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Immunological evidence for a *Physarum* β-tubulin polypeptide possessing an α-tubulin-like carboxyl terminus

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A number of well-characterised monoclonal antibodies have been produced which recognise either α- or β-tubulin. One of these antibodies, YL1/2, has been regarded as a specific probe for α-tubulin in that it recognises the linear amino acid sequence of the extreme C-terminus of the α-tubulin polypeptide. We now report that in the slime mould *Physarum polycephalum*, YL1/2 recognises the α-tubulin isotype (α_1) that is expressed in the myxamoeba, but that it discriminates between the α_1-tubulin sub-types of the plasmodium – the other major cell type in the *Physarum* life cycle; furthermore, YL1/2 does not recognise the plasmodium-specific α-tubulin isotype (α_2). Intriguingly, with plasmodial tubulin YL1/2 preferentially recognises a β-tubulin, the plasmodium-specific β_2 isotype. Reactivity with this one specific member of the β-tubulin family is abolished by treatment of the tubulin with carboxypeptidase. Thus, the plasmodium-specific β_2-tubulin of *Physarum* appears to be a β-tubulin with an α-tubulin-like carboxyl terminus.

Isotype heterogeneity; Tubulinyl-tyrosine; Differential gene expression; Monoclonal antibody; Tubulin; (*Physarum polycephalum*)

1. INTRODUCTION

Microtubules are composed of two closely related polypeptides, α- and β-tubulin, which in most organisms are encoded by a multi-gene family [1].

Recent studies of *Physarum polycephalum* have revealed a cell-type-dependent expression of tubulin isotypes in this slime mould; the tubulin isotypes are distinct, electrophoretically separable species [2,3]. The cell-type-dependent expression of tubulin isotypes in *Physarum* becomes of particular interest in view of the differential cell-type-dependent occurrence of microtubules in this organism. Essentially, the plasmodium contains no microtubules other than those of the transient mitotic apparatus; the myxamoeba possesses cytoplasmic and centriolar-associated microtubules; in the plasmodium, but not the myxamoeba, the mitotic spindle is intranuclear. This poses the question of whether there is selective isotype usage, or even function, amongst the various *Physarum* tubulins.

A number of well-characterised monoclonal antibodies have been produced which recognise either the α- or β-tubulin polypeptides. One of these antibodies, YL1/2, was raised against yeast tubulin [4] but has since been shown to have wide cross-reactivity with the tubulins of other organisms. It has been viewed as a specific probe for α-tubulin in that it is now known that it recognises the linear amino acid sequence of the extreme carboxyl terminus of the α-tubulin polypeptide [5]. The key amino acid in the YL1/2 epitope is the C-terminal residue itself. In most organisms so far studied this is a tyrosine residue and can be subject to enzymic cleavage and re-ligation [6], a cycle of post-translational modification apparently unique to α-

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Abbreviations: 2D, two-dimensional; IEF, isoelectrofocussing; PAGE, polyacrylamide gel electrophoresis

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tubulin and events which perhaps pertain to the assembly dynamics of the tubulin in vivo. YL1/2 does not bind to the detyrosinated form of α-tubulin and so is a useful probe for the tyrosinated form. We now report the unusual finding that in the slime mould *P. polycephalum* the YL1/2 monoclonal antibody recognises a unique member of the β-tubulin family, the plasmodium-specific β_2-tubulin isotype.

2. MATERIALS AND METHODS

2.1. *Culture of Myxomycetes*

Details of the particular strains of *P. polycephalum* myxamoebae (CLd_{AXE}) and plasmodia (M_3CVIII), and the methods used for their culture have been described elsewhere [2].

2.2. *Protein preparation and enzymatic cleavage*

Tubulin was purified from *Physarum* myxamoebae by two cycles of temperature-dependent assembly [7] and from *Physarum* microplasmodia by DEAE chromatography followed by a single cycle of temperature-dependent assembly [2]. Since the protein was destined for Western-blotting experiments utilising specific probes for tubulin, the plasmodial tubulin was not further purified by repeated cycling, since there is a concomitant drop in the overall yield of tubulin and the amount of the principal contaminant actin, is not easily lowered.

When required, *Physarum* microtubule protein at 2 mg/ml in 0.1 M Pipes buffer, pH 6.9, containing 2 mM EGTA, 1 mM $MgCl_2$, 0.1 mM EDTA, was treated with carboxypeptidase A (Sigma) at a protein/enzyme ratio of 100:1. Prior to use the carboxypeptidase was washed 3 times by suspension in distilled water and centrifugation, and solubilised in 0.1 M NH_4HCO_3. Digestion was allowed to proceed at 37°C for 30 min with gentle agitation and terminated by the addition of concentrated SDS gel sample buffer [8] and boiling for 2 min.

