Use of monoclonal antibodies to analyse the expression of a multi-tubulin family

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We have used a panel of monoclonal antibodies in a study of the expression of multiple tubulins in *Physarum polycephalum*. Three anti-β-tubulin monoclonal antibodies, DM1B, DM3B3 and KMX-1 all reacted with the β_1-tubulin isotypes expressed in both myxamoebae and plasmodia. However, these antibodies showed a spectrum of reduced reactivity with the plasmodial β_2-tubulin isotype – the competence of recognition of this isotype was graded DM1B > KMX-1 > DM3B3. The anti-α-tubulin monoclonal antibody, YOL 1/34 defined the full complement of *Physarum* α-tubulin isotypes, whilst the anti-α-tubulin monoclonal antibody, KMP-1 showed a remarkably high degree of isotype specificity. KMP-1 recognises all of the myxamoebal α_1-tubulin isotypes but only recognises 3 out of the 4 α_1-tubulin isotypes expressed in the plasmodium (which normally focus in the same 2D gel spot). KMP-1 does not recognise the plasmodial specific α_2-tubulin isotype. This monoclonal antibody reveals a new level of complexity amongst the tubulin isotypes expressed in *Physarum* and suggests that monoclonal antibodies are valuable probes for individual members of multi-tubulin families.

Monoclonal antibody *Tubulin* Physarum *Microtubule* *Western blotting* *Gene family*

1. INTRODUCTION

Tubulins from different species demonstrate a remarkably high degree of evolutionary conservation of their primary structure. However, there is an increasing number of reports relating to the numbers of microheterogeneous tubulin isotypes that are encountered in organisms, tissues and even single cells [1–6]. This microheterogeneous population of tubulin isotypes can result from both post-translational modifications and the differential expression of often numerous tubulin genes [1–5]. Owing to the diversity of tubulin isotypes which can be identified in a single organism, much speculation has arisen as to the possibility of there being microtubule populations composed of individual isotypes of tubulin and further, that such differences may have implications for specific microtubule functions.

The slime mould *Physarum polycephalum* is well suited to such an investigation of the isotype variability of tubulin. It has already been established that this organism exhibits cell-type specific [7,8] and cell cycle-phase dependent tubulin expression [9]. In addition, cell biological studies have revealed that very different microtubular structures are elaborated in the different cell types of the organism's life cycle. The organism is par-

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Abbreviations: BSA, bovine serum albumin; IEF, isoelectric focussing; PEG 1500, polyethylene glycol, average M_r 1500; Pristane, 2,6,10,14-tetramethylpentadecane

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ticularly useful since the tubulin isotypes have defined coordinates on 2D polyacrylamide gels. Myxamoebae express α_1- and β_1-tubulin isotypes, whilst the plasmodium expresses α_1-, α_2-, β_1- and β_2-tubulin isotypes. The same range of isotypes is also produced as the product of in vitro translation of mRNAs isolated from the plasmodium or myxamoebae.

A major aim of studies on tubulin gene families has been to link a particular gene with a tubulin polypeptide that has unique electrophoretic coordinates. In this context, the use of mutational analysis of selectable markers has been of great value. For example, in *Drosophila* sterile males which possess a mutation [10] in a particular β-tubulin gene (expressed only in the testis) and selection of *Aspergillus* mutants resistant to anti-microtubule drugs [11] are 2 routes which have proved to be of great value in recognising individual members of these organisms' multi-tubulin families at the gene and protein level. Here we report the use of a novel approach which seeks to use monoclonal antibodies to recognise specific tubulin isotypes expressed within one organism. This approach has general relevance; however it is particularly useful when applied to the *Physarum* system since 2 of the tubulin isotypes are expressed only in the plasmodium, a syncytium which is unsuited to selectional genetics.

