Incorporation of tubulin from an evolutionarily diverse source, Physarum polycephalum, into the microtubules of a mammalian cell

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Summary

Physarum myxamoebal tubulin was injected into PtK₂ cells to determine whether tubulin from this eukaryotic microbe could act as a reporter for microtubule growth and dynamics in a mammalian cell. The distribution of Physarum tubulin was determined by the use of a monoclonal antibody specific for Physarum tubulin and unable to detect mammalian tubulin. Physarum tubulin was incorporated into the microtubules of both interphase arrays and the mitotic spindle. Measurements of microtubule turnover kinetics were found to be similar to those of other studies in which chemically modified brain tubulin has been used. Results using this heterologous system demonstrate that tubulin from an evolutionarily diverse organism can be used as a marker for microtubule growth in mammalian cells. Furthermore, the Physarum tubulin was able to endow the injected cells with novel properties. Resistance to colchicine-induced microtubule disassembly, a characteristic of Physarum tubulin, was conferred on the injected PtK₂ cells. Use of this heterologous reporter tubulin system has also revealed features of variation in microtubule dynamics both within individual cells and between cells.

Key words: Physarum, tubulin, microtubule dynamics.

Introduction

An assessment of the in vivo dynamics of cell-biological processes can sometimes be made using biochemical approaches. However, a more complete description can be obtained if the kinetics of the process can be visualized directly in the cell. A standard approach used in this context is to purify the protein of interest and then label it with a convenient tag, for example a fluorophore or biotin, that will enable the chemically modified protein to be uniquely recognized by a specific detection system. The modified protein is injected into a living cell and enters the structure of interest. Subsequently, its position can be detected and in this way it acts as a reporter for the spatial and temporal dynamics of the process (reviewed by Kreis & Birchmeier, 1982; Wang et al. 1982). Such modified proteins must be carefully analysed to determine the extent of any changes brought about by the chemical modification.

In this study we have used a novel approach to produce a reporter protein that can be recognized specifically by a monoclonal antibody after injection into a mammalian cell. The method relies on the use of a protein from an evolutionarily diverse organism in conjunction with a monoclonal antibody that is highly specific for the protein to be injected. Whilst the functionally important sites of proteins are likely to be highly conserved, small non-conserved regions may occur at sites with fewer functional constraints. Such sites would prove ideal targets for specific monoclonal antibodies, which then provide the differential probe required in order to identify the injected protein against a background of host cell protein.

The microtubule network has been extensively studied by microinjection techniques and recent developments have enabled the dynamics of individual microtubules to be analysed in vivo. In these studies tubulin has been modified by biotinylation (Schulze & Kirschner, 1986, 1987) or by the addition of a fluorophore (Soltys & Borisy, 1985; Sammak et al. 1987; Sammak & Borisy, 1988). The majority of the microtubules in the various cell types studied were found to turn over rapidly, with half-lives in the range of 5–20 min. Furthermore, individual microtubules showed different rates of elongation (Schulze & Kirschner, 1987).

Multiple α and β-tubulin genes are present in most organisms and it is now clear that mixing of individual tubulin gene products can occur within single microtubules in an animal cell (Lewis et al. 1987; Lopata & Cleveland, 1987). It is not known whether this tubulin isotype heterogeneity is a result of functional specialization of the isotypes, and it is therefore of interest to determine the extent to which differences between tubu-
Physarum has been extensively characterized both biochemically (Roobol et al. 1980a,b, 1984) and immunologically (Birkett et al. 1985). Tubulin for this study has been purified from the myxamoebal cell-type and is composed of α1 and β1-tubulin isotypes (Burland et al. 1983; Roobol et al. 1984); the α1-tubulin isotype shows approximately 85% homology with five different mammalian tubulins (Singhofer-Wowra et al. 1986a) and the β1-tubulin isotype shows 85–89% homology with mammalian tubulins (Singhofer-Wowra et al. 1986b). The in vitro assembly kinetics of Physarum tubulin are similar to those for tubulin purified from mammalian sources in terms of both self-assembly (Roobol et al. 1980b) and nucleated assembly from isolated Physarum MTOCs (Roobol et al. 1982). The antibody KMP-1 is highly specific for the α1-tubulin isotype of Physarum (Birkett et al. 1985) and thus can be used to discriminate between Physarum tubulin and any other tubulin in a heterologous system.