2.3. *Electrophoresis and immunoblotting*

Single-dimension SDS-PAGE was performed according to Laemmli [8] on resolving gels containing 10% acrylamides. 2D gels (IEF/SDS-PAGE), essentially similar to those of O'Farrell [9] were run as in [10]. The ampholyte gradient was generated by mixing pH 3.5–10 and pH 5–7 Ampholines (LKB) in the ratio 3:2 (v/v) [3]. Second-dimension resolving gels contained 10% acrylamides. Sample preparation and gel staining with Coomassie blue R250 were as described in [3,10].

Electrophoretic transfer of proteins [11] from gels to 0.45 μm pore nitrocellulose membrane (BA 85), and the conditions used for immunoprobing such 'blots' were essentially as described in [10] with the exception that the antibody diluent was 10 mM Tris-HCl, pH 7.2, 0.5 M NaCl, 0.25% (v/v) Tween 20. Peroxidase-conjugated, species-specific secondary antibodies were employed in conjunction with 4-chloro-1-naphthol as substrate.

2.4. *Materials*

Pipes and GTP used in the purification of tubulin, Tween 20, 4-chloro-1-naphthol and carboxypeptidase A were obtained from Sigma (England). SDS, acrylamide and methylenebisacrylamide were from Fisons (England). Urea for isoelectric focussing was from Bio-Rad (England). Ampholyte mixtures were obtained from LKB. Schleicher and Schull BA85 nitrocellulose membrane was purchased from Anderman (England). Peroxidase-conjugated anti-mouse, anti-rat and anti-rabbit immunoglobulin antisera were obtained from Dakopatts (England).

Purified YL1/2 was the kind gift of Dr J.V. Kilmartin (MRC, Cambridge, England). DM1A was the kind gift of Dr S. Blose (Cold Spring Harbor Laboratory, USA). A rabbit polyclonal antiserum specific for tyrosinated α-tubulin ('Tyr-Ab') was the kind gift of Dr J.C. Bulinski (University of California, Los Angeles).

3. RESULTS AND DISCUSSION

Tubulin was prepared from *Physarum* myxamoebae by 2 cycles of assembly-disassembly, and from plasmodium, using the methods of Roobol et al. [2,7]. Actin invariably co-purifies with tubulin when using these methods of tubulin purification and always contaminates plasmodial tubulin to a greater extent than it does myxamoebal tubulin. In order to maximise the amount of plasmodial tubulin in this instance, its purification schedule was curtailed at the initial assembly stage, resulting

in a greater actin content than may be otherwise achieved. For immunoblotting purposes this sample can be beneficial since the presence of a large amount of actin acts as an internal control for non-specific antibody binding.

A comparison of the tubulin-containing regions of Coomassie blue-stained 2D gels reveals the relative abundance of the α and β-tubulin isotypes found in these two cell types of *Physarum* (fig.1a,d). The myxamoeba expresses α_1- and β_1-tubulin isotypes, whilst the plasmodium expresses, α_1-, α_2-, β_1- and β_2-tubulin isotypes, thus the α_2- and β_2-tubulin isotypes represent plasmodium-specific tubulins. Other, identical 2D gels were subjected to Western blotting with various anti-tubulin monoclonal antibodies; the anti-α-tubulin monoclonal antibody DM1A gave a characteristic reaction pattern [12]; it detected the α_1-tubulin isotype in the myxamoebae and both α_1- and α_2-tubulin isotypes of the plasmodium (fig.1b,e). The same pattern of reactivity was shown by another anti-α-tubulin monoclonal, YOL34 [4,10]. Thus, both of these well-characterised antibodies react with all of the α-tubulin isotypes and none of the β-tubulin isotypes found in *Physarum*.

When we probed the Western blots of *Physarum* tubulins with YL1/2 we found an intriguing and unique result. As might be expected, with the myxamoebal tubulins YL1/2 detected the α_1 isotype and did not react with the β_1-tubulin isotype (fig.1c). However, with the plasmodial tubulins, YL1/2 gave only a weak reaction with the α_1-tubulin isotype staining only the periphery of the 2D gel spot; it did not detect the α_2- or β_1-tubulin isotypes, yet gave a very strong reaction with the β_2-tubulin isotype (fig.1f).

