IDENTIFICATION OF CYTOPLASMIC TUBULIN IN *DICTYOSTELIUM DISCOIDEUM*

Piero CAPPUCCINELLI[+], Giovanna MARTINOTTI and B. David HAMES*,
Department of Biology, University of Essex, Wivenhoe Park, Colchester, CO4 3SQ, England

Received 21 April 1978

1. Introduction

Microtubules have been found in all eukaryotes examined and are important for a wide range of cellular activities such as mitosis, cell shaping, secretion and motility. Detection and quantitation of tubulin, the subunit protein of microtubules, is thus of importance in attempts to understand differentiation processes.

Microtubules have been described in the mitotic apparatus of *Dictyostelium discoideum* [1] and it has been shown that colchicine will inhibit not only growth [2] but also development [3] in this organism. A colchicine binding activity in extracts of vegetative *D. discoideum* amoebae has been characterised [4] and evidence presented that this is caused by tubulin [4]. We report here that *D. discoideum* amoebae contain tubulin remarkably similar to that from a mammalian source, pig brain, both in terms of molecular weight of the tubulin subunits and conversation of amino acid sequence as demonstrated by immunological cross reactivity.

2. Methods

2.1. *Growth of myxamoebae and preparation of high speed supernatant*

Myxamoebae of *D. discoideum* strain AX2 were grown axenically in HL5 medium containing 86 mM glucose and harvested in the exponential phase of growth [5]. In order to prepare cytoplasmic tubulin washed cells were suspended in 0.1 mM GTP, 1 mM $MgCl_2$, 0.24 M sucrose, 0.01 M sodium phosphate, pH 6.8, at 2.5 g wet wt/10 ml buffer and the suspension homogenised with ~ 40 strokes in a Teflon glass homogeniser; cell breakage was greater than 90%. The homogenate was centrifuged for 30 min at 5000 × *g* and the supernatant then centrifuged at 100 000 × *g* for 60 min.

2.2. *Immunological characterisation*

Pig brain tubulin was purified by repeated cycles of polymerization and phosphocellulose chromatography [6], and anti-tubulin antibodies raised by repeated injection of pig brain tubulin (0.4 mg tubulin in 0.2 ml Freunds complete adjuvant per injection) into the footpads of New Zealand rabbits. Booster injections were made intravenously in the absence of adjuvant.

Samples of the high speed supernatant from *D. discoideum* and known amounts of pig brain tubulin were subjected to rocket-immunoelectrophoresis using anti-pig brain tubulin in agarose plates prepared in 0.02 M barbitol buffer, pH 8.6. Immunoelectrophoresis was carried out at ~ 2–3 V/cm overnight in the cold and then after extensive washing to remove unprecipitated protein, the plates were stained with 0.5% Coomassie brilliant blue in 45% ethanol–10% acetic acid and destained in 45% ethanol–10% acetic acid.

2.3. *Copolymerisation with pig brain tubulin*

Aerobacter aerogenes was labelled with ^{35}S by overnight growth in minimal medium supplemented

[+] Permanent Address: Istituto di Microbiologia Medica, Universita di Sassari, Italy

* Present Address: Department of Biochemistry, 9 Hyde Terrace, University of Leeds, Leeds, LS2 9LS, England

with $Na_2{}^{35}SO_4$, harvested by centrifugation at 50 000 × *g* for 30 min, resuspended in 0.03 M phosphate buffer, pH 6.4, at 10 A_{650} units/ml and autoclaved. *D. discoideum* AX2 was inoculated into the bacterial suspension at 10^4 amoebae/ml and allowed to grow to 3×10^6 amoebae/ml in shaking culture at 22°C. The cells were washed twice with cold distilled water and suspended in ice-cold polymerisation buffer (0.1 M MES, 0.5 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP, pH 6.5) to give final conc. 1.5×10^8 cells/ml. Cell suspensions were disrupted by sonication with a 100W MSE ultrasonic disintegrator (peak-to-peak amplitude 10 μM) for four 15 s periods with continuous cooling in an ice-salt bath. Sonicates were stirred in an ice-bath for 30 min and then centrifuged at 100 000 × *g* for 60 min at 4°C. High speed supernatant was mixed with an excess of purified pig brain tubulin (gift from Dr M. Jacobs, MRC Cell Biophysics Unit, London) in polymerisation buffer containing phenylmethylsulfonylfluoride as protease inhibitor and incubated at 35°C for 30 min. The polymerised tubulin was recovered by centrifugation (100 000 × *g* for 60 min), depolymerised by incubation at 0°C for 30 min, and recentrifuged to remove insoluble material. After the addition of further pig brain tubulin in polymerisation buffer the sample was incubated once more at 35°C for 30 min to repolymerise the tubulin which was then recovered by centrifugation as above. Proteins present in the pellets after the first and second polymerisation steps were examined by SDS 5–20% polyacrylamide gradient gel electrophoresis. The gel was stained with Coomassie brilliant blue R-250 for photography and then prepared for fluorography by the method in [7].