Materials and methods

Preparation of tubulin

Tubulin was prepared from the myxamoebal cell type of Physarum polycephalum as described (Foster et al. 1987). The strain used was the wild-type axenic strain CLd-AXE. Tubulin was purified by DEAE chromatography followed by concentration and then by a cycle of assembly and disassembly. Pellets of assembled microtubules were frozen in liquid nitrogen and stored at −80°C until further use. Sheep brain tubulin was prepared according to the method of Roobol et al. (1976).

The purity of tubulin preparations was assessed by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). Protein concentrations were determined according to the method of Bradford (1976) using the Bio-Rad dye reagent and bovine serum albumin as a standard.

Electron microscopy

The ability of purified tubulin to assemble was assessed by electron microscopy of polymerized samples. Samples (50 μl) of tubulin were incubated at 30°C for 30 min and then 10 μl samples were placed on carbon-coated Formvar grids and negatively stained with aqueous uranyl acetate. Grids were examined in a Philips 410 electron microscope operating at 80 kV.

Electrophoresis and immunoblotting

Electrophoretic gels were run according to the method of Laemmli (1970). The transfer of proteins to nitrocellulose sheets and the antibody coupling procedure were as described (Birkett et al. 1985). Antibody binding was detected using a rabbit anti-mouse horseradish-peroxidase-conjugated second antibody and 4-chloro-1-naphthol as a substrate.

Gel samples were prepared as follows: a confluent flask of PtK2 cells (approx. 106 cells) was taken, the medium was decanted and the cells were washed briefly with phosphate-buffered saline (PBS). The cells were then scraped into 500 μl of sample buffer (62.5 mM-Tris-HCl, pH 6.8, 10% glycerol, 5% mercaptoethanol, 2% SDS) using a rubber policeman and boiled for 2 min. CLd-AXE myxamoebae (5 × 106) were harvested and resuspended in 50 μl sample buffer containing leupeptin (50 μg/ml); Peptide Institute, Japan) and phenylmethylsulphonyl fluoride (0.1 mM; Sigma) protease inhibitors and boiled for 2 min. The SDS was supplied by Fisons (UK) and leads to the inversion of Physarum tubulins on gels so that the α-tubulin subunit has the greater mobility (Clayton et al. 1980).

Antibodies

The distribution of microinjected Physarum tubulin was detected using a monoclonal antibody (KMP-1) that is specific for the myxamoebal tubulin isotype α1 (Birkett et al. 1985). The entire microtubule network was visualized using a monoclonal antibody (KMX-1) that recognizes the β-tubulin subunit from all species tested to date (Birkett et al. 1985).

The second-stage antibodies were preabsorbed against methanol-fixed and acetone-extracted PtK2 cells in suspension to reduce background staining. A rhodamine-conjugated rabbit anti-mouse immunoglobulin serum (Dakopatts) was used to identify KMP-1 staining and an affinity-purified fluorescein-conjugated goat anti-mouse IgG serum (Sigma) was used to identify KMX-1 binding. This staining procedure gave bright uniform fluorescence with no detectable patchiness in the KMP-1 staining microtubules. Similar KMP-1 staining patterns were observed in samples that had been singly stained with KMP-1 or double stained with KMP-1 and KMX-1.

DM1A, an anti-α-tubulin antibody (Blose et al. 1984) was a gift from Dr S. Blose (Protein Databases Inc. Huntington Station, NY).

