Quantitative determination, isolation and characterization of pig lung tubulin

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Tubulin from pig lung was quantitatively determined, isolated and characterized. It accounted for about 0.3–0.4% of the total soluble protein of pig lung, as measured by colchicine binding or radioimmunoassay. Purified tubulin was obtained by several cycles of polymerization and depolymerization in the presence of dimethyl sulphoxide and $^2\text{H}_2\text{O}$ as stabilizing agents. The proteolytic cleavage patterns of the lung tubulin subunits closely resembled those of other mammalian cytoplasmic tubulin subunits, such as those of brain and kidney. However, the pattern of lung isotubulins on isoelectric focusing differed substantially from that of brain isotubulins. These differences did not appear to be the result of major lung tubulin post-translational modifications, since approximately the same pattern of isotubulins was found for the tubulin synthesized by lung poly(A)-containing mRNA in a reticulocyte system *in vitro*.

Microtubules are present in all eukaryotic cells, where they are vital for the separation and correct distribution of chromosomes during mitosis. They also play an important role in morphogenesis, intracellular transport, secretion and motility (Dustin, 1978). The building block of microtubules is the protein tubulin, a heterodimer consisting of an α -chain and a β -chain (Ludueña *et al.*, 1977). Both chains of pig brain tubulin have M_r 50000 and are about 42% homologous (Ponstingl *et al.*, 1981; Krauhs *et al.*, 1981).

A comparison of the pig brain α - and β -tubulin amino acid sequences with the tubulin messenger nucleotide sequences of chick brain α - and β -tubulins (Valenzuela *et al.*, 1981) and rat brain α -tubulin (Lemischka & Sharp, 1982) shows that tubulin is a very conservative molecule, with a mutation rate comparable with that of the histones (Little *et al.*, 1981a). Nevertheless, functional differences have been shown between brain tubulin and lower-eukaryotic tubulins with respect to their cold-stability and drug-sensitivity (Kuriyama & Sakai, 1974; Roobol *et al.*, 1980; Kilmartin, 1981). Some differences have been found between the properties of mammalian and fish brain tubulins (Langford, 1978). Fulton & Simpson

(1976) postulated that different tubulins may be required for different functions. More recently, evidence has been put forward that functionally similar tubulins from widely different species may be more closely related than functionally dissimilar tubulins within the same organism (Little *et al.*, 1981b, 1983). Structural differences between tubulin molecules as analysed by isoelectric focusing have been found (Gozes & Littauer, 1978; Dahl & Weibel, 1979; George *et al.*, 1981; Denoulet *et al.*, 1982a). These differences appear to be related to the source of tubulin (i.e. liver and brain) (Gozes & Littauer, 1978; Denoulet *et al.*, 1982b). Lung microtubules have been implicated in the secretion of the phospholipid component of surfactants (Delahunty & Johnston, 1976), and their tubulin may be therefore quite different to that of brain, which is involved in other processes, i.e. dendrite formation (Solomon, 1980).

In the present work we have isolated tubulin from lung extracts, a source that contains 70-fold less tubulin than does brain. We have found that, although the proteolytic-cleavage pattern of lung tubulin closely resembled that of other mammalian cytoplasmic tubulins, large differences were found in the pattern of isotubulins on isoelectric focusing. These differences did not appear to be the result of post-translational modifications.

A part of this work has been reported in preliminary form elsewhere (Díez *et al.*, 1982).

Abbreviations used: poly(A)⁺ mRNA, poly(A)-containing mRNA; Pipes, 1,4-piperazinediethanesulphonic acid.

Experimental

Protein preparation

Preparation of lung extract. Pig lung was dissected, care being taken to avoid the bronchial tree, minced into small pieces and homogenized in isotonic buffer (0.32M-sucrose/1 mM-MgCl₂/1 mM-EDTA/10 mM-sodium phosphate buffer, pH 7.0) in the proportions 0.3 ml of buffer per g of tissue. The homogenate was centrifuged at 85000g for 45 min at 2°C, and the supernatant was used for radioimmunoassay, colchicine binding, vinblastine precipitation or taxol-induced polymerization.