In the application of a panel of both novel and well characterised existing monoclonal antibodies we have encountered various levels of discriminatory ability. The monoclonal antibody YOL 1/34 [12] defined the full complement of *Physarum* α-tubulins. Three anti-β-tubulin monoclonal antibodies, DM1B, DM3B3 [13] and KMX-1 all reacted with the β_1-tubulin isotype but produced a spectrum of reduced reactivity with the plasmodial β_2-tubulin isotype. One anti-α-tubulin monoclonal antibody, KMP-1 produced in this study, demonstrated a remarkably high degree of species and isotype specificity. KMP-1 is able to discriminate between myxamoebal and plasmodial α_1-tubulin isotypes and is completely unable to recognise the plasmodial α_2 isotype. In the plasmodium, KMP-1 can discriminate between α_1-tubulin isotypes that normally focus in the same 2D gel spot. In this context KMP-1 reveals new levels of complexity amongst the tubulin isotypes expressed by *P. polycephalum*.

2. MATERIALS AND METHODS

2.1. *Materials*

Tissue culture media and foetal calf serum (FCS) were obtained from Gibco, the plasticware was Falcon and Nunc. Male mice of the BALB/cByJ strain were obtained from the Jackson Laboratory, Bar Harbor, ME. Acrylamides and SDS were the electrophoretic purity grades from Fisons, England.

2.2. *Myxomycete culture and tubulin preparation*

Growth conditions for the slime mould and the preparation of tubulin were as described [7,14].

2.3. *Production of monoclonal antibodies*

BALB/c mice were inoculated with 20–50 μg of purified *P. polycephalum* tubulin per injection. For initial inoculations, the antigen was emulsified with an equal volume of Freund's complete adjuvant but repeat injections were made in phosphate-buffered saline (PBS) alone, each injection having a total volume of 0.2 ml, applied intraperitoneally. Splenectomy was performed 4 days after the final inoculation and the splenocytes fused with approx. 1/3 of their number of Sp2-O/Ag-14 hybridoma cells using PEG 1500, essentially by the method of Galfre et al. [15] as described by Reading [16]. ^{137}Cs-irradiated normal BALB/c splenocytes were used as feeder cells. The medium in each well was sampled for a tubulin binding assay between 14 and 21 days postfusion. Cloning of successful wells was performed by limiting dilution and thrice-cloned cells were used to prepare ascites fluid in BALB/c mice primed with Pristane (Aldrich). Typing of the subclass of each antibody was carried out by double immunodiffusion against specific antisera obtained from Miles Scientific, England.

The binding assay used for all the screening stages was essentially that of Reading [16] adapted for use with the 10 μl wells of Terasaki plates. The plates were prepared by drying 10 μl of a 10 μg/ml protein solution in each well. Before use any excess protein was removed by washing with PBS (pH 9.0) and blocking performed with polyvinylpyrrolidone-BSA as described [16]. Culture supernatants were incubated for 60 min at 25°C in wells containing *Physarum* myxamoebal tubulin, sheep-brain tubulin or bovine haemoglobin (control).

Peroxidase conjugated anti-mouse IgG and IgM antisera (Tago) were mixed, each at a dilution of 1:300, and incubated for 10 min at 25°C. The substrate used for colour development was 2,2-azinobis(3-ethylbenzthiazolinesulphonic acid) (ABTS, Sigma).

2.4. *Electrophoresis and immunoblotting*

IEF-SDS 2D gels were prepared and run according to the method of O'Farrell [17] as previously modified [8]. Gels which were not to be Western-blotted were stained with Coomassie brilliant blue R250.

Transfer of proteins from SDS-containing gels onto nitrocellulose membrane (BA85, 0.45 μm pore) (Schleicher and Schüll) was performed electrophoretically at 100 mA overnight followed by 400 mA for 30 min (electrode separation of approx. 10 cm), in 192 mM Tris, 25 mM glycine (pH 8.3), containing 20% (v/v) methanol [18]. The resulting blots were not blocked with an excess of protein but instead treated throughout in the presence of the non-ionic detergent Tween 20 (Sigma). Each nitrocellulose sheet was washed twice for 10 min with 10 mM Tris-HCl (pH 7.4), 140 mM NaCl (TBS) containing 0.1% (v/v) Tween 20 (TBS-Tween). Ascites preparations of antibodies KMX-1, KMP-1 (see section 3 for details) and YOL 1/34 were diluted 1:1000, DM1B and DM3B3 were diluted 1:500 in a high salt/Tween buffer (HST buffer) – 10 mM Tris-HCl (pH 7.4), 1.0 M NaCl, 0.5% (v/v) Tween 20; the antibody solutions were applied to the blots in sealed plastic bags and incubated at room temperature, with shaking, for 45–60 min. The nitrocellulose was washed over a 45 min period with at least 6 changes of TBS-Tween and one wash with HST buffer. Peroxidase conjugated rabbit anti-mouse Ig antiserum (P260, from Dakopatts, England) was diluted 1:500 in HST buffer, applied to the blots and subsequently washed out as described for the primary antibodies. A final 5 min wash in TBS was given before colour development with the substrate 4-chloro-1-naphthol; 6 ml of a 0.3% (w/v) methanolic solution diluted to 100 ml with TBS containing 25 μl of 30% H_2O_2. Both virgin electroblots and, where applicable, those processed immunologically were stained with naphthol-blue black [18].

