Accumulation of delta 2-tubulin, a major tubulin variant that cannot be tyrosinated, in neuronal tissues and in stable microtubule assemblies

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SUMMARY

Tubulin is the major protein component of brain tissue. It normally undergoes a cycle of tyrosination-detyrosination on the carboxy terminus of its α-subunit and this results in subpopulations of tyrosinated tubulin and detyrosinated tubulin. Brain tubulin preparations also contain a third major tubulin subpopulation, composed of a non-tyrosinatable variant of tubulin that lacks a carboxy-terminal glutamyl-tyrosine group on its α-subunit (delta 2-tubulin). Here, the abundance of delta 2-tubulin in brain tissues, its distribution in developing rat cerebellum and in a variety of cell types have been examined and compared with that of total α-tubulin and of tyrosinated and detyrosinated tubulin. Delta 2-tubulin accounts for approximatively 35% of brain tubulin. In rat cerebellum, delta 2-tubulin appears early during neuronal differentiation and is detected only in neuronal cells. This apparent neuronal specificity of delta 2-tubulin is confirmed by examination of its distribution in cerebellar cells in primary cultures. In such cultures, neuronal cells are brightly stained with anti-delta 2-tubulin antibody while glial cells are not. Delta 2-tubulin is apparently present in neuronal growth cones. As delta 2-

tubulin, detyrosinated tubulin is enriched in neuronal cells, but in contrast with delta 2-tubulin, detyrosinated tubulin is not detectable in Purkinje cells and is apparently excluded from neuronal growth cones.

In a variety of cell types such as cultured fibroblasts or primary culture of bovine adrenal cortical cells, delta 2-tubulin is confined to very stable structures such as centrosomes and primary cilia. Treatment of such cells with high doses of taxol leads to the appearence of delta 2-tubulin in microtubule bundles. Delta 2-tubulin also occurs in the paracrystalline bundles of protofilamentous tubulin formed after vinblastine treatment. Delta 2-tubulin is present in sea urchin sperm flagella and it appears in sea urchin embryo cilia during development.

Thus, delta 2-tubulin is apparently a marker of very long-lived microtubules. It might represent the final stage of α-tubulin maturation in long-lived polymers.

Key words: tubulin, post-translational modification, tyrosination, nontyrosinatable tubulin, delta 2-tubulin, cerebellum

INTRODUCTION

The carboxy-terminal region of α-tubulin is the site of specific post-translational modifications, which are not shown by other proteins. The carboxy-terminal amino acid of α-tubulin, tyrosine, can be cyclically removed or re-added to the protein. This results in tubulin that is either tyrosinated or detyrosinated, usually abbreviated as Tyr- or Glu-tubulin, respectively (Gundersen et al., 1987). Another remarkable reversible post-translational modification that occurs on the α-tubulin carboxy terminus is polyglutamylation. It consists of the addition of a branch chain of a variable number of glutamyl residues linked to a glutamate residue close to the carboxy terminus of the main chain (Eddé et al., 1990; Audebert et al., 1993).

Post-translationally modified tubulin, either detyrosinated or

polyglutamylated, is enriched in long-lived microtubules and this is most probably why it is abundant in neuronal cells (Gundersen and Bulinski, 1986; Eddé et al., 1990; Baas and Black, 1990; Wolff et al., 1992).

Brain tubulin preparations contain another major variant of α-tubulin that cannot be tyrosinated. The structure of this variant has been recently elucidated. It differs from Tyr-tubulin in that it lacks the two carboxy-terminal amino acids of α-tubulin (Paturle-Lafanechère et al., 1991). Before its structure was known, it was referred to as 'non-substrate' or 'non-tyrosinatable' tubulin. We now propose the name Δ2-tubulin for this isoform, a designation that relates to its structure rather than to a functional property.

In mammalian brain tubulin preparations, Δ2-tubulin represents about 35% of total tubulin (Paturle et al., 1989).

However, it is not known at present whether this is representative of its abundance in vivo and its tissue distribution has not been determined. Apart from obvious descriptive interest, these data are central to the investigation of the mechanisms that lead to the appearance of Δ2-tubulin and to the elucidation of its physiological significance. Here, we have examined the distribution of Δ2-tubulin in a variety of cell types. We find that Δ2-tubulin is a widespread isoform of tubulin. It is apparently remarkably abundant in neurons in which it is probably the predominant variant of α-tubulin as compared to Tyr- and Glu-tubulins. In other cell types, Δ2-tubulin is normally confined to very long-lived microtubule structures. The potential implications of these findings are discussed.

MATERIALS AND METHODS

Materials

The buffer used for microtubule protein isolation was 100 mM MES (2-(*N*-morpholino) ethanesulphonic acid), 1 mM MgCl$_2$, 1 mM EGTA (ethylene glycol bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid), pH 6.75 (designated MME). The sucrose/MME buffer was constructed by addition of 50% sucrose (w/v) to previously prepared MME buffer.