3. Results

3.1. *Copolymerisation with mammalian tubulin*

A common criterion for tubulin identity is the ability of the protein to copolymerise with authentic mammalian tubulin [8]. High speed supernatant from *D. discoideum* steady-state labelled with ^{35}S was incubated under conditions favourable for tubulin polymerisation, in the presence of pig brain tubulin. The polymerised material was separated by centrifugation, depolymerised, and allowed to repolymerise once more as described in section 2.3. Examination of the protein components was carried out by SDS–polyacrylamide gel electrophoresis followed by Coomassie brilliant blue staining and fluorography. Two *D. discoideum* proteins with similar molecular weights to α and β-subunits of pig brain tubulin were enriched in the copolymerised sample (fig.1d,e), whereas most other proteins had been removed by the polymerisation process. However three additional proteins of lower molecular weight were also observed after the second copolymerisation step (fig.1d). These are possibly degradation products of *D. discoideum* tubulin since, even though a protease inhibitor was used throughout these experiments, proteolysis is a severe problem in extracts of *D. discoideum*.

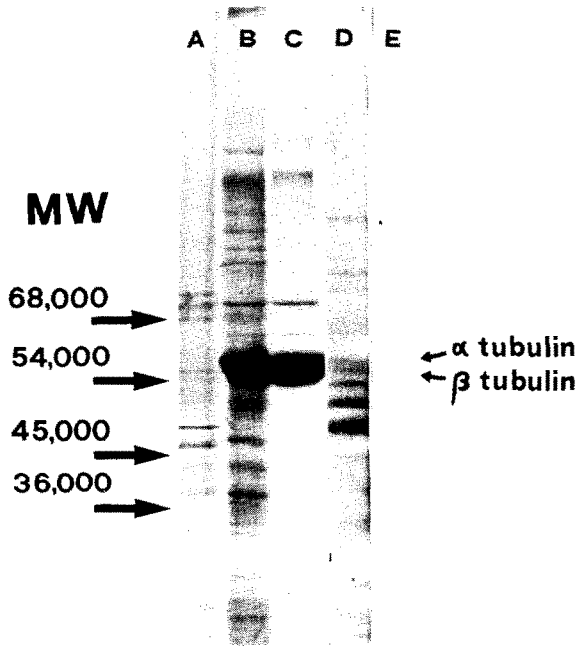

Fig.1. Copolymerisation of ^{35}S-labelled *D. discoideum* proteins with pig brain tubulin and analysis by SDS–polyacrylamide gel electrophoresis. After one cycle of copolymerisation of ^{35}S-labelled *D. discoideum* proteins with pig brain tubulin the post-100 000 × *g* supernatant (A) and pellet (B) were analysed on SDS–polyacrylamide gels. The latter was taken through a second cycle of copolymerisation with pig brain tubulin and high-speed centrifugation to yield pellet C. Track D shows the autoradiogram of track C whilst track E contains marker α- and β-subunits of pig brain tubulin.

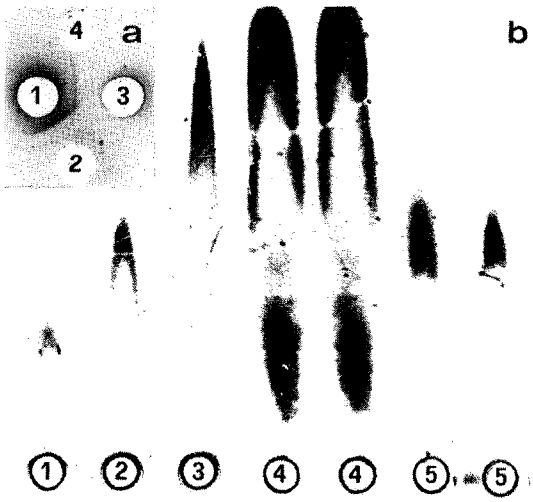

Fig.2. Immunological characterisation of *D. discoideum* tubulin. (a) Immunodiffusion pattern obtained after incubation of rabbit anti-pig brain tubulin antibody (well 1) with pig brain tubulin (well 2), *D. discoideum* 100 000 × *g* supernatant (well 3) and *D. discoideum* 100 000 × *g* supernatant pre-treated with vinblastine (well 4). (b) Rocket immunoelectrophoresis [10] of pig brain tubulin (track 1 = 100 μg/ml; 2 = 200 μg/ml; 3 = 400 μg/ml) and *D. discoideum* 100 000 × *g* supernatant (4 = 20 mg protein/ml; 5 = 10 mg protein/ml) in agarose containing 2.5% rabbit anti-pig brain tubulin antiserum. Sample size in each case was 5 μl.