3. RESULTS

Several monoclonal antibodies to tubulin have been used here, two, KMX-1 and KMP-1 were novel to this study, the others were gifts as follows: DM1B and DM3B3 were the gift of Dr S. Blose (Cold Spring Harbor). Both were raised against chick brain microtubules and both react specifically with the β-subunit of tubulin but differ in that they bring about different intracellular events when micro-injected into mammalian tissue culture cells [13]. YOL 1/34 was a gift from Dr J.V. Kilmartin (MRC Laboratory of Molecular Biology, Cambridge). Although raised against tubulin from *Saccharomyces*, YOL 1/34 demonstrates a high degree of species cross-reactivity and is specific for the α-subunit of tubulin [12]. KMX-1 and KMP-1 were generated during the course of this study using standard hybridoma techniques after inoculating mice with *Physarum* myxamoebal tubulin. KMX-1 showed anti-β-tubulin specificity on western blots of 1D gels and was typed as an IgG2b immunoglobulin. KMP-1, an IgM antibody, bound to α-tubulin on 1D blots of *Physarum* tubulin but not at all to mammalian brain tubulin samples. Subsequent protracted examination by Western blotting has confirmed that KMP-1 appears to be specific for myxomycete tubulin and does not recognise tubulin from various fungi, algae, higher plants, avian, insect or several mammalian sources (C.R. Birkett et al., unpublished). In contrast, and more typically for anti-tubulin antibodies, KMX-1 does react with tubulin from these same sources.

3.1. *Myxamoebal tubulins*

The panel of monoclonal antibodies was used to probe electroblotted 2D gels of myxamoebal tubulin (fig.1). Using the IEF gradient described, *Physarum* myxamoebal tubulin migrates as 2 major spots, α_1 and β_1; the α-tubulin has greater mobility than the β in the SDS dimension (fig.1a). For the 2 anti-α-tubulin and 3 anti-β-tubulin antibodies the results were as expected and no cross-recognition of the alternative tubulin subunit was detected. YOL 1/34 and KMP-1 detected only the myxamoebal α_1-tubulin isotype whilst KMX-1, DM3B3 and DM1B all detected the myxamoebal β_1-tubulin isotype only.