Ingredients used for the construction of phosphate buffered saline (PBS: 137 mM NaCl, 8 mM sodium phosphate, 1.5 mM potassium phosphate, 2.7 mM KCl, pH 7.4); MES, EGTA, magnesium chloride, SDS, bovine serum albumin (BSA), leupeptin, aprotinin, deoxyribonuclease (DNase), trypsin inhibitor and poly-L-ornithine were from Sigma.

Phenylmethylsulphonyl fluoride (PMSF), sucrose, paraformaldehyde (PFA), glucose and gelatin were purchased from Merck. Glycerol and chloral hydrate were from Fluka; adenosine 5′-triphosphate (ATP), 1,4-dithiothreitol (DTT) were from Boehringer Mannheim; isopentane was from Prolabo; Tween-20 (EIA grade), alkaline phosphatase-conjugated antibodies and the color development substrates were from Bio-Rad; trypsin was from Worthington; penicillin and streptomycin were from Eurobio; HEPES was from Gerbu; methanol was from Carlo Erba; pentobarbital was purchased from Sanofi, France. Nonidet P40 (NP40) was from Fluka. Taxol and vinblastine sulfate were, respectively, purchased from Molecular Probes and Aldrich.

The monoclonal Tyr-tubulin antibody (clone YL$\frac{1}{2}$) was a generous gift from Dr J. V. Kilmartin.

The monoclonal anti-α-tubulin antibody (DM1A) and fluorescein-conjugated phalloidin were purchased from Amersham.

The monoclonal anti-glial fibrillary acid protein (GFAP) and the monoclonal anti-microtubule-associated protein 2 (MAP2) were purchased from Boehringer Mannheim, France.

The fluorescein-conjugated secondary antibodies were from Cappel (goat F(ab′)$_2$ fragment to mouse IgG and F(ab′)$_2$ affinity-purified antibody to rabbit IgG) and from Jackson (goat anti-mouse with minimal cross-reaction to rat and goat F(ab′)$_2$ fragment to rat IgG).

The rhodamine-conjugated secondary antibodies were from Jackson (goat anti-rat with minimal cross-reaction to mouse and goat anti-rabbit).

Methods

Brain homogenates

Male OFA rats (Iffa Credo, l'Arbesle, France) were anaesthetized and killed. Brains were removed and homogenized in various conditions. For quantification of Δ2-tubulin in rat brain homogenates, brains were homogenized in MME buffer (1/2.5, w/v) containing 2 mM DTT, 10 μg/ml leupeptin, 10^4 units/ml aprotinin, 1 mM PMSF and diluted with an equal volume of 10% boiling SDS. Following homogenization, the sample was boiled for 3 minutes, sonicated and either immediately processed for SDS-PAGE or stored at −80°C for further use. For study of Δ2-tubulin formation in rat brain homogenates, brains were homogenized in MME buffer (1/1.25, w/v) containing 2 mM DTT, 10 μg/ml leupeptin, 10^4 units/ml aprotinin, 1 mM PMSF and 1% NP40. One half of the homogenate was diluted with an equal volume of homogenization buffer. The other half was diluted in boiling homogenization buffer containing 10% SDS. Such homogenates were then incubated at 30°C. At various time points, samples were taken and immediately boiled for 2 minutes in SDS-polyacrylamide gel sample buffer, prior to processing for immunoblot analysis.

Preparation of once recycled microtubule protein from rat brain (1X)

Four rats were anaesthetized and killed. The brains were removed and homogenized in MME buffer (0.75 ml/g of tissue). The homogenate was centrifuged (30 minutes, 4°C, 200,000 *g* average) in an ultracentrifuge (Optima TL, Beckman). The cytosol was supplemented in glycerol (20%) and in ATP (2 mM) and microtubule assembly was induced by incubation at 30°C. After completion of microtubule assembly, the solution was layered onto sucrose-MME buffer and centrifuged for 40 minutes (200,000 *g* average, 30°C) in a fixed angle rotor (TLA 100.3, Beckman). Pellets were stored at −80°C for further use.

Preparation and characterization of anti-peptide antibodies

These antibodies were obtained following the procedure described by Gundersen et al. (1984). The sequences of the peptides used as immunogens were: GEEEGEE (replica of the carboxy-terminal seven amino acid residues of Glu-tubulin) and EGEEEGE (replica of the carboxy-terminal seven amino acid residues of Δ2-tubulin). These peptides were conjugated to keyhole limpet haemocyanin (KLH), injected to rabbits (Fauves de Bourgogne, Elevage Scientifique des Dombes, France) and the sera were monitored for antibody activity with an ELISA as described by Gundersen et al. (1984). Purified Tyr-tubulin, Glu-tubulin or Δ2-tubulin (Paturle et al., 1989) were used as coating antigens in the microtiter wells.

IgGs were purified according to McKinney and Parkinson (1987) and their specificity against tubulin isoforms was checked by western blot analysis.

