MICROTUBULE PROTEINS IN THE YEAST, *SACCHAROMYCES CEREVISIAE*

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1. Introduction

Microtubule proteins, particularly tubulin, have been isolated and characterised from a range of higher eukaryote tissues [1,2] and cultured cells [3]. In virtually all the higher eukaryotes the main protein tubulin occurs in its native state as a heterodimer. The two constituent subunits, α and β tubulin, of ~55 000 mol. wt, can be separated on SDS–polyacrylamide gel electrophoresis. The microtubules and microtubule proteins of non-flagellated lower eukaryotes have received very little attention. However, organisms such as the yeast *Saccharomyces cerevisiae* have great potential as systems for the study of microtubule function. Studies with these organisms are hampered by the low tubulin concentration in cell extracts [4]. Tubulin from various cell types will copolymerise with brain microtubules. However, it is known that cell extracts inhibit the assembly of microtubules in vitro [5,6]. We have markedly reduced the inhibitory effects of yeast cell extracts by using RNase and DNase treatment, proteolysis inhibitors and a strain of yeast which posesses very reduced protease activity. Using the above precautions with brain microtubule protein depleted in microtubule-associated proteins we have been able to identify radiolabelled yeast proteins which, by their behaviour during repeated cycles of copolymerisation with brain microtubule and chromatography on phosphocellulose, are candidates for yeast tubulins and MAPs. These include a yeast protein comigrating with brain α-tubulin on SDS–polyacrylamide gels. The major possible yeast MAP has mol. wt 49 000. There is apparently no yeast protein which comigrates with brain β-tubulin.

Abbreviations: DMF, dimethyl formamide; DMSO, dimethyl sulphoxide; EGTA, ethylene glycol-bis (β-amino ethyl ether) tetra-acetic acid; MAPs, microtubule-associated proteins; PIPES, piperazine-*N*,*N*'-bis(2-ethane-sulphonic acid); PMSF, phenyl methyl sulphenyl fluoride; SDS, sodium dodecyl sulphate; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone HCl

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2. Materials and methods

Saccharomyces cerevisiae strain 20B-12, a mutant deficient in proteases A,B and C [7] was grown in a defined medium using $(NH_4)_2SO_4$ as the nitrogen source. Cultures were incubated for 16 h at 25°C on an orbital shaker, harvested by filtration onto a 1.2 μm Millipore membrane, washed with warm sulphate-free medium (a medium in which all the sulphates in the defined medium were replaced by chlorides) and finally resuspended in 50 ml sulphate-free medium containing 1 mCi Na_2 $^{35}SO_4$ (Radiochemical Centre, Amersham). Cells were incubated in this medium for 4 h at 30°C in a reciprocal shaking water bath.

Cells were collected by filtration, washed in sulphate-free medium and finally suspended in twice the pellet volume of PEMG buffer (0.1 M PIPES, 2 mM EGTA, 1 mM $MgSO_4$, 1 mM GTP, pH 6.9) containing 1 mM PMSF in 1% (v/v) DMF; 200 μM TLCK; 50 μg/ml leupeptin. The cells were broken by vortex mixing with an equal volume of cold 0.45 mm diam. glass beads. The extract was pipetted away from the beads and centrifuged at 130 000 × *g* for 30 min at 4°C.

Microtubule protein was prepared from fresh sheep brain as in [8]. Immediately before use

twice-polymerised microtubule protein was resuspended in cold PEMG buffer, depolymerised at 0°C for 30 min, and centrifuged at 130 000 × *g* at 4°C for 30 min. Tubulin was prepared from this supernatant using a modification of the method in [9] in which protein was polymerised in 0.4 M PIPES, 1 mM EGTA, 0.5 mM $MgSO_4$, 1 M glycerol, 10% DMSO, 0.5 mM GTP (pH 6.9) at 37°C for 20 min and collected by centrifugation at 130 000 × *g* for 20 min. Most of the MAPs remained in the supernatant and were discarded. The tubulin enriched pellet was resuspended in PEMG buffer, depolymerised and centrifuged as for the whole microtubule protein. The supernatant was added to ^{35}S-labelled yeast extract. At this stage ribonuclease A (EC 3.1.4.22) (2 μg/ml) and deoxyribonuclease 1 (EC 3.1.4.5) (1 μg/ml) (Sigma) were added.

Co-polymerised microtubules were formed by warming the mixture at 37°C for 30 min and centrifugation at 130 000 × *g* for 30 min at 26°C. This first 'hot pellet', HP_1, copolymer was resuspended in PEMG minus glycerol, depolymerised at 4°C for 30 min and centrifuged at 130 000 × *g* for 30 min at 4°C. The supernatant was adjusted with glycerol to final conc. 4 M, and the process repeated twice more to produce copolymerised pellets, HP_2 and HP_3. Protease inhibitors were present throughout at the above concentrations.