Recently Burland et al. [19] have described a

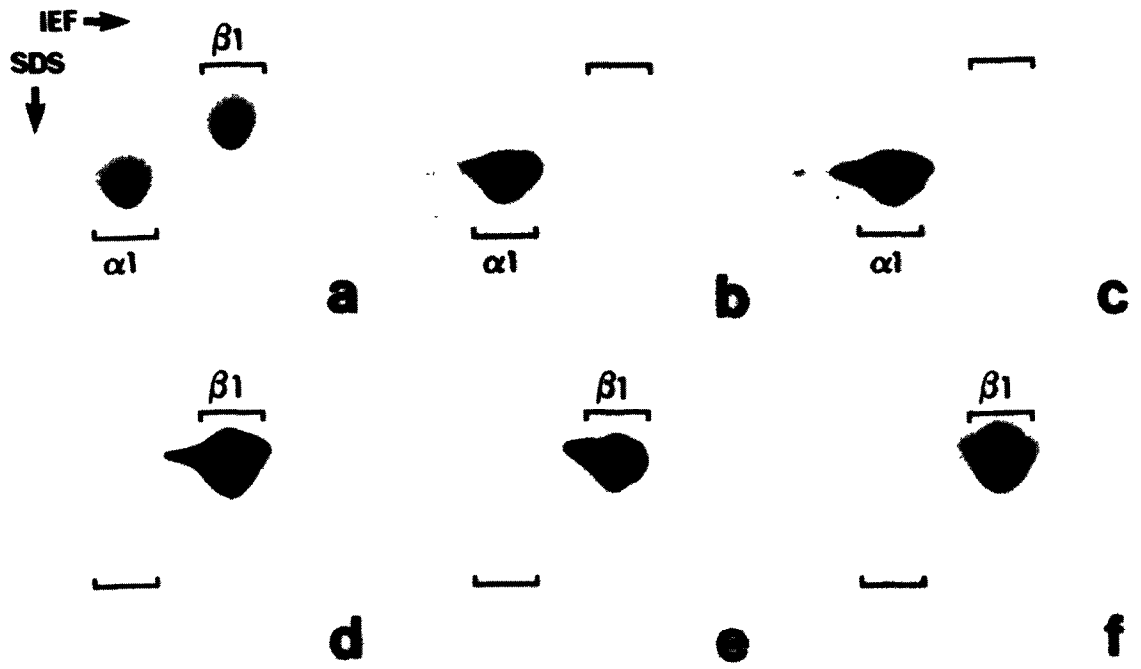


Fig.1. Purified tubulin from *Physarum* myxamoebae separated by 2D gel electrophoresis; only the tubulin-containing region is shown. Panel (a) is a gel stained with Coomassie brilliant blue showing the orientation of the tubulin subunits and a small amount of actin which inevitably remains. Panels (b–f) each represent gels identical to that in (a) but electroblotted onto nitrocellulose and then probed with monoclonal antibodies as follows: (b) YOL 1/34, (c) KMP-1, (d) DM1B, (e) KMX-1 and (f) DM3B3. None of the antibodies recognises the alternative available tubulin subunit or actin. The direction of migration in the IEF (acidic protein on the right) and SDS dimensions are indicated.

Physarum myxamoebal mutant BEN 210 which expresses a β-tubulin with novel electrophoretic mobility in addition to a β-tubulin with the wild-type mobility. This and other evidence suggests that at least 2 β-tubulin genes are expressed in the *Physarum* myxamoebae [19,20]. In wild-type myxamoebae the products of these genes are β_1-tubulins that possess identical 2D gel coordinates. Fig.2a shows the 2D gel pattern of the tubulins in an axenic strain carrying the *benD210* mutation (strain BEN 210). The anti-β-tubulin monoclonals KMX-1, DM3B3 and DM1B cannot distinguish between these myxamoebal β_1 subtypes.

3.2. *Plasmodial tubulins*

The 2D gel profile of plasmodial tubulin differs from that of the myxamoebae in that it contains 4 distinct tubulin isotypes, α_1, α_2, β_1 and β_2 of which the α_1 and β_1 isotypes have mobilities similar to those of the myxamoebal α_1 and β_1 polypeptides, respectively (fig.3a). The relative abundance of the 4 isotypes is $\beta_2 \geqslant \alpha_1 > \alpha_2 > \beta_1$.

The antibody YOL 1/34 detected both α_1- and α_2-tubulin isotypes and the intensity of the reactions is a reflection of the relative abundance of these 2 α-tubulin isotypes ($\alpha_1 > \alpha_2$) (fig.3). Electroblots probed with KMP-1 revealed a novel pattern. α_2-tubulin was not detected at all by KMP-1 and moreover, the α_1-tubulin gel spot was stained as 2 distinct zones. Post-staining of the probed immunoblot revealed that the antibody bound only at the lateral extremities of the expected α_1-tubulin gel spot, the major central region remaining undetected. The presence of protein in this central region was confirmed by the post-staining of KMP-1 immunoblots with amido black, or more conclusively by re-probing KMP-1 probed immunoblots with YOL 1/34, which resulted in the staining of this central zone. As a control, blots