Existing information regarding the YL1/2 epitope suggests that the linear amino acid sequence recognised must occur at the extreme carboxyl terminus; it is not enough for the sequence to occur within the polypeptide chain [5]. To check that the epitope that YL1/2 recognises on the plasmodial β_2-tubulin isotype did occur at the carboxyl terminus we subjected the *Physarum* tubulins to mild digestion using carboxypeptidase.

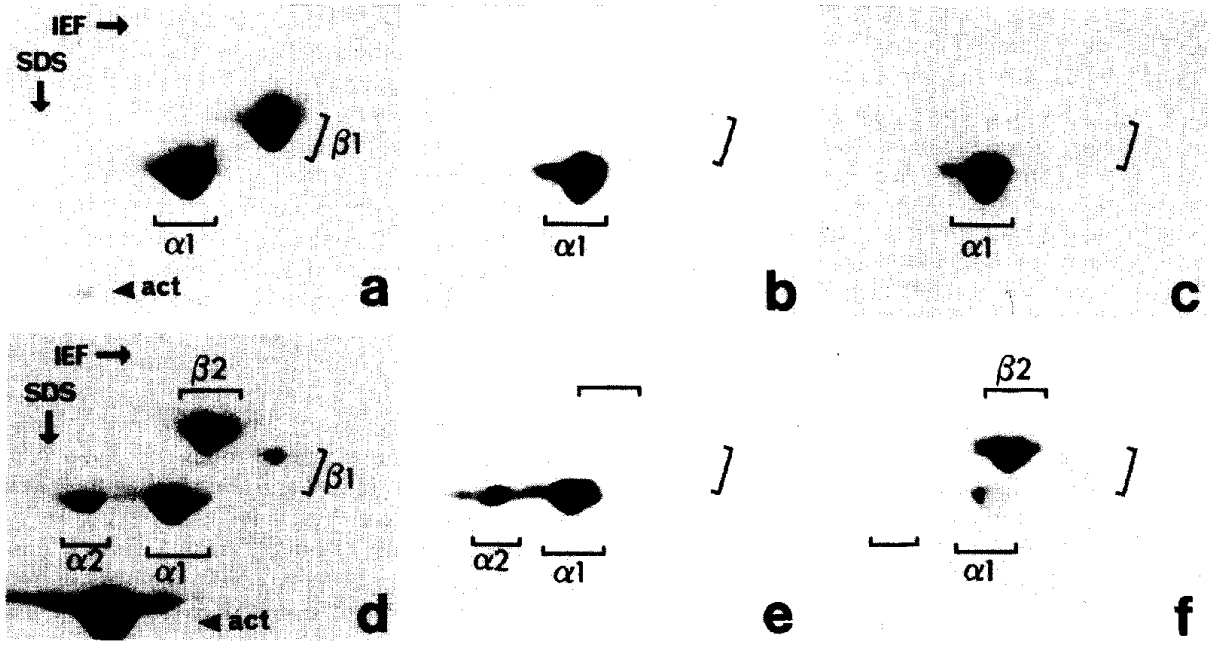

Fig.1. The panels represent either myxamoebal (a–c) or plasmodial (d–f) tubulin samples separated by 2D gel electrophoresis. Spots marked 'act' are actin; the α- and β-tubulin subunits are marked accordingly. Only the tubulin-containing regions are shown. (a,d) Gels stained with Coomassie blue R250; (b,e) immunoblots probed with the monoclonal antibody DM1A; (c,f) immunoblots probed with the monoclonal antibody YL1/2.

Fig.2 shows that treatment of myxamoebal tubulin with carboxypeptidase removed the YL1/2 epitope (but not that of DM1A, see [13]). Likewise, treatment of the plasmodial tubulins with carboxypeptidase stopped YL1/2 binding to the β_2-tubulin isotype.

