 2-fold irrespective of the age of the rat from which they were cultured (Table 2). Western blot analysis suggested that >90% of the immunoreactive material was the 13 kDa galaptin at all ages. In fibroblasts grown from 7-day-old rat lung elastin synthesis and secretion was unaffected by the presence of lactose or anti-galaptin in the culture medium. Similarly galaptin synthesis and secretion were unaffected by β-aminoproprionitrile or anti-elastin in the culture medium.

Immunocytochemical staining of formaldehyde (0.5%)-fixed fibroblasts revealed little galaptin but acetone-fixed fibroblasts showed strong intracellular staining (Fig. 4).

Cultured fibroblasts only secreted 3–6% of newly synthesized galaptin and 5–8% of total galaptin at confluence, irrespective of the age of the rat lung of the original culture. The amount of secreted galaptin compares closely with the amount of secreted lactate dehydrogenase and acid phosphatase (Table 3). In post-confluent cultures increased amounts of galaptin could be recovered from the dishes with 4 M-urea (Table 3).

When fibroblast cultures were challenged with 10^{-5} M-iodoacetamide (2 h), and then supplemented with [^{35}S]methionine or [^{14}C]phenylalanine, total protein synthesis and elastin synthesis was greatly decreased (Table 4). However, galaptin synthesis was only marginally depressed in the presence of 10^{-5} M-iodoacetamide (Table 4).

Table 3. Proteins secreted by rat lung fibroblasts

Fibroblasts from 7-day-old rat lung were used at 3rd passage and cultured in the presence of β-aminoproprionitrile (25 μg/ml) and lactose (5 mM). Trivial amounts of galaptin were recovered from the extracellular matrix, but in cells at 7 days post-confluence, significant amounts of galaptin, equal to the secreted protein, were recovered from the extracellular matrix with 4 M-urea.

Protein	Secreted protein [% of total protein (soluble + cellular)]		
	Cells at confluence	Cells at 2 days post-confluence	Cells at 7 days post-confluence
Galaptin	4.6	5.9	11.4
Acid phosphatase	5.2	7.3	12.2
Lactate dehydrogenase	4.9	4.9	8.6
Elastin	70.3	84.0	82.1

DISCUSSION

Immunocytochemistry is a technique which yields important information but it is also a technique which introduces artefacts of staining, sectioning and fixation. The experiments described in this paper were performed in the light of immunocytochemical evidence associating galaptin with the organization or synthesis of elastic fibril components. Elastic fibrils contain both elastin and microfibrillar glycoproteins [26] and these microfibrillar proteins appear to be present during elastic secretion perhaps forming a scaffold onto which amorphous elastin is deposited. The microfibrillar proteins contain carbohydrate, bind concanavalin A [26] and can be isolated from tissue with guanidinium chloride in the presence of reducing agents [5,6]. I could not detect galaptin amongst the microfibrillar glycoproteins isolated with guanidinium chloride [5]. Since the lung parenchyma was washed and sieved extensively before to guanidinium chloride extraction and 4 M-urea appears to remove galaptin from the extracellular matrix any extracellular galaptin was probably removed before guanidinium chloride extraction and therefore did not appear to be closely associated with the microfibril. Further, none of the components of the microfibril isolated by electrophoresis bound ^{125}I-galaptin although several bands bound ^{125}I-concanavalin A. Galaptin is therefore neither a microfibrillar protein nor one that interacts strongly with microfibrillar glycoproteins.

In developing rat lung elastin synthesis has been studied in tissue slices and appears to be maximal in 7–12-day-old rat lungs [12]. Total protein synthesis and galaptin synthesis have been studied in a similar system [12,14]. Here I have developed a technique of preparing thin slices of expanded lung, to permit optimal preservation of anatomical relationships and optimal nutrient access to cells. The results obtained for total protein synthesis are dissimilar from both previous investigations using collapsed thick lung slices. In the former investigation, where [^{14}C]valine was the isotope used and protein synthesis on days 12–15 was 3–4-fold greater than protein synthesis on days 0–4 or day 20 [12]. In the more recent study, using [^{14}C]phenylalanine as a tracer, protein synthesis declined steadily from birth [14]. In thin lung slices protein synthesis using [^{14}C]-phenylalanine as a tracer was maximal at day 10 and then declined slowly to half this value at day 20. In determining total protein synthesis I have included secreted protein and this differs from the previous studies. The concentration of [^{14}C]phenylalanine used was sufficient to saturate intracellular pools [14]. Agar inflation of the smallest lungs may produce more cellular damage than in older lungs and account for the low level of protein synthesis in 2–4-day-old rat lungs. The peaks of elastin and galaptin synthesis are not coincident. Elastin synthesis peaks before total protein synthesis and this is in agreement with previous work [12], but galaptin synthesis largely parallels total protein synthesis. Elastin synthesis and secretion were not affected by concentrations of lactose sufficient to solubilize cell-surface galaptin or by anti-galaptin. Therefore galaptin at the cell surface does not appear to be necessary for elastin synthesis and secretion.

Both smooth-muscle cells and fibroblasts synthesize elastin and galaptin [27]. Cells in culture therefore provide a simpler system in which to study possible interrelationships between galaptin and elastin synthesis and secretion. Lung fibroblasts were cultured from 7-day-old rat lung to provide cultured cells with maximum elastin and galaptin biosynthetic potential. In these cultured cells elastin synthesis and secretion were unaffected by lactose or anti-galaptin. The secretion of galaptin was

Table 4. Effect of iodoacetamide on protein synthesis in rat lung fibroblasts

Fibroblasts from 7-day-old rat lung were used at third passage and cultured in the presence of β-aminoproprionitrile (25 μg/ml) and lactose (5 mM).

Protein synthesis	Fibroblasts cultured in the presence of:		
	Control	10^{-6} M-Iodoacetamide	10^{-5} M-Iodoacetamide
Total protein (pmol of [^{14}C]Phe/day per μg of DNA)	656±73	361±66	167±43
Galaptin (pmol of [^{14}C]Phe/day per μg of DNA)	4.3±0.4	3.5±0.4	3.0±0.5
Elastin (pmol of [^{14}C]Val/day per μg of DNA)	8.8±0.5	2.3±0.4	< 0.03

very low and comparable with the release of other intracellular enzymes from senescent or leaky cells. Further, when cells were cultured in the presence of iodoacetamide, a known cellular toxin [28], elastin biosynthesis ceased but galaptin synthesis was maintained. Under such conditions of cellular stress protein synthesis is reduced but the maintenance of galaptin synthesis would suggest that galaptin has an important intracellular function.

The evidence presented here suggests that galaptin is not associated with the synthesis or organization of the elastic fibril. Current evidence could also suggest that the function of these galaptins is intracellular and that their secretion to the extracellular matrix occurs only with cell lysis. Such evidence is suported by the requirement of the galaptins to maintain reduced cysteine residues [17] and by acetylation of the *N*-terminus [29]. The recent elucidation of the mechanism of action of ricin [30] might suggest that endogenous lectins, too, could be involved in protein synthesis.

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