Polyacrylamide gel electrophoresis and western blots

These were as described by Paturle et al. (1989). The dilution used for antibodies were 1/4,000 for anti-α-tubulin antibody (DM1A), 1/1,000 for anti-Tyr-tubulin antibody (YL$\frac{1}{2}$), 1/100,000 for anti-Glu-tubulin antibody, and 1/250,000 for anti-Δ2-tubulin antibody.

Protein concentrations

These were determined by the BCA procedure (Pierce).

Immunofluorescence on cerebellum sections

Tissue preparation

For adult rats, experiments were performed on male OFA rats weighing 250-300 g. The animals were anaesthetized with intraperitoneal injections of a mixture containing chloral hydrate and pentobarbital. After rapid transcardial perfusion of 100 ml saline (NaCl 0.9%) with a perfusion pump (Mastoflex), fixative followed (PBS containing 4% PFA, pH 7.4) at a constant rate (50 ml/min).

A 600-700 ml portion of fixative was perfused per rat. The brain was removed, and the cerebellum dissected and transferred to the same fixative for 3 hours at 4°C. Cerebellum was then incubated overnight at 4°C in a 20% sucrose solution made in the same buffer, frozen by isopentane in dry ice and parasagittal sections (20 μM thick) were then cut in a cryotome at −18°C (Microm Kryostat HM500, Germany).

For rat pups, the procedure was slightly modified. A small canula

(seringe needle cut and pumiced) was used for transcardial perfusion. Fixative (25-30 ml) was perfused at a slow rate (1 ml/min).

Only brains showing optimal fixation were selected and processed for immunofluorescence staining. Postfixation was overnight in PBS, 10% sucrose, 4% PFA. The brains were then incubated for 12 hours in PBS, 20% sucrose, then in PBS, 30% sucrose for another 12 hours. The procedure was then the same as in the case of adult rat cerebellum.

Immunofluorescence procedure

All the steps of the immunofluorescence procedure were performed on floating tissue sections placed in wells of 2 ml in volume (Disposo trays 96CV, Linbro) containing the various reagents.

After extensive washing in PBS, the sections were incubated in PBS, 1% BSA, 0.1% Tween for 30 minutes followed by DM1A (1/1,000), YL$\frac{1}{2}$ (1/1,000), anti-Glu-tubulin antibody (1/500), anti-GFAP antibody (1/100) or anti-Δ2-tubulin antibody (1/1,000) overnight at 4°C. The sections were then rinsed 3 times in PBS, 0.1% Tween and incubated in either anti-mouse fluorescein, anti-rat fluorescein or anti-rabbit fluorescein (1/250) as appropriate, for 60 minutes at room temperature.

All antibodies were diluted in PBS, 1% BSA. The sections were washed in PBS, 0.1% Tween, mounted on gelatin-coated slides and coverslipped in glycerol-potassium hydrogen carbonate.

Cerebellar cell cultures
Culture preparation

Cell cultures were prepared from the cerebellum of foetal rats by enzymic and mechanical dissociation. Embryos were taken after laparotomy from anaesthetized pregnant OFA rats at embryonic day 18. Cerebella were dissected out in PBS supplemented with 10 mM glucose (PBSG) and collected in ice-cold PBSG. Pooled tissue pieces were incubated for 6 minutes with PBS containing 1% trypsin and 0.1% DNase at room temperature. Following incubation, tissues were rinsed with PBSG and trypsinization was stopped with a Ca^{2+}-free culture medium (MEM, Gibco BRL) supplemented with 0.02% trypsin inhibitor and 0.1% DNase. Cellular suspensions were obtained by repeated aspiration through small-bore Pasteur pipettes. After a brief centrifugation (10 minutes, 100 *g*), dissociated cells were resuspended in culture medium, counted and plated at a final density of 10^5 cells/cm^2 onto poly-L-ornithine (3 μg/ml)-coated tissue culture chamber slides (4 wells, Labtek).

The culture medium was composed of a 1:1 mixture of minimal essential medium (Gibco BRL) and F12 nutrient (Gibco BRL) supplemented with glucose (33 mM), glutamine (2 mM), sodium bicarbonate (18 mM), HEPES (10 mM), penicillin (5 i.u./ml), streptomycin (5 μg/ml) and 10% foetal calf serum (FCS, Gibco BRL). Cultures were incubated at 37°C in a humidified atmosphere with 5% CO$_2$. The culture medium was changed twice a week.

At appropriate times, cells were prepared for immunofluorescence.

Cell fixation

Before fixation, cells in tissue culture chamber slides were briefly rinsed in PBS at 37°C. In experiments involving actin or MAP2 labelling, cells were fixed using 4% paraformaldehyde in PBS for 10 minutes at room temperature.

In experiments involving GFAP labelling, cells were fixed in −20°C anhydrous methanol for 6 minutes. After fixation, cells were rinsed 3 times in PBS, 0.1% Tween and then processed for double-staining experiments.