The three times polymerised copolymer pellet (HP_3) was resuspended in cold buffer (0.025 M PIPES, 0.5 mM EGTA, 0.25 mM $MgSO_4$, 0.1 mM GTP, pH 6.9) and applied to a Whatman P11 phosphocellulose column equilibrated in this resuspension buffer. Material bound to the column was eluted with a stepwise gradient of KCl in column buffer (further details in legend to fig.3). Fractions eluted from the column were collected, mixed with PCS scintillation cocktail (Hopkin and Williams Ltd, Chadwell Heath, Essex) and the radioactivity determined in a Packard 3003 Tricarb Liquid Scintillation Counter.

Phosphocellulose was prepared by initial swelling and de-fining in distilled water (15 ml/g powder) followed by successive 1 h equilibration in 0.5 M NaOH and 0.5 M HCl and washed with 1 litre of distilled water after each stage. The ion-exchanger was finally resuspended and stored in 0.025 M PIPES adjusted to pH 6.9 with NaOH.

SDS–polyacrylamide gel electrophoresis was performed by the Laemmli method [10] in 1 mm thick slabs of 6% acrylamide with a 2.5% acrylamide stacking gel. Samples were prepared by dilution of protein into Laemmli sample buffer, or by addition of sample buffer to acetone precipitates. These were then heated at 100°C for 90 s, cooled, loaded onto gels and subjected to electrophoresis at 12 mA for 2 h. Gels were stained with 0.1% Coomassie brilliant blue in glacial acetic acid : isopropanol : water (10:25:65), dried and exposed to X-ray film (Kodirex) for 5–25 days.

Protein was determined by the Lowry method [11] using bovine serum albumin as a standard.

3. Results and discussion

Brain microtubule protein depleted in high molecular weight MAPs by polymerisation in DMSO was used for copolymerisation experiments in order to expose the maximum possible MAP binding sites to the yeast extract.

Copolymerisation was performed in 4 M glycerol to facilitate the polymerisation of the MAP-depleted tubulin. Preliminary experiments have shown that yeast extracts inhibit the assembly of tubulin to a lesser extent in glycerol buffer. Also, the inhibitory activity of the extract is substantially reduced by the use of RNase and a strain of yeast deficient in proteases.

Figure 1 shows autoradiograms of SDS–polyacrylamide gels of the first and second copolymer pellets (HP_1 and HP_2, respectively). HP_1 contains a large number of yeast proteins, most of which disappear during the second cycle of assembly-disassembly. The major yeast proteins remaining associated with the microtubules throughout two cycles of assembly have mol. wt ~49 000 and ~55 000. Other less abundant proteins have mol. wt ~73 000, ~110 000, ~130 000, ~200 000 and ~230 000.

The 55 000 mol. wt yeast protein migrates as a diffuse band in the same position as brain α-tubulin. Direct comparison of the stained gel and its autoradiogram (fig.2) suggests that this yeast protein is homologous to the brain α-tubulin. Figure 2 also shows that one of the less abundant yeast proteins has a mobility just greater than the brain β-tubulin and mol. wt ~52 000. There is, however, apparently no

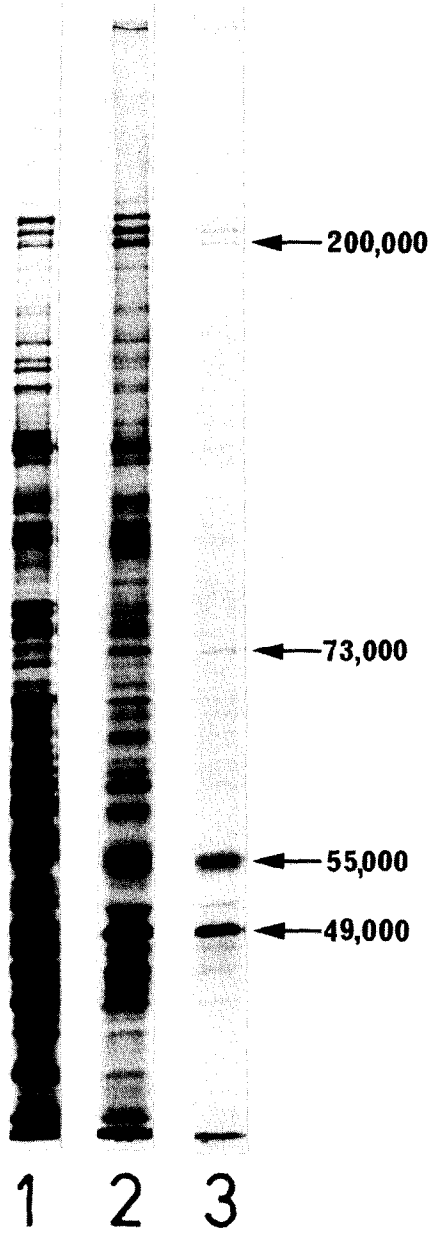

Fig.1. Autoradiograms of SDS–polyacrylamide gels of: (1) yeast soluble proteins; (2) HP_1 copolymer; (3) HP_2 copolymer. Figures denote molecular weights of major bands.