Preparation of tubulin for microinjection

Pellets of microtubules, which had been stored at −80°C, were thawed and depolymerized on ice in a minimal volume (approximately 1 ml) of PEMEG buffer (0.1 M-Pipes (piperazine N,N′-bis(2-ethanesulphonic acid)), 2 mM-EGTA, 1 mM-MgSO4, 0.1 mM-EDTA and 1 mM-GTP, pH 6.9) for 30 min. The sample was then centrifuged at 100 000 g and 4°C for 30 min to remove aggregated material, the supernatant was divided into samples and stored in liquid nitrogen until required. Before injection a sample was taken and dialysed against microinjection buffer (100 mM-glutamic acid, 140 mM-KOH, 1 mM-MgSO4, 1 mM-dithiothreitol and 1 mM-GTP at pH 7.2 with citric acid; Ianz et al. 1983) for 1 h. Samples were taken for protein assays, electrophoresis and electron microscopy so that the purity and assembly competence of the tubulin preparation could be assessed.

Cell culture

PtK2 cells were cultured in Eagle’s Minimum Essential medium.
Microinjection

Microinjection of PtK2 cells was as described (Prescott et al. 1988). Tubulin was introduced into the cell in injection buffer. Injected cells were localized by their proximity to an ink mark on the coverslip and the presence of KMP-1 staining. Cells were injected in a single line, the time of injection being recorded to the second with a stop watch.

For microinjection at 37°C the cells were perfused with warm medium and the temperature monitored using a thermometer probe at the edge of the coverslip; temperatures remained within ±1 deg C of the desired temperature during the microinjection and the subsequent incubation period. For microtubule-disassembly experiments Physarum tubulin or sheep brain tubulin was injected into cells, which were then incubated at 37°C for 60 min to permit incorporation of the injected tubulin into the microtubule network. The cells were then transferred to MEM with 10% FCS containing 15μg/ml colchicine for a further 3 h at 37°C, treated with 1% Nonidet P40 in PEMEG buffer for 45 s and fixed as below.

Immunofluorescence

At varying recorded times following injection, cells were fixed in 90% methanol in buffer (100 mM-Mes (2-(N-morpholino)-ethanesulphonic acid), 1 mM-EGTA, 1 mM-MgSO4, pH 6.9) at −20°C for 5 min followed by extraction with acetone at −20°C for 30 s and then washed three times in PBS-BSA (0.02%) (Weber et al. 1975a).

All antibodies were diluted in PBS-BSA and all washes were with the same. The antibody staining regime was as follows: KMP-1 (1/100) for 4 h, three 5-min washes, TRITC-conjugated rabbit anti-mouse immunoglobulins for 4 h, three 5-min washes, KMX-1 (1/100) for 4 h, FITC-conjugated goat anti-mouse IgG for 4 h followed by three 5-min washes. Antibody incubations were at 37°C. The coverslips were mounted in Citifluor (City University, London). The cells were observed using a Zeiss Standard microscope equipped with epifluorescence optics using planapochromat ×63 and ×100 lenses. Photographs were taken using an Olympus OM2N camera on Kodak T-Max 400 film.

Calculation of microtubule growth rates

In order to measure accurately the lengths of growing microtubule ends incorporating Physarum tubulin the slides were viewed with an image-intensification camera (RCA model 47 7909) mounted on a Zeiss Universal microscope. Images from the microscope were magnified using a ×4 adaptor (Zeiss 47 79 09) before entering the camera. Image quality was improved by using the ‘Crystal’ image-enhancement system (Quintel, Newbury). The final images were recorded on a U-matic video recorder (Sony, Tokyo). The lengths of growing microtubules were measured using either the ‘Seescan’ image processor or by projecting enlarged 35 mm negatives of the replayed television image onto paper, tracing the fragments and measuring them with a CTS tablet and custom-written software for a BBC microcomputer. The calculated microtubule growth rates were found to be identical, using either method.

Results

Tubulin assembly in vitro

Tubulin comprises less than 1% of the soluble protein of P. polycephalum (Roobol et al. 1980b) and thus must be isolated by a series of biochemical techniques before purification by cycles of assembly and disassembly in vitro.

The characteristics of a typical preparation of tubulin from Physarum are shown in Fig. 1. Analysis by SDS–PAGE and Coomassie Blue staining of the gel shows that tubulin is the only major component of the sample (Fig. 1A) and extensive numbers of microtubules with normal morphology were formed when microtubule assembly was induced in vitro (Fig. 1B).