Double staining of actin and of the different tubulin isoforms

Fixed cells were first incubated with fluorescein-conjugated phalloidin (1/20), then with either anti-Tyr-tubulin antibody (YL$\frac{1}{2}$; 1/1,000), anti-Glu-tubulin antibody (1/500) or anti-Δ2-tubulin antibody (1/1,000). A third incubation with rhodamine-conjugated

anti-rat (for YL$\frac{1}{2}$) or anti-rabbit secondary antibodies (1/250) followed. Cells were then processed for DNA staining with Hoechst (10 μg/ml). Finally, the slides were mounted with Aquamount.

Double immunofluorescence staining of GFAP or MAP2 and of the different tubulin isoforms

Cells were processed as above, except that a first incubation with primary monoclonal antibodies (anti-GFAP 1/100; anti-MAP2 1/100), instead of phalloidin, was followed by a second incubation with fluorescein-conjugated anti-mouse secondary antibodies (1/250).

Other cell cultures

BAC cells were primary cultures from bovine adrenal cortex and were kindly provided by Dr C. Blanc-Brude. They were grown in Ham's F10 medium plus 10% FCS.

HeLa and NIH/3T3 cells were generous gifts from Drs V. Chevrier and R. L. Margolis, respectively. HeLa cells were cultured in RPMI 1640 medium complemented with 10% FCS. NIH/3T3 cells were grown in DME complemented with 5% FCS.

Each culture medium was supplemented with 100 i.u./ml penicillin and 100 μg/ml streptomycin and all cell types were incubated at 37°C in a humidified incubator with 6% CO$_2$. Culture media were from Boehringer Mannheim Diagnostic, Houston, USA.

Cell treatments

These treatments were performed at 37°C. Taxol (5 μM, 10 μM or 50 μM) or vinblastine (10 μM) were added to growth medium. In control cells, the vehicle (DMSO for taxol treatment or methanol for vinblastine treatment) was added to growth medium.

Indirect immunofluorescence of non-neuronal cells

Cells grown on glass coverslips were briefly rinsed in PBS at 37°C and fixed by plunging the coverslips into −20°C anhydrous methanol for 6 minutes.

They were sequentially incubated with the different primary antibodies (DM1A 1/1,000; YL$\frac{1}{2}$ 1/1,000; anti-Glu-tubulin 1/500; or anti-Δ2-tubulin 1/1,000, in PBS containing 0.1% BSA), then with FITC-conjugated secondary antibodies (anti-mouse, anti-rat or anti-rabbit 1/250). Finally, the coverslips were mounted with Aquamount.

Sea urchin spermatozoa and embryos

Sea urchin sperm cells and embryos were obtained and processed for immunofluorescence as described by Multigner et al. (1992).

RESULTS

Immunoblot analysis of antibody specificity

In the present study, we use antibodies developed against Tyr-tubulin, Glu-tubulin and Δ2-tubulin. To a large extent, our results rely on the specificity of such antibodies.

We have previously designed methods that yield homogeneous preparations of Tyr-, Glu- and Δ2-tubulin (Paturle et al., 1989). We have used such preparations to check the specificity of our antibodies. Samples of protein solutions containing purified tubulin isoforms were run into SDS-gels and immunoblotted. The three isoforms were then reacted with monoclonal anti-total α-tubulin antibody (DM1A), with monoclonal anti-Tyr-tubulin antibody (YL$\frac{1}{2}$) or with anti-peptide polyclonal antibodies directed against Glu-tubulin or Δ2-tubulin, revealing excellent specificity of the antibodies. The anti-Tyr-tubulin, anti-Glu-tubulin and anti-Δ2-tubulin antibodies reacted specifically with the corresponding tubulin isoform,

no cross-reactions with the other tubulin isoforms were detectable (Fig. 1).

Immunoblot analysis of the abundance of Δ2-tubulin in rat brain extracts

It is known that in recycled brain tubulin preparations, Δ2-tubulin accounts for about 35% of total tubulin.

We wondered whether this percentage reflected the true proportion of Δ2-tubulin in brain tissues or if Δ2-tubulin was generated during the recycling procedure. To test which of these possibilities was correct, we compared the abundance of Δ2-tubulin in rat brain homogenates prepared in boiling SDS to that of Δ2-tubulin in 1×-recycled tubulin preparations (1X proteins).

We expected boiling SDS to block any putative enzymatic system capable of generating Δ2-tubulin from Glu- or Tyr-tubulin, in vitro. Therefore, such a homogenization procedure was expected to eliminate the possibility of artefactual generation of Δ2-tubulin during extract preparation. To confirm that no artefactual generation of Δ2-tubulin occurred in SDS homogenates, we made a systematic survey of the presence of Δ2-tubulin in various brain extracts.