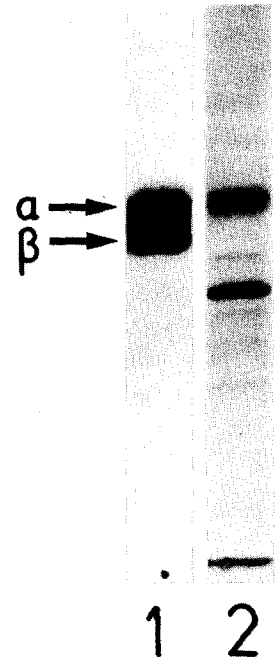

Fig.2. The tubulin area of an SDS–polyacrylamide gel of a HP_2 copolymer: (1) is the protein stained with Coomassie brilliant blue; (2) is the autoradiogram of the stained gel (1).

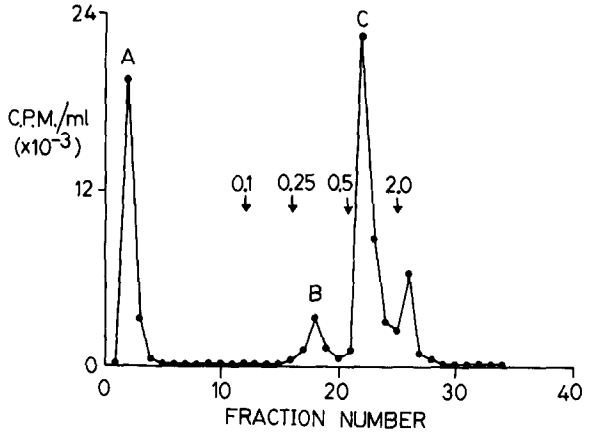

Fig.3. Separation of radioactive yeast proteins in a HP_3 copolymer. 2.4 mg protein in 1.6 ml were applied to a 3 × 1 cm phosphocellulose column at 4°C. Flow rate was ~ 20 ml/h; 2 ml fractions were collected. Arrows denote the addition of 10 ml each of 0.1 M, 0.25 M, 0.5 M and 2.0 M KCl.

yeast protein that comigrates with brain β-tubulin. The other major protein in this area of the gel has mol. wt ~49 000.

The elution of radioactivity from phosphocellulose after application of a copolymer HP_3 is shown in fig.3. The pattern of yeast proteins appearing in each peak, as compared with the starting material, is shown in fig.4. Peak A in fig.3 is the unbound fraction which contains the brain tubulin and two radioactive yeast proteins. These are the proteins which comigrate with the brain α-tubulin and the 52 000 mol. wt protein. The behaviour of these two proteins in copolymerisation and ion exchange chromatography makes them strong candidates to be yeast tubulins. Similar results in *Aspergillus nidulans* were shown [12] where the α-tubulins show homology with brain α-tubulin. There is, however, less homology between the *Aspergillus* β-tubulin and brain β-tubulin.

Peaks B and C from the phosphocellulose column contain the majority of the associated proteins. The 49 000 mol. wt protein is eluted with 0.5 M KCl and thus behaves in a similar way to the 49 000 mol. wt protein identified as a MAP in neuroblastoma, C_6 glioma and CHO cells [3]. The behaviour of these proteins during repeated cycles of polymerisation and phosphocellulose chromatography suggests that the 49 000 and 110 000 mol. wt proteins may be yeast MAPs. Preliminary experiments indicate that the critical concentration for polymerisation of the copolymer is lower than that of a control. Also, we have evidence of ring-like structures in the cold depolymerisation copolymer whereas none are visible in our MAP-depleted brain protein preparations.

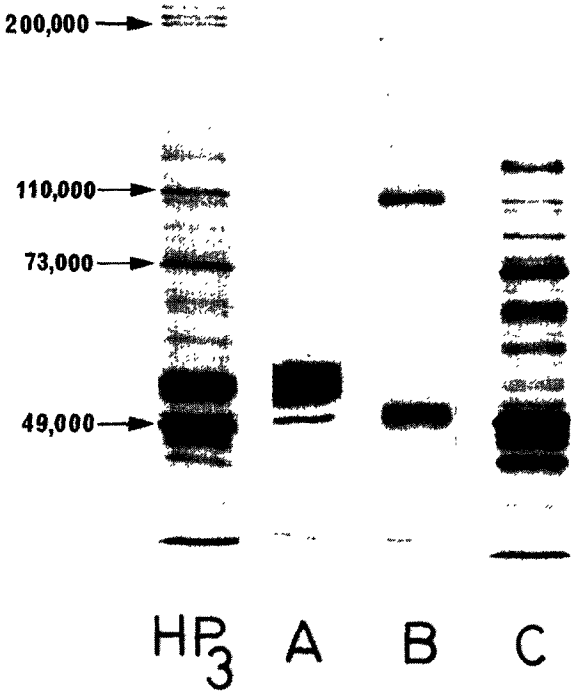

Fig.4. Autoradiograms of SDS–polyacrylamide gels of yeast proteins in the HP_3 copolymer and peaks A–C from the phosphocellulose column. Protein was precipitated from the column fractions by addition of an equal volume of cold acetone and a precipitate allowed to form overnight at –20°C. 50 μl Laemmli sample buffer was added to the resulting precipitate and treated as in section 2.

Acknowledgements

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