Specificity of the antibody KMP-1

The ability to identify Physarum tubulin (P-tubulin) injected into PtK2 cells relies on the high degree of specificity of the monoclonal antibody KMP-1, which was raised against Physarum myxamoebal tubulin and is specific for the α tubulin isotype of Physarum. The cross-reactivity of KMP-1 with the tubulins of many other organisms has been tested (Birkett & Gull, 1985; R. Sasse, unpublished results) and no reactivity exists towards the tubulins of fungi, protozoa, insects, nematodes, plants, birds or mammals.

The specificity of KMP-1 towards P-tubulin was demonstrated by immunoblotting preparations of total cell proteins from PtK2 cells and Physarum myxamoebae (Fig. 2). KMP-1 clearly recognized α-tubulin from Physarum and no reactivity towards the PtK2 cell tubulin was observed (Fig. 2A). The α-tubulins of PtK2 cells and Physarum myxamoebae were identified by the cross-reactive anti α-tubulin monoclonal antibody DM1A (Fig. 2B).
Fig. 2. The reaction of whole cell extracts from PtK₂ cells (lane 1) and CLd-AXE (lane 2) with the monoclonal antibodies KMP-1 (2A) and DM1A (2B). KMP-1 clearly recognized the tubulin from CLd-AXE but no reaction with the PtK₂ cell extract was observed. DM1A recognized the α-tubulins of both organisms.

Incorporation of Physarum tubulin into the microtubule arrays of PtK₂ cells

Cells were fixed after known periods of time, ranging from 30 s to several hours, following injection of P-tubulin; tubulin concentrations between 0.7 and 5 mg ml⁻¹ were used.

Cells were sequentially stained with KMP-1 followed by the rhodamine-conjugated anti-mouse antibody and then with KMX-1 followed by the fluorescein-conjugated anti-mouse antibody. This protocol ensured that the microtubules containing P-tubulin were revealed by KMP-1 staining. KMX-1 recognizes the β-tubulin subunit and as such its detection of microtubules is independent of the α-tubulin subunit composition and so detects the complete microtubule network.

Initial experiments were designed to assess whether the P-tubulin was able to be incorporated into the microtubules of mammalian cells. Cells were injected with the P-tubulin and then incubated for periods of time between 15 and 20 min before fixation. This time period was chosen as several recent studies using injected labelled tubulin have indicated that about 80% of the microtubules showed turnover and incorporation of the injected tubulin throughout their length by these times (Schulze & Kirschner, 1986, 1987).

The result of such an experiment is shown in Fig. 3. Staining with KMP-1 clearly labels the microtubule network of the injected cell and the stained microtubules form a uniformly stained, extensive array (Fig. 3A). Counterstaining with KMX-1 also reveals the whole microtubule network (Fig. 3B). Cells injected with P-tubulin and left for 20 min were also closely examined for the coincident localization of microtubules staining with KMP-1 and the whole microtubule network. Fig. 3C, D are enlargements of an area of the cell shown in Fig. 3A, B, respectively. Examination of the microtubules at increased magnification shows co-localization of staining with KMP-1 and KMX-1.