Brain homogenates prepared in MME buffer, in the presence or absence of SDS were incubated at 30°C for up to 2 hours and the abundance of Δ2-tubulin in these extracts was quantified on immunoblots at various time points during incubation. We found no sign of Δ2-tubulin generation in such extracts (not shown). Δ2-Tubulin was present in extracts at time 0 and its abundance remained constant over time. However, we found that Δ2-tubulin generation could be detected in brain homogenates from newborn rat prepared in the presence of added NP40 (Fig. 2a,b). We used such extracts to test the inhibitory effect of SDS on Δ2-tubulin generation in vitro. Indeed SDS efficiently blocked Δ2-tubulin generation (Fig. 2c,d). Taken together, these results strongly suggest that Δ2-tubulin generation does not occur measurably in rat brain extracts prepared under standard conditions and that, in any case, systems that could potentially generate Δ2-tubulin are efficiently inhibited by SDS.
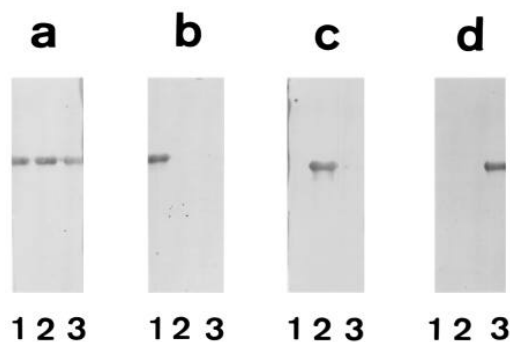
We compared the abundance of Δ2-tubulin in samples of 1X

Fig. 1. Immunoblot analysis of antibody specificity. Equal quantities (0.8 μg) of Tyr-tubulin (1), Glu-tubulin (2) or Δ2-tubulin (3) were run into 8% SDS-polyacrylamide gels, transferred onto nitrocellulose sheets and immunoblotted using monoclonal anti-α-tubulin antibody (DM1A, a), monoclonal anti-Tyr-tubulin antibody (YL$\frac{1}{2}$, b), polyclonal anti-Glu-tubulin antibody (c) or polyclonal anti-Δ2-tubulin antibody (d) as primary antibodies. The secondary antibodies were conjugated to alkaline phosphatase for enzyme visualization.

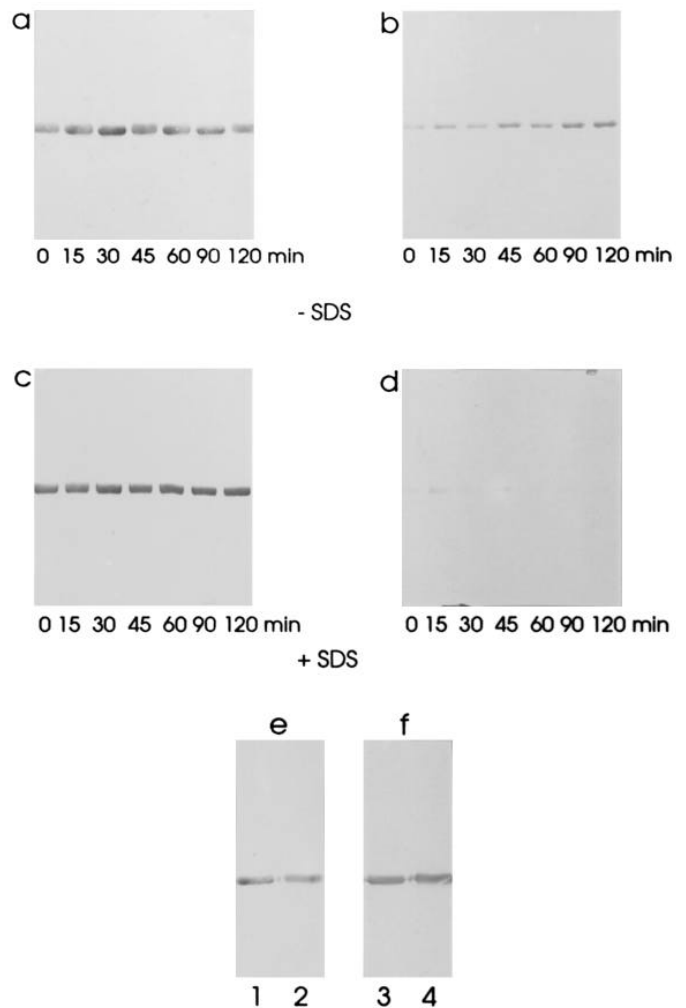**Fig. 2.** Immunoblot analysis of Δ2-tubulin abundance in rat brain extracts. (a-d) Inhibition of Δ2-tubulin formation in rat brain homogenates containing SDS. Brain homogenates from newborn rats were prepared as described in Materials and Methods. Samples (100 μl) were incubated at 30°C for the indicated times in the absence (a,b) or in the presence (c,d) of added SDS and further analysed on immunoblots for total α-tubulin content (a,c) and for Δ2-tubulin content (b,d) using monoclonal anti-α-tubulin antibody (DM1A) and polyclonal anti-Δ2-tubulin antibody as primary antibodies, respectively. The amount of homogenate protein loaded onto the gel was 5 μg. (e,f) Comparison of Δ2-tubulin abundance in rat brain homogenates and in 1X recycled microtubule protein. Samples of 1X proteins (lanes 1, 3) and of SDS brain homogenates (lanes 2, 4) were run into 8% SDS-polyacrylamide gels, transferred onto nitrocellulose sheets and immunoblotted with anti-α-tubulin antibody (DM1A, e) and anti-Δ2-tubulin antibody (f). The amounts of 1X protein and of homogenate proteins loaded onto the gel were, respectively, 1.7 μg and 10.4 μg. The secondary antibodies were conjugated to alkaline phosphatase for enzyme visualization.