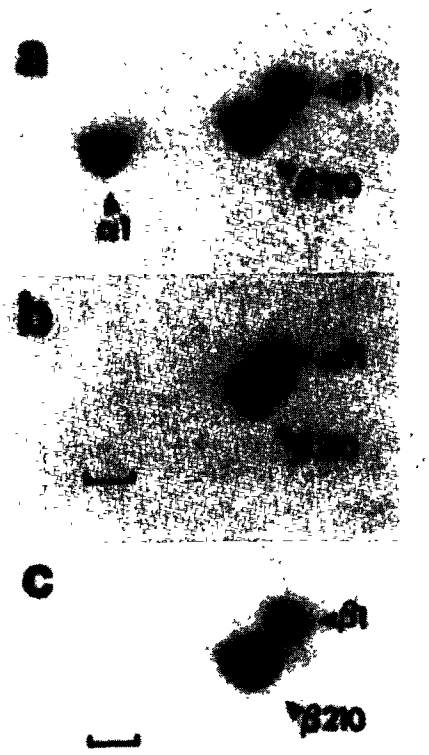


Fig.2. Tubulin containing regions of 2D gels. The sample was semi-purified tubulin from the mutant myxamoebal strain of *Physarum*, BEN 210. Panel (a) is a stained gel, (b) an immunoblot probed with KMX-1 and (c) an immunoblot probed with DM3B3. The tubulin subunits are marked accordingly, β210 being the β-tubulin novel to the mutant. The orientation of the gels is the same as in fig.1.

were probed twice sequentially with KMP-1 but this central area always remained unstained (not shown). To test that we were not merely observing a concentration-dependent phenomenon, gels which were heavily overloaded with plasmodial tubulin were probed in the same manner and with up to a 4-fold increase in the stated antibody concentration; in each case the same finding was confirmed. Immunoblotting by the method of Towbin et al. [18], substituting peroxidase secondary antibody instead of ^{125}I-labelled immunoglobulin, also returned the same result.

Probing western blots with KMP-1 therefore reveals the presence of multiple α-tubulin isotypes even within the plasmodial α_1-tubulin 2D gel spot. To obtain a more definitive analysis of these isotypes we subjected 1D IEF stick gels to electroblotting. Under these conditions the myxamoebal α_1-tubulin was defined by KMP-1 as a cluster of 3 α-tubulin isotype bands, a major species with one minor species on both the acidic and basic side. The grouping of these α_1 isotype bands was such that their fusion during second dimension SDS electrophoresis would be expected to produce the droplet-shaped 2D gel spot which was visualised by Coomassie staining and by immunoblotting with these 2 antibodies (fig.1). There was no detectable difference between the patterns defined by YOL 1/34 or KMP-1.

Plasmodial tubulins were also separated by 1D IEF and, after blotting, the nitrocellulose filters were reacted with KMP-1. In this case KMP-1 detected 3 discrete bands (1 major, 2 minor) in the α_1-tubulin region, with a significant gap between the 2 most basic bands (fig.4b). This gap provides the reason for the characteristic 'hollow spot' revealed by probing 2D gels with KMP-1 (fig.3c). When YOL 1/34 was used to probe blots of 1D IEF gels of plasmodial tubulins 4 α_1 isotype bands were revealed; the additional band revealed by YOL 1/34 was a major band occupying the space in the α_1 isotype cluster left after probing with KMP-1. KMP-1 did not detect the plasmodial α_2-tubulin isotype on the blots of the 1D IEF gels (fig.4b). Thus, the KMP-1 monoclonal antibody can be viewed as being highly isotype specific in that it reacts with only 3 of the 4 isotypes that migrate in the plasmodial α_1-tubulin 2D gel spot and it does not react with the plasmodial α_2-tubulin isotype.

The 3 anti-β-tubulin monoclonal antibodies, DM1B, DM3B3 and KMX-1 all recognised the plasmodial β_1-tubulin isotype. This was to be expected since it is known that one β_1-tubulin gene, *benD*, is expressed in both myxamoebae and plasmodia [19]. All 3 antibodies produced spots of approximately the same shape and relative intensity. However, all of these antibodies produced a differential result with the plasmodial β_2-tubulin isotype. In each case the reaction with β_2 was much less than that which would be expected on the basis of the β_1 reactivity and the relative proportions of these 2 isotypes (fig.3). (There is at least 5-times more of the β_2-tubulin isotype than the β_1 isotype

