IMMUNOLOGICAL CHARACTERISATION OF THE MICROTUBULE-ASSOCIATED PROTEIN MAP_2

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1. Introduction

When microtubules are assembled from mammalian brain extracts in vitro, several proteins other than tubulin copurify in constant stoicheiometry to tubulin as judged by SDS–electrophoresis [1]. Among these microtubule-associated proteins are two or more high molecular weight polypeptides with chain weights >250 000 and a group of polypeptides (tau) with chain weights of 55 000–70 000. Both a 280 000 mol. wt polypeptide(s), designated MAP_2 and the tau proteins apparently share the ability to increase the rate of assembly of pure tubulin in vitro [2], and further, both proteins have been shown to be present along the length of microtubules in situ by using specific antibodies [3,4]. It thus seems likely that some of these proteins at least are integral parts of the functional microtubule.

MAP_2 is a particularly interesting protein since it appears to be the major component of the side arms of microtubules seen by both negative staining and by transmission electron microscopy [2] where it appears to form an organised lattice on the microtubule surface [5,6]. Most models relating to the function of microtubules in cells imply interaction between microtubules and other subcellular organelles (see [5] for discussion), thus the role of a side arm protein may be crucial for the specificity and modulation of such an interaction. Indeed there is some evidence that microtubule-associated proteins are necessary for the interaction in vitro between actin filaments [7] or secretory granules [8] and microtubules. It may also be pertinent that MAP_2 is the preferred substrate for a microtubule-associated cyclic AMP-dependent protein kinase [9], although there is no evidence at present linking phosphorylation of MAP_2 to a change in its properties.

Biochemical characterisation of microtubule-associated proteins has been complicated by several factors; firstly different preparative procedures for the isolation of microtubules have given rise to different apparent stoicheiometries for these proteins in relation to tubulin (see [9]). These difficulties have been further exacerbated by the susceptibility of the high molecular weight polypeptides to proteolytic hydrolysis [10]. Thus there have been uncertainties as to whether the lower molecular weight proteins like the tau group are proteolytic degradation products of the high molecular weight polypeptides and also whether the high molecular weight polypeptides themselves, as separated by electrophoresis in the presence of SDS, are individual proteins with different functions or a group of proteins derived from one precursor.

Here, antibodies raised against electrophoretically pure MAP_2 have been used, together with direct immunochemical labelling of thin sections of SDS–polyacrylamide gels, to clarify some of these points. The data show that MAP_2 is an immunologically distinct protein from MAP_1 and from the tau group, and further that MAP_2 in brain microtubules from different mammalian species and in one avian species is immunologically indistinguishable. Considering that this antiserum crossreacted with cytoplasmic microtubules in pig ovarian granulosa cells [3] it seems likely that MAP_2 is a unique protein associated with microtubules from a variety of sources, and has maintained a high degree of evolutionary conservatism; thus strengthening the view that MAP_2 plays an important role in cytoplasmic microtubule function in vivo.

2. Materials and methods

2.1. *Materials*

Chemicals were obtained from BDH (Chemicals), Poole, Dorset, and materials for electrophoresis were of 'BDH electrophoresis grade'. Peroxidase coupled to goat anti-rabbit IgG (peroxidase-GARS) was purchased from Miles Labs., Stoke Poges, Slough, and mica sheet from Agar Aids, Stansted, Essex.

2.2. *Preparation of microtubule proteins, MAP_2 and antisera.*

Microtubules were prepared from the different brain samples by a single polymerisation step followed by centrifugation through a sucrose cushion as in [9]. Brain supernatants refer to the supernatant after the first centrifugation step for 1 h at $20\,000 \times g_{av}$. MAP_2 was isolated from microtubule proteins, and antibodies raised against it in rabbits, as detailed in [3].

2.3. *Direct immunochemical staining of SDS–polyacrylamide gels*

Samples were subjected to electrophoresis in the presence of SDS on 5% polyacrylamide gels as in [11]. The solvent front was only allowed to migrate 2–3 cm to facilitate subsequent sectioning. Gels were then fixed by immersion in 45% methanol, 7% acetic acid for 2 h at 23°C. The methanol/acetic acid was then removed by washing 2 × 30 min in 20 mM Tris–HCl (pH 7.6), 0.876% (w/v) NaCl, 0.02% (w/v) NaN_3 (TBS) prior to sectioning. The following procedure is modified from [12].