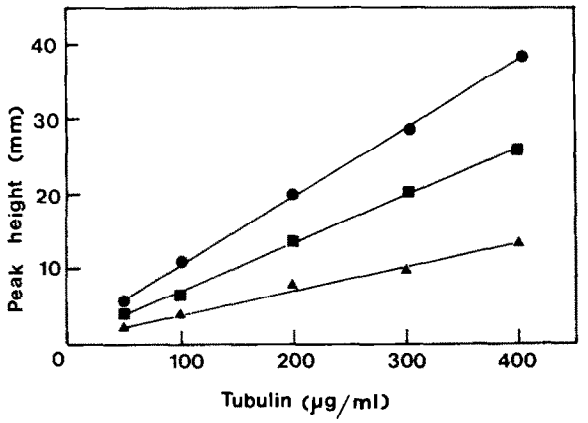

Fig.3. Standard curves for pig brain tubulin determined by rocket immunoelectrophoresis [10] using rabbit anti-pig brain tubulin antiserum at 3.2 μl/cm² gel (●), 5.1 μl/cm² gel (■) and 7.6 μl/cm² gel (▲). Sample volume was 5 μl in each case and electrophoresis was performed at 2–3 V/cm for 18 h at 5°C.

3.2. *Immunological characterisation*

Further evidence for tubulin in *D. discoideum* was provided by immunodiffusion of high speed supernatant from *D. discoideum* against anti-pig brain tubulin antibody. Precipitin lines were formed when the antigen was either *D. discoideum* high speed supernatant or authentic pig brain tubulin (fig.2). Control experiments in which the high speed supernatant was preincubated with 2 mM vinblastine (to precipitate any tubulin present [9]) and then centrifuged at 10 000 × *g* for 20 min before immunodiffusion, showed no precipitin lines indicating that the immunoprecipitation was due to tubulin in the *D. discoideum* extracts.

3.3. *Cellular content of tubulin*

Standard amounts of pig brain tubulin were subjected to immunoelectrophoresis according to the rocket technique [10] to define standard curves (fig.2b, fig.3) which were used to estimate the amount of cross reacting tubulin in *D. discoideum* high speed supernatant. Repeated determinations on different preparations of high speed supernatant from *D. discoideum* AX2 grown in the presence of glucose show that 2.3 ± 0.4% of high speed supernatant protein is tubulin. Given that ~ 40–50% of the total cell protein is usually found in this fraction, tubulin accounts for about 1% of the total cell protein of vegetative *D. discoideum* amoebae.

4. Discussion

Tubulin, the subunit protein of microtubules, has the property of binding alkaloids such as colchicine [11] and this binding usually interferes with microtubule function. Whilst colchicine is known to inhibit growth in *D. discoideum* it is especially interesting that this drug also inhibits development at a specific morphological stage [3]. Furthermore, recent evidence suggests that at this time a burst of mitosis normally occurs [12] which is therefore presumably of developmental significance. Hence, *D. discoideum* tubulin may play an important role during both growth and development in this organism.

Most tubulins, including pig brain tubulin, exist in

a dimer form with mol. wt of 115 000–120 000, consisting of non-identical α- and β-subunits [13–17]. The copolymerisation experiments reported here indicate that *D. discoideum* tubulin consists of two subunits similar in molecular weight to those of pig brain tubulin and common to most reported tubulins. This is in contrast to the tubulin from another simple eukaryote, the fungus *Allomyces neomoniliformis*, which has only a mol. wt 30 000 [18].

The immunological cross reactivity of *D. discoideum* tubulin against anti-pig brain tubulin antibody suggests a significant conservation of amino acid sequence in the tubulin of these evolutionary divergent species. A similar cross reactivity is observed between antibodies against sea urchin spermatozoon tubulin and that of mammalian sources [19] and between antibodies prepared against the tubulin β-subunit of *Chlamydomonas reinhardii* flagella with pig brain tubulin [20]. Furthermore the first 24 amino acids of α tubulin subunit from chick brain and sea urchin flagella are identical while there are 7 conservative substitutions in 25 positions of the β-subunit, demonstrating a high degree of evolutionary conservation [21]. On the basis of the results presented here one might expect *D. discoideum* tubulin to show a similar conservancy.