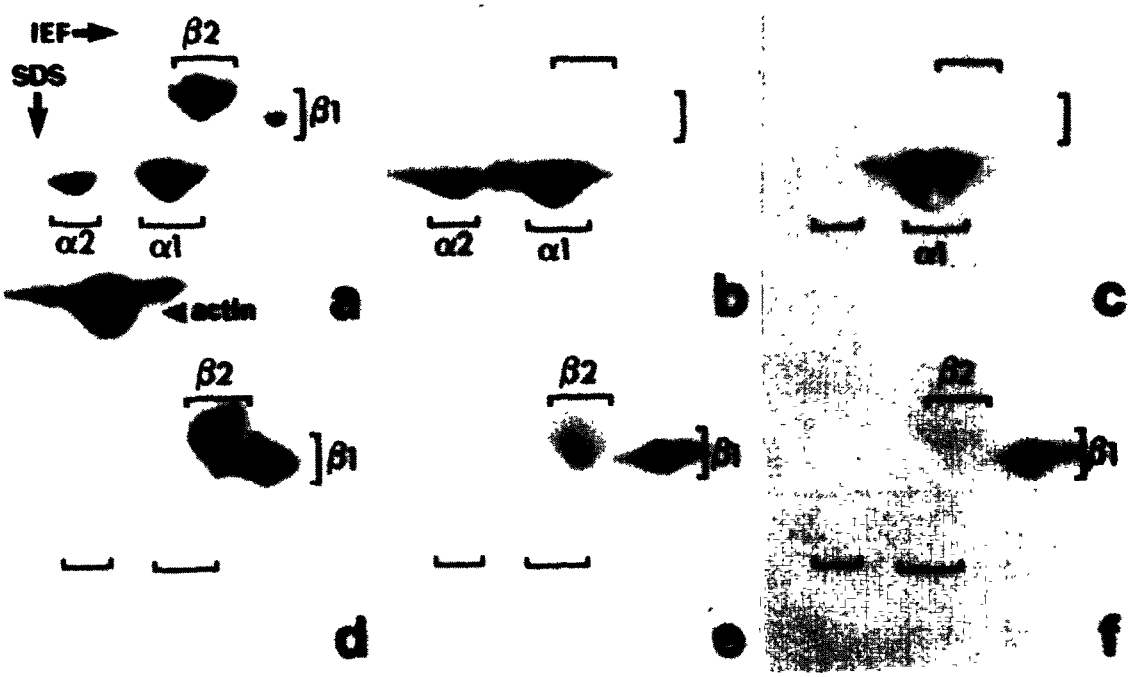
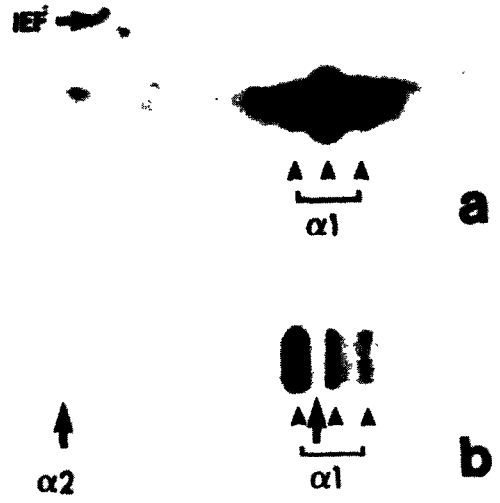


Fig.3. 2D gel and replica immunoblots of *Physarum* plasmodial tubulin. The sample consisted of only partially purified tubulin (to maximise the yield) and therefore there is an abundance of actin also present. Panel (a) is a stained gel demonstrating the characteristic coordinates of all 4 tubulin isotypes. Panels (b–f) are immunoblots of identical gels probed with the monoclonal antibodies: (b) YOL 1/34, (c) KMP-1, (d) DM1B, (e) KMX-1 and (f) DM3B3. The orientation of the gels is the same as in fig.1.



[7].) Although all 3 anti-β-tubulin antibodies recognised the β_1-tubulin more competently than β_2-tubulin, the extent of the distinction varied. DM1B was the best able to react with the β_2 isotype, whilst DM3B3 gave hardly any reaction (fig.3). The KMX-1 reactivity was intermediate between these two.