Cycled brain microtubule protein. Pig brain microtubule protein was obtained by cycles of temperature-dependent polymerization-depolymerization in buffer A (0.5 mM-MgCl₂/2 mM-EGTA/0.1 M-Pipes adjusted to pH 6.4 with NaOH) containing 4 M-glycerol and 0.5 mM-GTP in accordance with the protocol described by Shelanski *et al.* (1973), but 4 M-glycerol was included only in the first polymerization step.

Cycled lung microtubule protein. Assembly of lung tubulin *in vitro* was performed by the procedure of Shelanski *et al.* (1973), with some modifications. Minced lung was homogenized in buffer A prepared in ²H₂O in the proportions 0.1 ml of buffer per g of tissue. The homogenate was centrifuged at 95000g for 45 min at 2°C. Glycerol, dimethyl sulphoxide and GTP were added to the supernatant to final concentrations of 4 M, 10% (v/v) and 1 mM respectively. The solution was warmed at 35°C for 30 min and centrifuged at 65000g for 40 min at 25°C. The pellet was resuspended in buffer A, homogenized, kept at 4°C for 40 min and centrifuged at 20000g for 15 min at 4°C. The supernatant was removed and an additional polymerization cycle was carried out. Twice-cycled microtubule protein was stored at -70°C.

Taxol (a drug isolated from *Taxus brevifolia*) assembly of lung microtubules was performed as described by Vallee (1982) with the use of 20 μM-taxol and 1.5 mM-GTP.

Lung tubulin polymers were examined by electron microscopy as described previously (de la Torre *et al.*, 1981).

Precipitation of lung tubulin by vinblastine sulphate (Lilly) was performed by the method of Olmsted *et al.* (1970).

Proteolytic digestion. Isolated carboxymethylated kidney α- and β-tubulin subunits were from a previous investigation (Little *et al.*, 1981b). Pig lung tubulin was reduced with 2-mercaptoethanol and carboxymethylated with sodium iodoacetate in accordance with the method of Crestfield *et al.* (1963). The subunits were separated by electrophoresis on 7% (w/v) polyacrylamide slab gels with

the system of Yang & Criddle (1970), and they were located as translucent bands after incubation with 4 M-sodium acetate (Higgins & Dahmus, 1979). Elution of the subunits was performed as described previously (Little *et al.*, 1981b). Limited proteolytic digestion of the tubulin subunits with *Staphylococcus aureus* proteinase in a 5% (w/v) polyacrylamide stacker gel and electrophoresis of the peptides on a 15% (w/v) polyacrylamide gel were performed in accordance with the method of Cleveland *et al.* (1977). Approx. 1 μg of tubulin subunit and 0.1 μg of enzyme were applied per well. The gels were stained with silver by the procedure of Oakley *et al.* (1980).

The system of Laemmli (1970) was employed for polyacrylamide-gel electrophoresis.

Isoelectric focusing. Isoelectric focusing in polyacrylamide slab gels was done essentially as described by O'Farrell (1975), with some modifications, in an LKB Multiphor system. Acrylamide and *NN'*-methylenebisacrylamide concentrations were 4.88% (w/v) and 0.15% (w/v) respectively. The Ampholine concentration was 2% (w/v), and the mixture pH 3.5-5/pH 4-6/pH 5-7 (1:8:1, by vol.) was used. The gels contained 8 M-urea and were 0.5 mm thick. The samples, in a solution containing 6 M-urea and 10% (v/v) 2-mercaptoethanol, were applied on a square piece (1 cm × 1 cm) of Whatman no. 1 paper in the vicinity of the anode. After ½ h at 50 V and then ½ h at 100 V, the voltage was increased by 100 V every ½ h until a value of 600 V was reached. This voltage was maintained for another ½ h and then lowered to a constant voltage of 300 V and kept constant for about 18 h; for the last 2 h 600 V voltage was applied. After focusing, the gels were fixed, stained and destained as indicated by George *et al.* (1981).