In a second series of experiments cells were injected with P-tubulin and fixed between 30 s and 5 min after injection. The results from such experiments are shown in Fig. 4. In these cells incorporation of the P-tubulin into the whole microtubule network had not yet occurred and a progressive increase in the amount of microtubule polymer containing P-tubulin occurs with time. After short periods of time (Fig. 4A) only small fragments of KMP-1 staining microtubule are observed and in addition KMP-1 staining at the centrosome is bright. After longer periods of time more P-tubulin is incorporated into the microtubule network (Fig. 4C, E). The staining...
Fig. 4. *Physarum* tubulin incorporation after short time periods. A,C,E. Cells stained with KMP-1; B,D,F, the same cells stained with KMX-1. A. Two cells microinjected with P-tubulin and incubated for 0-83 and 0-88 min at 37°C before fixation. The KMP-1 antibody has stained the growing ends of the microtubule network as short bright fragments and there is bright fluorescence at the centrosomes (arrows). B. The total microtubule network of the microinjected cells in A is stained with KMX-1. C. Two cells injected with P-tubulin and incubated for 2-17 and 2-28 min at 25°C. The longer incubation time has enabled the P-tubulin to be incorporated into longer portions of microtubule ends. The centrosomal arrays contain longer fragments extending well into the cell periphery (arrows). The cell at the right-hand edge has also been microinjected. D. The cells in C counterstained with KMX-1. E. Three cells microinjected with P-tubulin and incubated at 37°C for 2-6, 2-75 and 2-82 min before fixation. In these cells the microtubule network has extensively incorporated P-tubulin although an area of incomplete incorporation can still be seen (arrow). F. The total microtubule network of the cells in E. Bars, 20 μm.

of the whole microtubule network with KMX-1 suggests that the microtubule array is not perturbed by the microinjection process, as a complete array is seen in all cells (Fig. 4B,D,F). The KMP-1-staining microtubule fragments appeared to co-localize with the ends of microtubules staining with KMX-1. Cells that had not been injected were clearly identified as they showed only background staining with KMP-1 but extensive micro-
Fig. 5. A cell microinjected with P-tubulin and incubated for 1-5 min at 37°C before fixation. The KMP-1-stained microtubule ends at the cell periphery (A) are at the distal ends of microtubules forming the microtubule array of the cell (B, see arrows). The length of microtubule fragment staining with KMP-1 varies between different microtubules, being considerably longer in some microtubules (large arrows) than others (small arrow). Bar, 5 μm.

Microtubule arrays when stained with KMX-1 (Fig. 4E,F). In cells fixed 2-6 to 2-8 min after injection (Fig. 4E,F) the microtubule network contains many microtubules labelled along their entire lengths, although inspection at the cell periphery showed that in some microtubule ends staining with KMP-1 persisted.

Microtubule ends are more clearly seen in Fig. 5, an enlarged area of a cell fixed 1-5 min after injection. The microtubule lengths that label with KMP-1 are clearly coincident with the ends of the existing microtubules as judged by the KMX-1 staining (compare Fig. 5A and B). The microtubule ends that stain with KMP-1 are very variable in length. Some microtubules are not labelled after this short incubation period, others have only short lengths labelled, whilst some microtubules have comparatively long labelled distal segments.

Dividing cells were also injected to determine whether Physarum tubulin could be incorporated into the mitotic apparatus of PtK2 cells. Cells in prophase and in metaphase were stained with KMP-1 and KMX-1. The prophase cell (Fig. 6A,C) was fixed 1-32 min after injection; two centrosomes can be seen with many microtubules radiating from them. These microtubules stain with both KMP-1 and KMX-1. Microtubules can be identified at the cell periphery and are stained with KMP-1 and KMX-1, indicating that although the microtubule array is being rearranged in preparation for mitosis there are dynamic microtubules at the cell periphery at this stage of mitosis. The cell in metaphase (Fig. 6B,D) was fixed 2-2 min after injection and the P-tubulin was incorporated into the spindle microtubules. Individual cells were also injected with P-tubulin and allowed to complete mitosis before fixation. The two daughter cells both contained complete networks of microtubules that stained with both KMP-1 and KMX-1 (data not shown).

Microtubule elongation rates in vivo

The pattern of incorporation of injected P-tubulin into the cellular microtubule array appeared to be very similar to that described in studies in which mammalian brain was used as the source of exogenous tubulin (Schulze & Kirschner, 1986, 1987; Soltys & Borisy, 1985). In order to assess further the efficacy of Physarum tubulin as a marker for microtubule growth, the kinetics of microtubule elongation were measured. The exact time of microinjection was recorded for each cell and measurements made of the lengths of the microtubule ends stained with KMP-1 in each cell. Measurements of microtubule growth rate were made at two different temperatures (25°C and 37°C) and using two different concentrations of P-tubulin (1 and 5 mg ml⁻¹). The length of microtubule polymer stained with KMP-1, i.e. containing P-tubulin, was determined for each microtubule end and the mean microtubule end length was determined for each cell. A mean rate of microtubule growth was then obtained from the results of many cells; at 25°C this was 2-3 μm min⁻¹ (s.d. = 1-03, n = 120) and at 37°C a value of 4-6 μm min⁻¹ (s.d. = 1-84, n = 189) was obtained. These results are significantly different from one another (P < 0-001) and demonstrate the effect of temperature on microtubule assembly in vivo.