Gels were frozen onto a modified chuck in a Cambridge microtome maintained at −30°C. The gel was sectioned parallel to its long axis, 50 μm sections were used for immunocytochemistry and were transferred to mica sheets, 150 μm sections were cut for staining with Coomassie blue and these were transferred to Whatman no. 1 filter paper strips. The sections were flattened by manipulation, after flooding the mica with distilled water. The flattened sections were then heated for 3–5 min on a hotplate at 120°C to fix the section to the mica sheet.

Mica sections were placed into a humidity chamber then layered with antiserum diluted into TBS containing 0.1% (v/v) Triton N-101 to reduce background. Incubation at room temperature for 1–20 h then followed, depending on the avidity or dilution of the antiserum. Unbound antibody was removed by washing in 3 changes of TBS containing 0.025% (v/v) Triton N-101.

The sections were then incubated with peroxidase–GARS, usually a 1:25 dilution of the commercial stock, into TBS containing 0.1% (v/v) Triton N-101 for 2 h at 23°C followed by a similar wash sequence and a final rinse in distilled water.

The visualise the peroxidase, the sections were incubated for a few minutes in 0.05 M Tris–HCl (pH 7.5) containing 0.5 mg/ml 3,3′-diaminobenzidine–HCl and 0.5 μl/ml 30% (w/v) hydrogen peroxide until the bands became visible. The sections were then rapidly transferred to distilled water to prevent overreaction. The contrast of faint bands could be increased by careful exposure of the section to OsO_4 vapour in a sealed container in a fume cupboard.

3. Results and discussion

The development of the technique of direct immunochemical staining of SDS–polyacrylamide gels [12–15] has made it possible to visualise antibody specificity or antigen relationships in a simple and elegant way. It is important to note however, that although this technique does not require a precipitating antiserum like the Converse and Papermaster technique [16] not all antisera will label the appropriate antigen band when it is presented in this way (unpublished). Thus interpretation of data in terms of antibody specificity should be made with caution.

Several methods have been described for direct immunochemical staining of SDS–polyacrylamide gels. The above method was developed for two main reasons.

(i) The thickness of gel determines how long the incubation and wash times are; for whole tube gels the total wash and incubation times are ~1 week. With thin sections the equivalent time can be reduced to <1 day.

(ii) Many identical sections may be cut from the same gel which means that several experiments can be performed with a few micrograms of protein, including a Coomassie blue control.

Fig.1 shows the labelling of (an SDS gel-containing) microtubule proteins isolated from pig brain, with anti-MAP_2 antiserum. This experiment shows clearly that MAP_1 and the tau proteins are not immunologically related to MAP_2. Thus MAP_2 is not a prote-