Isolation of tubulin antibodies. Phosphocellulose-purified brain tubulin was prepared as described by Weingarten *et al.* (1975) and used as an antigen to immunize rabbits. The immunized animals were bled, and the γ-globulin fraction was purified and concentrated 10-fold from the serum by (NH₄)₂SO₄ precipitation. When this serum was tested on fractionated brain proteins by immunautoradiography (Granger & Lazarides, 1979), it reacted only with the two tubulin subunits.

Assays

Radioimmunoassay. Increasing amounts of the unlabelled antigen were added to a mixture of 10 μl (18000 c.p.m.) of ¹²⁵I-tubulin-phosphocellulose (tubulin iodinated as indicated by Nieto *et al.*, 1981) and 50 μl of anti-tubulin γ-globulin (diluted 1:400) in a total volume of 0.32 ml by radioimmunoassay buffer, containing 5 mg of bovine serum albumin/ml, 0.5% (w/v) NaN₃ and 0.5% (v/v) Triton X-100 in phosphate-buffered saline

(0.15M-NaCl/25mM-sodium phosphate buffer, pH7.5). The mixture was allowed to react at 4°C overnight. Then 15 μ l of a 1:5 dilution of *Staphylococcus aureus* suspension (Pansorbin TM; Calbiochem-Boehringer Corp., La Jolla, CA, U.S.A.) were added. After 15min the total radioactivity was counted. The tubes were centrifuged and the radioactivity present in the pellet was determined. The background was determined in a test tube where no anti-tubulin antibody was added.

Colchicine binding. Colchicine binding was performed essentially as described by Sherline *et al.* (1974). Briefly, 0.2ml samples of brain and lung extract at different concentrations were mixed with 15 μ l of an incubation mixture containing 4.6M-NaCl, 1.4mM-GTP and 0.11mM-colchicine (Sigma Chemical Co.) containing 9.6 μ Ci of [³H]-colchicine/ μ mol (Amersham International). The samples were incubated for 90min at 35°C. Three different preincubation times were used (indicated in the Results section). The reaction was stopped by addition of 2ml of a solution of charcoal (3mg/ml) to the incubation samples. After 10min at 0°C, the mixture was centrifuged in an Eppendorf centrifuge and the radioactivity in the supernatant was counted.

Translation of lung mRNA. Lung poly(A)⁺ mRNA was prepared as described by Lombardero & Nieto (1981), except for an additional step of oligo(dT)-cellulose chromatography. Cell-free protein synthesis was performed in a reticulocyte lysate system as described by Lombardero & Nieto (1981), with L-[³⁵S]methionine (774 Ci/mmol; Amersham International) (0.16mCi/ml). The translated products were immunoprecipitated with an excess of anti-tubulin serum followed by addition of *Staphylococcus aureus* suspension. The pellet was washed twice with a buffer containing 0.5% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 1mM-methionine, 1mM-phenylmethane sulphonyl fluoride, 0.02% (w/v) NaN₃ and 1mg of bovine serum albumin/ml, and it was resuspended in 8M-urea. This solution was used for isoelectric-focusing analysis after the addition of purified lung tubulin as a carrier. After isoelectric focusing, the bands corresponding to different lung isotubulins were cut out from the gel and the associated radioactivity was counted.