These generalized statements of average rates, however, tend to disguise two important levels of variation; first, variation between individual microtubules within one cell; and second, variation between individual cells. These two forms of variation are illustrated by the data derived from the analysis of over 100 cells, displayed in Figs 7 and 8. Fig. 7 shows a graph of mean microtubule end length per cell plotted against time, for cells injected with P-tubulin at a concentration of 1 mg ml⁻¹ and at a temperature of 37°C. The straight line corresponds to a mean growth rate of 4-6 μm min⁻¹. The scatter of points about this mean growth rate is large and indicates variation between the different cells. The distribution of microtubule fragment lengths within a cell was analysed for all cells containing over 100 KMP-1 staining microtubules. Four such cells are denoted A, B, C and D; cells A and B were fixed 0-98 and 1-00 min after injection but have mean microtubule end lengths of 8-9 and 4-2 μm, respectively. Similarly, cells C and D were fixed 1-58 and 1-37 min after injection but have mean micro-
tubule end lengths of 10.0 and 4.5 µm, respectively. The histograms shown in Fig. 8 show the KMP-1 staining microtubule length distributions in these four cells and demonstrate that in cells that have been allowed to incorporate tubulin for the same amount of time the distribution of P-tubulin into microtubule ends can be very different. The modal value may be similar, as in C and D, although C contains many more long fragments than D resulting in a larger mean value. Alternatively, the modal values can differ, as in A and B.

The action of anti-microtubule agents on injected cells
Tubulin from Physarum shows little sensitivity to colchicine and its analogues (Roobol et al. 1980), whilst mammalian cell microtubules are highly sensitive to colchicine (Weber et al. 1975). This has provided an opportunity to determine whether the injected P-tubulin is passively incorporated into the host cell microtubule array or whether it is able to confer novel properties on the microtubule cytoskeleton. PtK2 cells were injected with P-tubulin and incubated for 1 h to permit complete steady-state incorporation of the P-tubulin into the microtubule network. The cells were then treated with colchicine for 3 h to determine whether colchicine had differential effects on injected and non-injected cells.

In the presence of colchicine (15 µg ml⁻¹) the microtubules of uninjected PtK2 cells were depolymerized. However, adjacent cells that had been injected showed an intact microtubule network that contained P-tubulin as judged by staining with KMP-1 (Fig. 9A, B). In order to ensure that protection from colchicine was not a consequence of the additional mass of tubulin in the injected cells, control experiments using sheep brain tubulin were performed. The injection of brain tubulin at 8 mg ml⁻¹ did not confer any resistance to the action of colchicine on PtK2 cells. The microtubule array was mainly disassembled in both injected and uninjected cells (Fig. 9D); only a few resistant microtubules persisted in both the injected and uninjected cells. Thus, the colchicine resistance conferred on cells injected with P-tubulin is most
Fig. 7. A graph of the mean fragment length of each individual cell plotted against time. Each cross represents a different cell, the mean fragment length was calculated from measurement of all the fragments in which both ends of the microtubule were clearly visible in each cell. Cells were injected with P-tubulin at a concentration of 1 mg ml⁻¹ and incubated at 37°C. The straight line corresponds to the mean microtubule growth rate of 4.6 μm min⁻¹. The points A, B, C and D are the four individual cells analysed in Fig. 8.

likely to be a consequence of the different drug sensitivity of the P-tubulin into the cells. Physarum tubulin is sensitive to the anti-microtubule drug nocodazole (Quinlan et al. 1981). To determine that the effect was colchicine-specific, cells injected with P-tubulin were treated with nocodazole at 5 μg ml⁻¹. There was no significant difference in the sensitivity of injected and uninjected cells to nocodazole.