proteins with that in SDS homogenates containing comparable amounts of tubulin, as judged by inspection of anti-total α-tubulin immunoblots. When such samples were immunoblotted using an anti-Δ2-tubulin antibody as a primary antibody, the signals were equivalent when comparing 1X proteins and homogenates (Fig. 2e,f). We conclude from these experiments

that Δ2-tubulin does not appear because of preparation artefacts, whether in crude extracts or in recycled tubulin preparations and that it truly accounts for 35% of total brain tubulin. Since it is specifically present in neurons (see below), its relative abundance must be even higher in these cells.

Immunofluorescence localization of Δ2-tubulin in adult and developing cerebellum

In sections of adult rat cerebellum (Fig. 3), the Δ2-tubulin antibody stained all types of neuronal cells. Purkinje cell bodies, Purkinje cell dendritic arborization and the cells of granular layer were brightly stained. This antibody gave intense particulate staining within the molecular layer, indicative of staining of granule cell axons (parallel fibers) as seen in cross-section (Cambray-Deakin and Burgoyne, 1987). In contrast, no staining of Bergman glial cells was detectable (Fig. 3e). In similar sections, as previously described (Cumming et al., 1984; Cambray-Deakin and Burgoyne, 1987; Cambray-

Deakin, 1991), anti-Tyr-tubulin antibody stained both Purkinje cells and Bergman glial cells, but did not stain parallel fibres (Fig. 3c). Anti-Glu-tubulin antibody stained parallel fibres and cells in the granular layer, but not Purkinje cells (Fig. 3d). Thus, Δ2-tubulin has a specific distribution in adult cerebellum that is not superimposable on that of either Tyr- or Glu-tubulin.

Previous work has shown variations in the proportions of Tyr- and Glu-microtubules during cerebellum development. Namely, parallel fibres contain Tyr-tubulin during early development, but this isoform disappears progressively at the time of synaptogenesis with Purkinje cell dendrites (Cumming et al., 1984).

Therefore, we examined the Δ2-tubulin antibody staining of sections of developing cerebella to see if the formation of this isotype was also developmentally regulated (Fig. 4). Two days after birth, Δ2-tubulin was absent from the external germinal layer but readily detectable in the subjacent cell layers (Fig. 4f). In ten-day-old rats, the germinal layer was still unstained
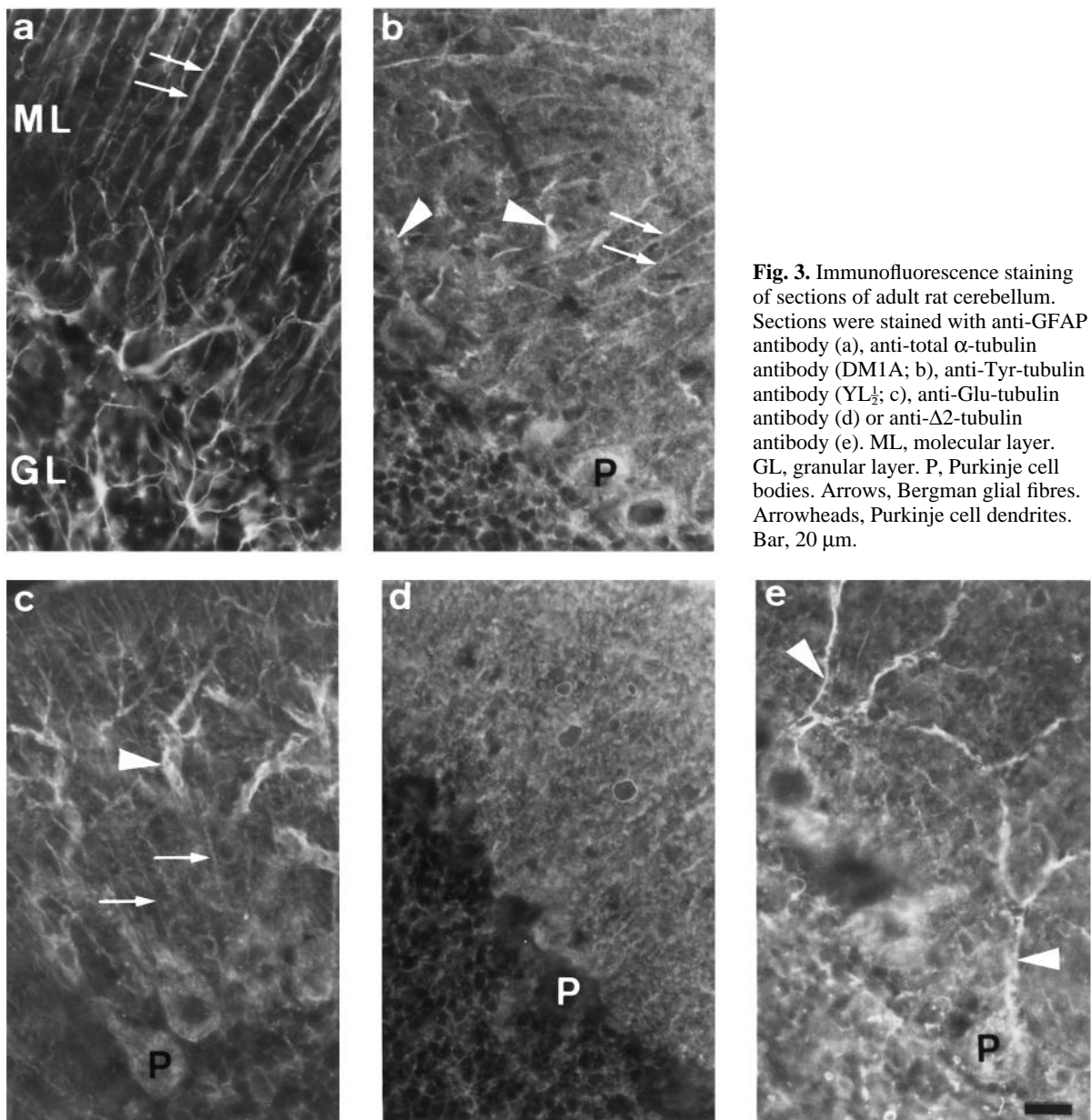