Results

Quantitative determination of tubulin in pig lung

The amounts of tubulin in the soluble fraction of lung and brain extracts were measured by radioimmunoassay. Fig. 1 shows that the tubulin content in lung extracts is about 70 times below that of brain extract. Since tubulin accounts for about 25% of the soluble protein in brains of pigs

Fig. 1. Quantitative determination of the concentrations of tubulin in pig brain and lung by radioimmunoassay. ¹²⁵I-tubulin-phosphocellulose was incubated with different amounts of protein from brain extract (●) and lung extract (○) as indicated in the Experimental section. The mixtures were allowed to react with anti-tubulin γ -globulin, and after incubation the antigen-antibody complexes were precipitated by adding *Staphylococcus aureus* suspension. After centrifugation, the radioactivity in the pellets was counted and the percentage of ¹²⁵I-tubulin sedimented with respect to total ¹²⁵I-tubulin was calculated.

Fig. 2. Colchicine-binding activity of lung extract. Samples of lung extract at three different protein concentrations (○, 2.5mg/ml; ●, 3mg/ml; △, 15mg/ml) were preincubated for three different times (0, 1 and 2h), and subsequently 15 μ l of the incubation mixture was added. The mixture was incubated for 90min and the bound colchicine was measured as indicated in the Experimental section.

(Hiller & Weber, 1978; J. C. Diez, unpublished work), we calculate that about 0.4% of the protein present in lung extract is tubulin. This estimation was checked by measuring the colchicine-binding activity of the lung protein. Since the binding of colchicine to tubulin decays with apparent first-order kinetics, and the rate of decay is dependent

on tubulin concentration (Wilson & Bryan, 1974), we measured the binding of colchicine to tubulin in lung extracts at three different protein concentrations. The samples were preincubated at 35°C for different time periods before addition of colchicine to correct for the decay in binding activity. The initial colchicine binding calculated from Fig. 2 indicates a proportion of tubulin with respect to total soluble protein of about 0.33%. The half-time of colchicine-binding decay under the conditions used was calculated to be 3 h by using the data shown in Fig. 2.

Polymerization of lung microtubules

The polymerization of tubulin into microtubules is dependent on tubulin concentration and requires

a minimal (critical) concentration (Gaskin *et al.*, 1974). This concentration can be lowered for brain tubulin by the addition of microtubule-associated proteins (MAPs) or by the presence of $^2\text{H}_2\text{O}$ (Olmsted & Borisy, 1973), glycerol (Shelanski *et al.*, 1973) or dimethyl sulphoxide (Himes *et al.*, 1977) (for review see Ponstingl *et al.*, 1983). The low content of tubulin in lung extracts and that of proteins related to brain microtubule-associated proteins, e.g. 90 ng of MAP₂/mg of soluble protein (Valdivia *et al.*, 1982) and 25 ng of τ -protein/mg of soluble protein (E. Montejó, unpublished work), indicated that highly concentrated extracts might be necessary to achieve microtubule polymerization. Lung extracts were accordingly used at protein concentrations of 40 mg/ml for the poly-

Fig. 3. Scheme of the procedure for the purification of lung tubulin

The method indicated in the Experimental section was followed, and the protein present in each step of purification was characterized by electrophoresis.

merization of tubulin at 35°C. The extracts also contained 10% (v/v) dimethyl sulphoxide, 4M-glycerol and 70% (v/v) $^2\text{H}_2\text{O}$. Microtubule protein was further purified by an additional polymerization cycle. Fig. 3 shows the protocol for the isolation of lung tubulin by successive cycles of polymerization-depolymerization and the protein composition at the different steps of purification. After two polymerization-depolymerization cycles more than 80% of total polymer protein was tubulin. An alternative method for the isolation of lung tubulin was based on the incubation of lung extracts at 35°C in the presence of taxol to form microtubules. Fig. 4 also shows the morphology of tubulin polymers obtained by these two procedures. Many open microtubules were observed with both methods of microtubule polymerization.

We have routinely chosen the dimethyl sulphoxide/glycerol procedure for the isolation of lung tubulin. In our hands the taxol procedure results in a lower yield of tubulin and does not allow further purification by recycling.