Discussion

In this study we have assessed whether tubulin from an evolutionarily diverse source would be utilized by a mammalian cell and so become integrated into the microtubule network. These experiments address two questions: first, whether there is evolutionary conservation of important assembly sites within the tubulin molecule; and second, whether they relate to the potential application of such a system to the study of microtubule dynamics in mammalian cells.

A clear finding from our experiments is that Physarum tubulin can act as an efficient marker for microtubule dynamics in mammalian cells. The distribution of Physarum tubulin after microinjection into PtK² cells was found to follow a time course similar to that observed by Schulze & Kirschner (1986) for biotinylated mammalian brain tubulin injected into BSC1 cells. After short periods of time (30 s to a few minutes) Physarum tubulin was located at the distal ends of microtubules and with increasing time more of the microtubule network contained incorporated Physarum tubulin. This result is consistent with the concept that microtubules grow by an end-dependent elongation process. We found no examples of microtubules in which Physarum tubulin was proximal to endogenous tubulin. Also, there appeared to be no spontaneous formation of Physarum microtubules in the cytoplasm as all the fragments observed at early time points could be traced to pre-existing microtubules when the counterstained total microtubule image was examined.

After periods of time of 15 min or more after injection most of the microtubule array could be stained with the monoclonal antibody KMP-1. We did, however, observe a few microtubules that were unlabelled by KMP-1 even after times in excess of 15 min. These microtubules often possessed a curly profile and closely resembled those described as the stable sub-population by Schulze & Kirschner (1987) and Schulze et al. (1987). PtK² cells are known to possess this population of microtubules (Schulze et al. 1987). The distinct result of this analysis is that Physarum tubulin is indeed able to participate in the assembly of microtubules within a mammalian cell. The patterns of assembly that we have found in this work using Physarum tubulin are remarkably similar to those revealed by other studies using biotinylated (Schulze & Kirschner, 1986) or fluorescently derived (Soltys & Borisy, 1985) brain tubulin. Thus it would seem that the conclusions from these microinjection studies are unlikely to be greatly influenced or altered by the type of injected reporter tubulin used. A more detailed analysis of the kinetics of microtubule polymerization using

Fig. 8. Histograms to show the distribution of microtubule fragment lengths in the cells denoted A, B, C and D in Fig. 7. The incubation period (t), the mean microtubule end length (x) and the number of microtubule ends (n) are indicated.
Fig. 9. The effect of microtubule inhibitors on *Physarum* tubulin incorporation. A, B. Cells following incubation in the presence of 15 μg ml⁻¹ colchicine for 3 h. The arrowed cell was injected with P-tubulin at 1 mg ml⁻¹ and shows an extensive microtubule array. The periphery of an un.injected cell is denoted by the arrowheads. A. KMP-1 staining; and B, KMX-1 staining; C, D, the control experiments using brain tubulin. Several cells in a field were injected with brain tubulin (8 mg ml⁻¹) and their positions recorded by video (C), the arrow shows a cell being injected. After colchicine treatment the cells were fixed and stained with KMX-1 (D); the injected cells are denoted by arrows. Bar, 20 μm.

*Physarum* tubulin as a probe has given very similar results to existing data using biotinylated tubulin (Schulze & Kirschner, 1986). Injections were carried out at *Physarum* tubulin concentrations in the range 0.7 to 5 mg ml⁻¹ and we found no evidence that this sevenfold change in concentration produced any major effect on the rates of tubulin accumulation into microtubules after injection. Assuming injection volumes of 10% of the total cell volume (Izant *et al.* 1983) and a total cell tubulin concentration of 2 mg ml⁻¹ (Hiller & Weber, 1978), the cellular concentration of tubulin after injection would be increased by about 3.5% at 0.7 mg ml⁻¹ and 25% at 5 mg ml⁻¹.