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Fig.4. Immunoblotting of *Physarum* tubulin samples subjected to IEF in stick gels (2 mm diameter cylinders). The portion shown in each case is the region containing α-tubulin: (a) myxamoebal tubulin sample and (b) plasmodial tubulin sample. Both electroblots were probed with the monoclonal antibody KMP-1. The blots are positioned with the acidic end to the right-hand side. α-Tubulin isotypes recognised by KMP-1 are marked with arrowheads and the positions of the isotype bands recognised additionally by YOL 1/34 are marked with arrows.

4. DISCUSSION

We have been able to establish a variety of immunological identities amongst the tubulin isotypes of *P. polycephalum* through the use of a panel of both established and novel monoclonal antibodies. Our findings are relevant to the interpretation of the *Physarum* multi-tubulin family and signify that the tubulin polypeptides demonstrate greater microheterogeneity than previously described [7,8]. Antibody-antigen interaction in vitro is governed largely by the availability of the requisite epitope region of the antigen. For a polypeptide antigen the smallest size of an epitope with good immunogenicity is reportedly around 6 sequential amino acid residues [21] but it is possible for binding to be dictated by as little as a single side chain of an amino acid [22]. Consequently, a monoclonal antibody which has the specificity for a small linear oligopeptide has the potential for discrimination due to a single amino acid substitution within that peptide, or a single post-translationally modified amino acid member of its epitope. We have been able to show that it is possible to use this discriminatory potential of monoclonal antibodies to discern immunological relationships even between the members of the multi-tubulin family expressed within one organism.

The 3 monoclonal anti-β-tubulin antibodies KMX-1, DM3B3 and DM1B recognise *Physarum* myxamoebal β_1-tubulin as a homogeneous isotype. Mutational analysis has recently revealed that at least 2 β-tubulin genes are expressed in the myxamoebae [19] and it will be of interest to determine whether the products of the 2 genes are identical, making *Physarum* similar to *Chlamydomonas* in this respect [23], or whether there are immunologically cryptic differences which nevertheless allow the 2 polypeptide species to comigrate so exactly on 2D gels. Certainly, as regards the KMP-1, DM1B and DM3B3 epitopes on tubulin of the BEN 210 mutant myxamoebae, the β_1 polypeptides are indistinguishable.

Plasmodial β_1-tubulin is produced by the *benD* gene [19] and so its recognition by the same panel of anti-β-tubulin antibodies is not surprising. However, the very faint detection of the substantially more abundant plasmodial β_2-tubulin isotype is intriguing. Visual comparison of the different immunoblots revealed that the competence of binding was graded, DM1B > KMX-1 > DM3B3 so it is possible that multiple sites are involved in the differences between the β_1- and β_2-tubulin isotypes. At least 3 β-tubulin loci have been identified in the *Physarum* genome [20] and so it is possible that β_2-tubulin is the product of a gene that is evolutionarily distinct from the β_1-tubulin genes.

Myxamoebal α_1-tubulin was recognised by YOL 1/34 and KMP-1 as a single spot on 2D gels. Immunoblotting of 1D IEF gels revealed that this myxamoebal species is, in fact, composed of 3 distinct sub-types, all of which are detected by both antibodies. However, with respect to the plasmodial α_1-tubulin, KMP-1 does not detect the full repertoire of sub-types. We have detected 4 sub-types which focus in the plasmodial α_1-tubulin 2D gel spot. KMP-1 does not react with one of these sub-types, nor does it detect the plasmodial specific α_2-tubulin isotype. For KMP-1 to miss a plasmodial α_1-tubulin sub-type and the α_2-tubulin isotype completely its recognition epitope is likely to be completely blocked or simply not present in these α-tubulin polypeptides. Schedl et al. [20] have shown that there are at least 4 α-tubulin loci in the *Physarum* genome so allowing for the possibility that many of these immunologically defined sub-types may be directly encoded by this α-tubulin gene family.

There are particular advantages in this immunological approach to studies of the individual isotypes expressed by a multi-tubulin family. Isotype-discriminating monoclonal antibodies will be of use in identifying the products of cDNA expression libraries and at this level it should be possible to match tubulin isotypes with their individual genes. Our results show that this use of monoclonal antibodies is a viable approach and has already revealed distinct relationships within the *Physarum* multi-tubulin family.

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