Characterization of lung tubulin

(a) *Peptide analysis.* The proteolytic-cleavage patterns of pig lung α - and β -tubulins were almost identical with those of bovine kidney α - and β -tubulins used as a reference (Fig. 5). Since the cleavage patterns of kidney tubulin subunits have also been shown to be identical with those of brain tubulin subunits (Little *et al.*, 1981b), and since

Fig. 5. Proteolytic-digestion patterns of α - and β -tubulin subunits from lung and kidney

Tubulin subunits from pig lung (L) and bovine kidney (K) were fractionated and digested as indicated. For details see the Experimental section. Arrowhead shows the position of the proteinase used.

Fig. 4. Characterization of lung tubulin polymers

Lung tubulin was assembled in the presence of dimethyl sulphoxide (a) or taxol (b), and the polymers obtained were characterized by electron microscopy and electrophoresis in sodium dodecyl sulphate/polyacrylamide gels containing 7.5% acrylamide. For details see the Experimental section.

cleavage patterns of bovine and pig brain tubulin subunits are identical (results not shown), it seems that lung tubulin is very closely related to the tubulins of brain and kidney.

(b) *Isoelectric-focusing analysis.* Fig. 6 shows the isoelectric-focusing analysis of lung and brain tubulins. A different pattern was obtained for both proteins. Lung tubulin appears to contain a lower number of isoforms than does brain tubulin, and quantitative differences in the proportion of possibly common isotubulins were also found. Such differences could be due to different efficiencies of assembly for the various isotubulins, since lung tubulin was isolated by polymerization through several cycles and the first one was performed at a low tubulin concentration. However, this appears not to be the case, since (a) the pattern of brain isotubulins polymerized near the critical concentration is similar to that of brain isotubulins polymerized from more concentrated tubulin solutions (J. C. Díez & J. Avila, unpublished work) and (b) Fig. 7 shows a similar pattern for lung tubulin isolated by polymerization-depolymerization (Fig. 7a) and for that obtained by vinblastine precipitation (Fig. 7b).

Translation of lung tubulin in vitro

The translation of total poly(A)⁺ mRNA from lung in a reticulocyte-lysate system resulted in the presence of two polypeptides with the same mobilities as those of α - and β -tubulin subunits. The ³⁵S-labelled tubulin was precipitated with anti-tubulin serum followed by the addition of *Staphylococcus aureus* suspension and further analysed by isoelectric focusing (Fig. 7c). The pattern of radioactivity closely paralleled the isotubulin pattern of carrier lung tubulin, although the proportions of some isotubulins to one another are different (Fig. 7c). This could be due (a) to different affinities of the dye for the various isotubulins (Bryan & Wilson, 1971) compared with that of the methionine content of these subunits (Ponstingl *et al.*, 1981; Krauhs *et al.*, 1981) or (b) to a differential expression of the α -tubulin mRNA and β -tubulin mRNA in our system *in vitro*.

Discussion

We have isolated lung tubulin from pigs and characterized some of its structural and functional features. One functional characteristic of tubulin is

Fig. 6. *Characterization of isotubulins from lung and brain*

(a) Isoelectric-focusing gel pattern of cycled lung tubulin (upper pattern) and brain tubulin (lower pattern); (b) and (c) densitometric profiles of the brain tubulin and lung tubulin gels shown in (a) respectively. For details see the Experimental section.

Fig. 7. Densitometric isoelectric-focusing patterns of lung tubulin isolated by different procedures (a) Lung tubulin obtained by polymerization cycles; (b) lung tubulin obtained by vinblastine precipitation; (c) polypeptides corresponding to the products translated by lung poly(A)⁺ mRNA and immunoprecipitated by anti-tubulin serum. For details see the Experimental section. The densitometric pattern corresponding to carrier lung protein isolated by polymerization cycles is shown together with the associated ³⁵S radioactivity indicated by the vertical lines.