Three proteins of smaller molecular weight also copolymerised with the pig brain tubulin. Although proteins other than tubulin are known to be associated with tubulin in microtubules (microtubule-associated proteins) it is unlikely that these are responsible since microtubule associated proteins usually have mol. wt > 70 000 [6,22]. Since proteases are extremely active in *D. discoideum* and two cycles of copolymerisation require a total of 60 min incubation at 35°C, the proteins are more likely to be tubulin degradation products even though a protease inhibitor was present throughout these experiments. Unfortunately, copolymerisation data cannot provide an accurate indication of the cellular content of tubulin since it is not clear what proportion of *D. discoideum* tubulin is capable of copolymerisation. However, using the rocket technique of immunoelectrophoresis [10] we estimate that ~ 1% of vegetative *D. discoideum* amoebal protein is tubulin. This compares reasonably with a figure of about 0.1–0.3% of the protein as tubulin in mammalian liver and 0.4% in sea urchin eggs. In more specialised organs such as adult brain, tubulin may represent as much as 25% of the total protein [23].

Acknowledgements

We thank the SRC for financial support and Professor J. M. Ashworth for helpful discussion and valuable criticism. P. C. was supported by an EMBO fellowship.

References

[1] Moens, P. B. (1976) J. Cell Biol. 68, 113–122.
[2] Zada-Hames, I. M. (1977) J. Gen. Microbiol. 99, 201–208.
[3] Cappuccinelli, P. and Ashworth, J. M. (1976) Exp. Cell Res. 103, 387–393.
[4] Cappuccinelli, P. and Hames, B. D. (1978) Biochem. J. 169, 499–504.
[5] Hames, B. D. and Ashworth, J. M. (1974) Biochem. J. 142, 301–315.
[6] Weingarten, M. D., Lockwood, A. H., Hwo, S. Y. and Kirschner, M. W. (1975) Proc. Natl. Acad. Sci. USA 72, 1858–1862.
[7] Bonner, W. M. and Laskey, R. A. (1974) Eur. J. Biochem. 46, 83–88.
[8] Water, R. D. and Kleinsmith, L. J. (1976) Biochem. Biophys. Res. Commun. 70, 704–708.
[9] Patzelt, L., Singh, A., Marchand, Y. L. and Jeanrenand, B. (1975) J. Cell. Biol. 66, 609–620.
[10] Laurell, C. B. (1966) Anal. Biochem. 15, 45–54.
[11] Wilson, L., Bamburg, J. R., Mizel, S. B., Grisham, L. M. and Cresswell, K. M. (1974) Fed. Proc. Fed. Am. Soc. Exp. Biol. 33, 158–166.
[12] Zada-Hames, I. M., Ashworth, J. M. (1978) in: Development and Differentiation in the Cellular Slime Moulds (Cappuccinelli, P. and Ashworth, J. M. eds) pp. 69–79, Elsevier/North-Holland, Biomedical Press, Amsterdam.
[13] Haber, J. E., Peloquin, J. G., Halverson, O. and Borisy, G. G. (1972) J. Cell Biol. 55, 355–367.
[14] Bryan, R. G. (1972) Biochem. 11, 2611–2616.
[15] Owellen, R. J., Owens, A. H. and Donigian, D. W. (1972) Biochem. Biophys. Res. Commun. 47, 685–691.
[16] Hart, J. W. and Sabnis, D. D. (1973) Planta 109, 147–152.
[17] Wilson, L. and Meza, J. (1973) J. Cell Biol. 58, 709–719.
[18] Olson, L. W. (1973) Arch. Microbiol. 91, 281–286.
[19] Weber, K., Pollack, R. and Bibring, T. (1975) Proc. Natl. Acad. Sci. USA 72, 459–463.

[20] Piperno, G. and Luck, D. J. L. (1977) J. Biol. Chem. 252, 383–391.
[21] Luduena, R. F. and Woodward, D. O. (1973) Proc. Natl. Acad. Sci. USA 70, 3594–3598.
[22] Keates, R. A. B. and Hall, R. H. (1975) Nature 257, 418–421.
[23] Rapoport, E., Bekley, P. D. and Bucher, N. L. R. (1975) Anal. Biochem. 69, 92–99.