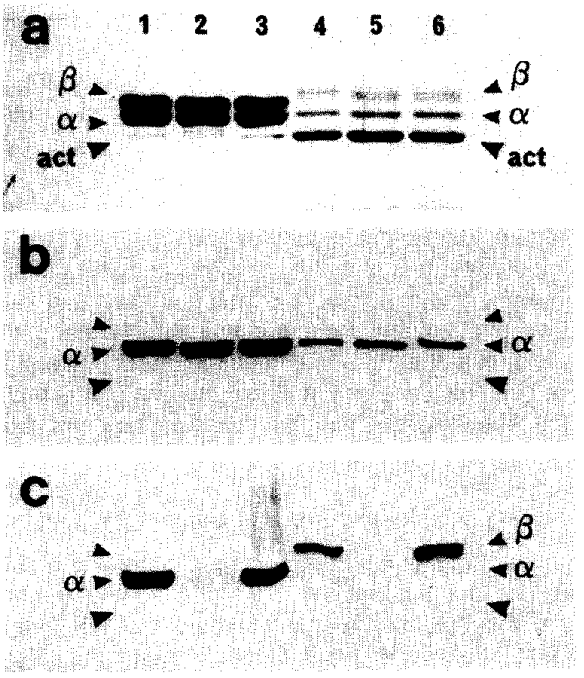

Fig.2. Single-dimensional SDS gel electrophoresis and corresponding immunoprobing of carboxypeptidase A-digested *Physarum* tubulins. Each of three panels (a–c) represents an identical gel on which the samples were: (1) normal myxamoebal tubulin; (2) myxamoebal tubulin digested with carboxypeptidase A (Sigma) for 30 min at 30°C at a protein/enzyme ratio of 100:1 (w/w); (3) myxamoebal tubulin treated as in lane 2 but without the addition of enzyme; (4) normal, partially purified plasmodial tubulin; (5) plasmodial sample digested with carboxypeptidase A as indicated above for the amoebal sample; (6) plasmodial protein digest control, treated as in lane 5 but without the addition of carboxypeptidase. (a) Coomassie R250-stained gel; (b) immunoblot probed with DM1A; (c) immunoblot probed with the monoclonal antibody YL1/2. The tubulin samples are the same as those used for the 2D gel analysis and the same disproportionate levels of actin are present. Labelling of the polypeptide bands is as described in the legend to fig.1. On such 1D SDS-PAGE gels the α_1- and α_2-tubulin bands of *Physarum* plasmodial tubulin comigrate as a single band, the β_1 and β_2 isotypes as a closely spaced doublet, but for clarity they are marked here as a single species.

One other antiserum exists that recognises the extreme C-terminus of a tubulin and is known as the 'Tyr antibody' [14] (Tyr-Ab) in that it can also bind only to the tyrosinated form of α-tubulin and not to the truncated, detyrosinated form. We used this as a further check on the specificity of the YL1/2 reactivity and as with YL1/2, β_2-tubulin was labelled strongly by the Tyr-Ab.

It was pertinent to address the question of whether there is an active tyrosination cycle in *Physarum*, and if so whether β_2-tubulin might act as a substrate. An approach used successfully in *Trypanosoma* has been to measure the incorporation of radiolabelled tyrosine in the presence of inhibitors of protein synthesis [15,16]. Protein synthesis in actively growing *Physarum* macroplasmodium could maximally be inhibited by 98.5% as judged by following the incorporation of [^{3}H]tyrosine or [^{3}H]phenylalanine in the presence of 50 μg/ml cycloheximide, 25 μg/ml puromycin and 25 μg/ml chloramphenicol. However, no significant post-translational incorporation of either radiolabelled amino acid could be detected in either α- or β-tubulins after they had been separated by gel electrophoresis and examined by fluorographic or liquid scintillation counting procedures. The residual amino acid incorporation (1.5% of the uninhibited levels) was not susceptible to carboxypeptidase A treatment. Naturally synchronous macroplasmodia were examined in this manner at 8 points throughout the cell cycle but myxamoebae could not be subjected to this approach because of their inability to utilise exogenous amino acid. However, previous work utilising purified microtubule proteins, semi-purified sheep-brain tubulin-tyrosine ligase and subcellular fractions of *Physarum* myxamoebae failed to demonstrate endogenous tubulin-tyrosine ligase activity in *Physarum*; also, *Physarum* microtubule protein was not a substrate for the sheep-brain ligase (Roobol, A. and Gull, K., unpublished). Therefore, at this juncture we have been unable to identify an active tyrosination cycle in *Physarum*.

The novel binding pattern of YL1/2 is not due to misidentification of the *Physarum* tubulin isotypes which is based on extensive experimental evidence: the myxamoebal tubulins α_1 and β_1 were