Fig. 3. Immunofluorescence staining of sections of adult rat cerebellum. Sections were stained with anti-GFAP antibody (a), anti-total α-tubulin antibody (DM1A; b), anti-Tyr-tubulin antibody (YL½; c), anti-Glu-tubulin antibody (d) or anti-Δ2-tubulin antibody (e). ML, molecular layer. GL, granular layer. P, Purkinje cell bodies. Arrows, Bergman glial fibres. Arrowheads, Purkinje cell dendrites. Bar, 20 μm.

(Fig. 4h). Otherwise, the distribution of Δ2-tubulin was similar to that observed in adult cerebellum (data not shown). These results suggest that Δ2-tubulin occurs early during neuronal maturation but only in differentiated non-mitotic cells.

We wondered whether this apparent developmental regula-

tion of Δ2-tubulin applied to the whole brain as well as to cerebellar neurons. We quantified the abundance of Δ2-tubulin on immunoblots of rat brain homogenates at various time points during development, and found a progressive enrichment of Δ2-tubulin in such homogenates up to day 22 of the postnatal
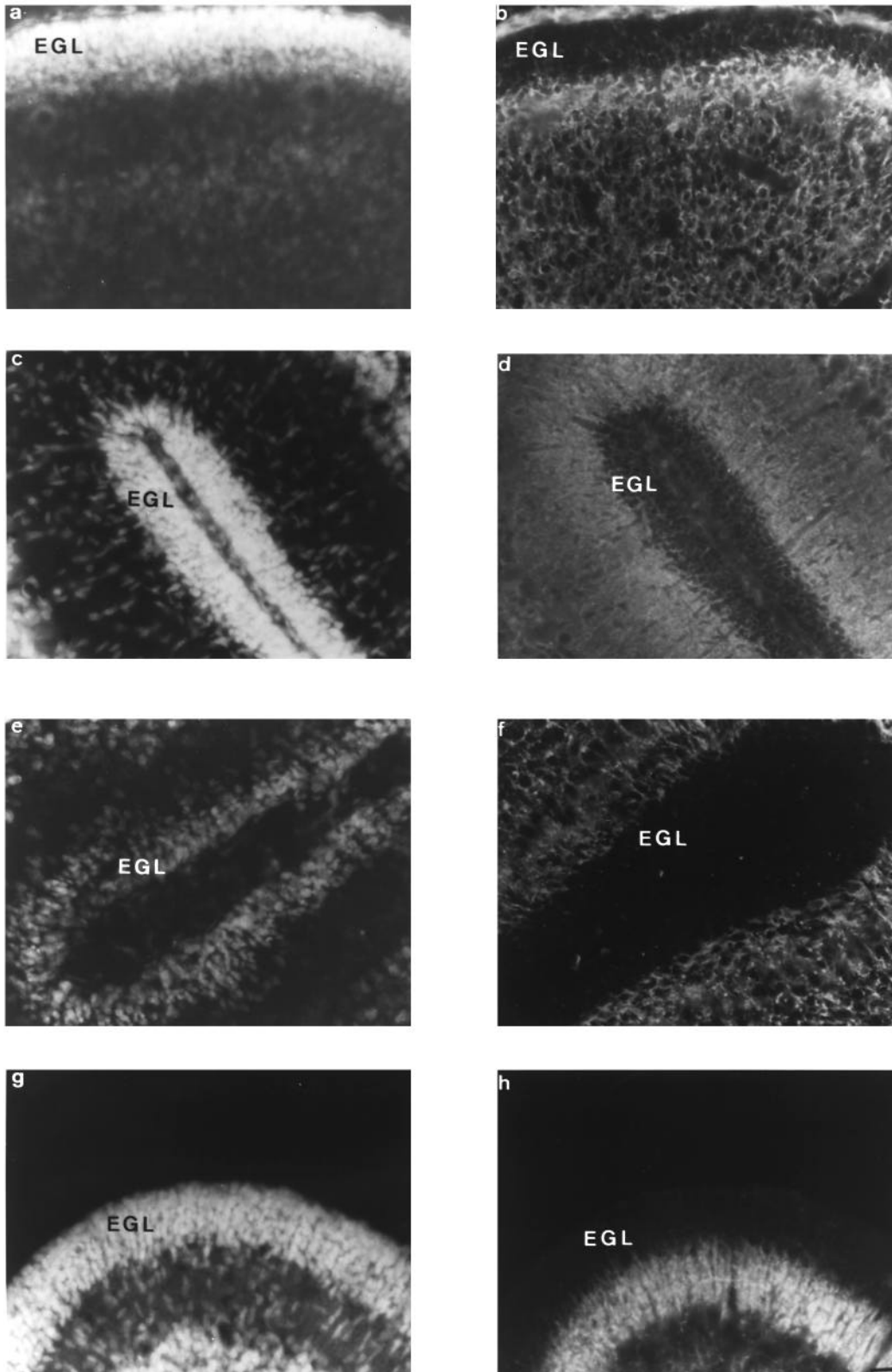
Fig. 4. Immunofluorescence staining of the cerebellar cortex during postnatal development. Sections of 2-day-old (a,b,e,f) and 10-day-old (c,d,g,h) rat cerebellum were stained with anti-total α-tubulin antibody (DM1A; b,d) or with anti-Δ2-tubulin antibody (f,h). (a,c,e,g) Corresponding staining of the DNA with Hoechst. EGL, external germinal layer. Bar, 20 μm.

period (Fig. 5). This result strongly suggests that Δ2-tubulin is developmentally regulated during brain maturation.

Immunofluorescence staining of cerebellar cells in primary culture