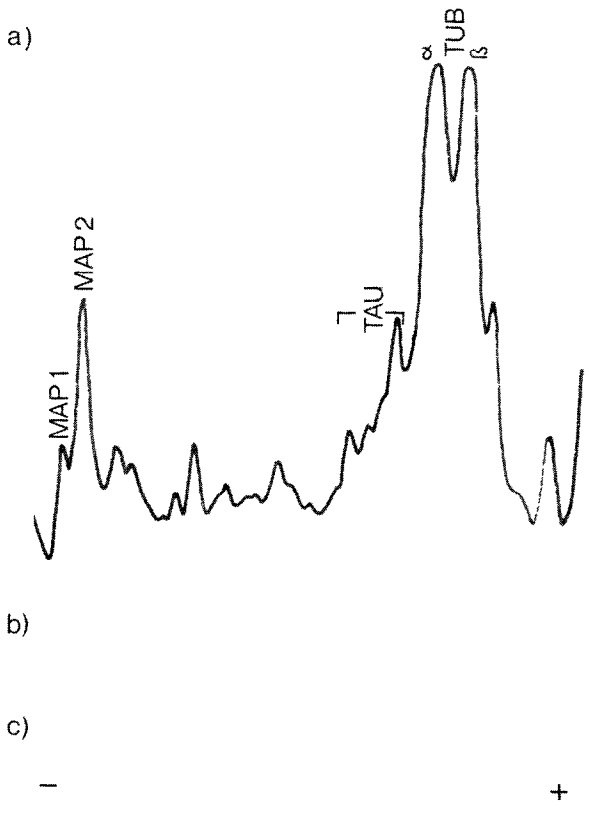

Fig.1. Direct immunochemical labelling of SDS gels containing pig brain microtubule proteins with anti-MAP_2 antiserum. Pig brain microtubules were prepared using a single polymerisation cycle, subjected to electrophoresis on 5% SDS–polyacrylamide gels and the gels subsequently labelled with anti-MAP_2 antiserum as in section 2.2. (a) Densitometric scan at 600 nm of a whole Coomassie blue-stained gel. (b) 50 μm section of parallel gel treated with anti-MAP_2 antiserum. (c) 150 μm section of same gel as in (b) stained with Coomassie blue. Gels were run from left to right.

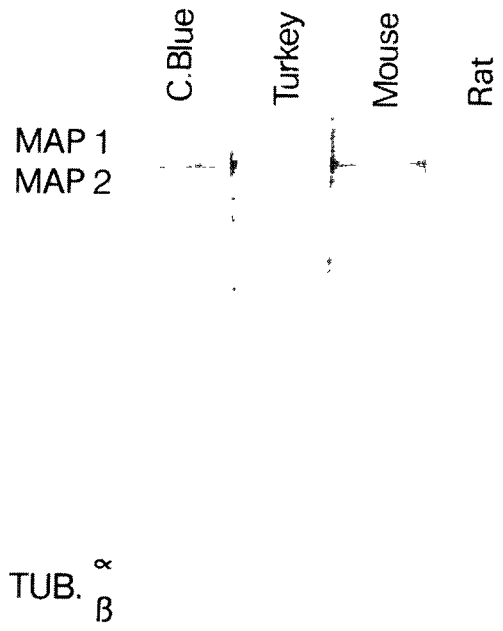

Fig.2. Direct immunochemical labelling of SDS gels containing microtubule proteins prepared from different species with anti-MAP_2 antiserum. Sections were prepared from 5% SDS–polyacrylamide gels containing microtubule proteins isolated from brain of mouse, rat and turkey. The sections were labelled with anti-MAP_2 antiserum as in section 2.2. The left gel section (turkey microtubule proteins) was stained with Coomassie blue and the right gel sections labelled with anti-pig MAP_2 antiserum.

olytic fragment of MAP_1 nor are the tau proteins derived from MAP_2 since one would expect the precursor or the proteolytic fragments respectively to contain some of the antigenic sites of the MAP_2 protein.

The role of MAP_1 in microtubule structure is far from clear. It represents on stained gels <5% of the total protein yet its presence during assembly of microtubules appears to alter the spacing between adjacent microtubules, from that seen after assembly in the presence of MAP_2 alone, suggesting that it may play some role in the orientation or structure of the MAP_2 side arms [5].

Fig.2 shows similar experiments to those shown in fig.1 using microtubules prepared from mouse, rat and turkey brain. The intensity of staining is subjectively indistinguishable from that of pig MAP_2 against which the antisera were raised, suggesting conservation of these antigenic sites on the MAP_2 of other species.

It is not known against which portion of the MAP_2 molecule the antibody population is directed, but since the antiserum will label fixed intact microtubules [3] some at least must be directed against the exposed portion of the side arm.

It is reasonable to believe that the high degree of evolutionary conservatism seen with tubulin and the actomyosin-related proteins is due in part to the constraints imposed by their requirement to assemble into functional multimolecular structures with each other. Since MAP_2 must interact with the tubulin

polymer, presumably at specific sites, then it is likely that this complementary site on MAP_2 will be conserved in parallel with the site provided by the tubulin polymer. The rest of the MAP_2 molecule, the exposed side arm, is of unknown function, but these results suggest that this portion also has antigenic sites conserved, which would imply in converse that it performs some specific recognition function.

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