Fig. 8. Comparison of the isoelectric focusing positions of tubulin from lung and brain

(a) Brain isotubulins; (b) lung isotubulins. For details see the Experimental section. Isotubulins are numbered by decreasing isoelectric points. ■, ▨ and □ represent successive decreasing intensity of staining of the isoforms.

its binding to colchicine. With a half-time of decay for colchicine binding of 3 h, lung tubulin resembles tubulins from other sources, which have half-time decays for colchicine binding between 2.8 h and 6.6 h (Green *et al.*, 1975; Pfeffer *et al.*, 1976; Barnes & Roberson, 1979).

The amount of protein with ability for colchicine

binding and a radioimmunoassay for tubulin indicated that only 0.3–0.4% of the soluble protein in lung extracts was tubulin. Similarly low amounts of tubulin are present in kidney (approx. 1%), where it was found necessary to add both glycerol and dimethyl sulphoxide to achieve tubulin polymerization (Barnes & Roberson, 1979). We found that, in addition, ²H₂O greatly increased the yield of microtubules in lung extract.

A typical preparation yielded 0.2 mg of microtubule protein/g wet wt. of lung tissue after two cycles of purification. The characterization of the polymerized protein indicates a high proportion of tubulin in such preparations. After resuspension of the pellet obtained after the second polymerization cycle and clarification of the solution by high-speed centrifugation, the resulting cold supernatant is composed essentially of homogeneous tubulin.

The proteolytic-cleavage patterns of lung tubulin subunits after digestion with *Staphylococcus aureus* proteinase were very similar to those of other mammalian (brain and kidney) tubulins. This indicated that possible structural differences between lung and brain tubulins may be minor ones. By analysing lung and brain tubulins by isoelectric focusing, however, we have found large differences in their isotubulin patterns. The isolation of lung tubulin by vinblastine precipitation results in a pattern of isotubulins similar to that of tubulin isolated by assembly–disassembly. This

means that all of the lung isotubulins present in a lung extract have the ability to polymerize.

It has been indicated that both subunits of brain tubulin have a high degree of heterogeneity when analysed by isoelectric focusing (Gozes & Littauer, 1978; Dahl & Weibel, 1979; George *et al.*, 1981; Denoulet *et al.*, 1982a). Some of these differences could be due to post-translational modifications or to artifact oxidation of thiol groups or the deamidation of asparagine or glutamine (Robinson & Rudd, 1974). Direct evidence of sequence heterogeneity, however, has been obtained from work on the amino acid sequences of both subunits from pig brain (Kraus *et al.*, 1981; Ponstingl *et al.*, 1981). Moreover, multiple dispersed sequences complementary to both α -tubulin mRNA and β -tubulin mRNA have been detected in different organisms (Sánchez *et al.*, 1980; Cleveland *et al.*, 1981; Valenzuela *et al.*, 1981), and the presence of several different β -tubulin mRNA species has been found in fibroblasts and lymphoid cells (Lopata *et al.*, 1983).

Post-translational modifications appear to be an unlikely explanation for the pattern of lung isotubulins observed on isoelectric focusing, since peptides reacting with anti-tubulin serum were synthesized by lung mRNA in a reticulocyte system *in vitro* and these co-migrated with the lung isotubulin purified by assembly-disassembly.

A comparison similar to that made by Denoulet *et al.* (1982b) for liver and brain tubulins has been made for brain and lung tubulins. Some of the isotubulins present in lung tubulin may be identical with those present in brain, although the brain isotubulins β_1 , β_4 and β_5 appear to be absent from lung microtubules (summarized in Fig. 8). The largest difference between brain and lung isotubulin patterns, however, is the relatively large proportion of the most basic lung α - and β -isotubulins in comparison with the amounts of other isotubulins. It is quite possible, therefore, that these two isotubulins are particularly important for a specific function of lung microtubules.

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