originally identified on the basis of their self-assembly into microtubules and by peptide mapping [7,17]. The plasmodial tubulins, α_1, α_2, β_1 and β_2 were identified by peptide mapping, immunoprecipitation and hybrid selection of specific mRNA by cloned tubulin DNA sequences, followed by in vitro translations [3]. Also, all four of these plasmodial tubulins are found in microtubules purified by in vitro assembly [2]. A previous immunological study utilising the well-characterised monoclonal antibodies DMIB, DM3B3, KMX-1 (all anti-β-tubulin antibodies) and YOL1/34 (anti-α-tubulin) has also served to confirm the identities of the myxamoebal and plasmodial tubulins [10], but has also revealed that there is microheterogeneity within at least the plasmodial α_1-tubulin isotype. The number of α- and β-tubulin loci in the *Physarum* genome has been examined by studies involving restriction fragment length polymorphisms and the results indicate four unlinked α-tubulin sequence loci and at least three unlinked β-tubulin sequence loci [18]. One of these loci, *betB* has been shown to be linked to an expressed β-tubulin structural gene, *benD* that encodes a β_1-tubulin isotype which is expressed in both myxamoeba and plasmodium [19]. However, no other connections have been made between tubulin DNA sequences and any of the electrophoretically defined tubulin polypeptides. Because of the low yields of *Physarum* tubulin preparations [2] and the excessive losses encountered during separation of the α- and β-subunits [20] it is unlikely that the protein sequence of plasmodial tubulins will ever be obtained directly but will be derived from the gene sequences. Ascribing the isotype identity to gene-derived polypeptide sequences will be greatly facilitated by the protein sequences of myxamoebal α- and β-tubulins [20,21]. Major differences between the isoelectric points of α_1- and α_2-tubulins are possibly indicative of non-conservative amino acid substitution between these polypeptides which may well be self-evident from primary sequences obtained in the future. Likewise, β_2-tubulin is relatively more basic than β_1 and similar identification may apply. Our present findings allow us to predict some of these relationships.

Although YL1/2 was raised against tubulin from *Saccharomyces uvarum* the epitope characterisation [5] made use of mammalian tubulin and peptides based on the C-terminal region of mammalian α-tubulin, which is -Gly-Glu-Glu-Glu-Gly-Glu-Glu-Tyr. Three major sites of importance within the YL1/2 recognition epitope were identified – the free carboxylate group must be carried by an aromatic residue, the penultimate residue which should bear a negatively charged side chain and the third residue from the end should also bear a carboxylate side chain. The corresponding C-terminus of *Physarum* myxamoebal α-tubulin does not exactly fit this model, being -Gly-Asp-Glu-Glu-Gly-Glu-Tyr) [20], but nevertheless it binds YL1/2. However, the α_1 isotype is only poorly identified in the plasmodium by YL1/2. The apparently single 2D gel spot of plasmodial α_1-tubulin in fact comprises 3 or 4 microheterogeneous subtypes [10], which merge to form the single spot during the course of the second electrophoretic separation. Plasmodial polypeptide(s) migrating at the centre of this α_1-tubulin cluster appear not to be recognised by YL1/2.

Of the several known constraints applicable to YL1/2 binding with α-tubulin perhaps the greatest is that the C-terminal amino acid should bear an aromatic side group. If certain of the plasmodial α-tubulin isotypes do lack a C-terminal aromatic amino acid rendering them YL1/2-unreactive, there are two ways in which this might occur: either (i) from the post-translational cleavage of the C-terminus or (ii) directly from the tubulin gene(s). So far we have not found any evidence in favour of (i) experimentally, but cannot discount the action of carboxypeptidases or any other possible modifications of the carboxyl terminus. The second option, that there might be gene(s) for structurally different α-tubulins which are expressed only in the plasmodium (ii) is probably of greater significance. Monteiro and Cox [22] have recently isolated a genomic clone of an α-tubulin gene which is expressed only in the plasmodium. Interestingly, this gene encodes an α-tubulin polypeptide with a divergent C-terminal region ending in a methionine residue. There is also an example of a vertebrate tubulin which possesses a divergent C-terminus, one ending with serine and localised to the chick testis [23].

Our finding that YL1/2 recognises the β_2-tubulin isotype represents a unique observation. In general YL1/2 has previously been considered

to be able to discriminate α- from β-tubulin. That carboxypeptidase A treatment removes the YL1/2 reactivity confirms the location of this epitope at the C-terminus of β_2-tubulin. However, it is likely that the altered primary sequence of β_2-tubulin that confers the unusual YL1/2 recognition property involves more than just the terminal residue itself and in fact encompasses a reasonable number of amino acid residues. Support for this interpretation comes from the original definition of the YL1/2 epitope [5] and our observation [16] that the possession of a C-terminal Tyr on the β-tubulin of *Trypanosoma brucei* [24] does not confer YL1/2 reactivity on this β-tubulin. *Physarum* β_2-tubulin must therefore contain a C-terminus which is α-tubulin-like. This characteristic is not a feature of the *Physarum* β_1-tubulin isotypes [21].

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