The average rates of microtubule elongation in PtK₂ cells were 4.6 μm min⁻¹ at 37°C and 2.3 μm min⁻¹ at 25°C. Thus, the rates of microtubule elongation in PtK₂ cells are influenced by the incubation temperature. Puriﬁed *Physarum* tubulin is able to assemble at 37°C in vitro (Foster *et al.* 1987) and the average rate of microtubule elongation in vitro revealed by the *Physarum* tubulin at 37°C is very similar to the value determined by Schulze & Kirschner (1986) using biotinylated brain tubulin. However, our data with *Physarum* tubulin also reveal an interesting feature regarding general cellular physiology. *Physarum* myxamoebae grow well at 25°C but are incapable of growth at 37°C. Thus, our experiments show that the tubulin from an organism that is normally grown optimally at 25°C can be recruited to the microtubules of an evolutionarily diverse cell at 37°C. Moreover, comparison with previous data suggests that the kinetics of the process appear to be dictated by the host mammalian cell, so reinforcing the concept that the
dynamics of microtubule polymerization in vitro are very much influenced by factors acting at the level of the individual microtubule. Also, the results of our approach emphasize the likelihood that these factors governing microtubule assembly dynamics have been conserved during evolution.

Our use of a marker tubulin from an evolutionarily diverse source enabled us to ascertain whether a heterologous tubulin could bestow unique properties on the microtubules of the host cells. Many eukaryotic microbes have microtubules that are insensitive to the action of colchicine. There is good evidence that this colchicine insensitivity is a function of the tubulin of these organisms, since purified tubulin is able to assemble in vitro in the presence of colchicine (Quinlan et al. 1981; Kilmartin, 1981). Our study of drug sensitivity demonstrates that the injected tubulin can confer this property on the host cell microtubules. Physarum tubulin conferred a high level of resistance to colchicine-induced depolymerization onto the microtubule network of injected Ptk₂ cells. This effect was most marked when higher concentrations of Physarum tubulin were used. Concentrations of less than 1 mg ml⁻¹ sometimes gave ambiguous results as, although increased numbers of microtubules remained after drug treatment, they appeared somewhat reduced in number compared to complete microtubule arrays. Thus, it seems likely that colchicine insensitivity may depend on a threshold level of Physarum tubulin being injected into the cell. However, the novel conclusion from these experiments is that heterologous tubulin can confer a specialized property on the microtubule cytoskeleton of the host cell.

Analysis of the microtubule dynamics of many individual cells after microinjection of Physarum tubulin revealed the general rates of microtubule elongation referred to previously. However, examination of the data from very many cells has revealed a large degree of cell-to-cell variation. It is clear that the nature of the dynamics of microtubules within one cell will lead to variation between individual microtubules (Schulze & Kirschner, 1986, 1987; Sammak et al. 1987; Sammak & Borisy, 1988). However, our data also suggest that there is cell-to-cell variation in the average dynamics of the microtubule cytoskeleton.

The results of our study and that of Schulze & Kirschner (1986) indicate that the scatter is not due to the injection of a variable amount of tubulin, as concentration has been shown to have no effect on the rate of tubulin assembly. We have also analysed our results in terms of separate experiments and we find the same degree of scatter in all cases. Staining of the whole microtubule network of injected cells with KNX-I shows no visible change in the microtubules in these cells, and injected cells are able to progress through the cell cycle and undergo mitosis; therefore, it is unlikely that the scatter is due to differential damage to cells. The cell-to-cell variation we observe is not large but may indicate a significant variation that is dependent on cell shape, motility or the position of the cell within the interphase period of the cell cycle. Variable rates of elongation have been suggested by direct observations of dynamic microtubules (Schulze & Kirschner, 1988) and it is known that the turnover of microtubules is much faster during mitosis (Saxton et al. 1984). Thus, the cell-to-cell variation that we detect may be understandable as a further cell-cycle modulation. Ptk₂ cells are relatively immotile cells and analysis of polarized motile cells may further complicate measurement of microtubule kinetics in vitro. Tests of this and other explanations will depend on future analyses involving knowledge of the cell cycle positions of individual cells or their motility at the time of injection.

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