The results presented in the preceding section strongly suggest that Δ2-tubulin is neuronal specific. We examined cerebellar cells in primary culture in order to confirm this observation.

Cultures of cells dissociated from the immature cerebellum were examined eight days after seeding (Figs 6 and 7).

Such cell cultures mainly contain neuronal cells and glial cells, namely astrocytes (Currie, 1980). Other cell types such as fibroblasts, macrophages or oligodendrocyte precursors represent less than 1% of the total cell population (Currie, 1980). These minor cell subpopulations have not been systematically examined in the present study.

We next compared the distribution of Tyr-tubulin, Glu-tubulin and Δ2-tubulin in neuronal cells and in glial cells. Neuronal cells and glial cells were identified both according to morphological criteria and by using immunochemical markers. These markers included MAP2, a neuronal-specific protein present in neurites (Alaimo-Beuret and Matus, 1985; Caceres et al., 1986) and GFAP, an intermediate filament protein present in astrocytes but not in neurons (Currie, 1980). Cerebellar cell preparations were double stained with either anti-MAP2 antibody and one of the anti-tubulin isoforms antibodies (Fig. 6) or with anti-GFAP antibody and one of the anti-tubulin isoforms antibodies (Fig. 7).

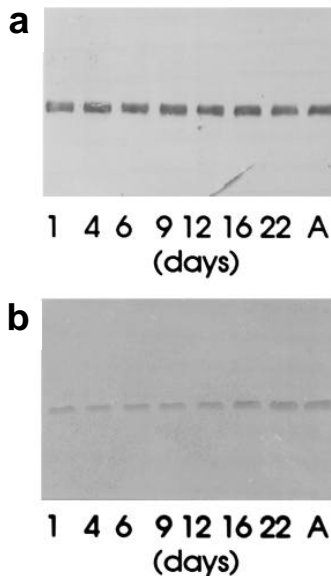As expected, both neuronal and glial cells were brightly

Fig. 5. Immunoblot analysis of Δ2-tubulin in developing rat brain. Brains from rats aged 1 to 22 days and from adult rats (A) were homogenized in SDS as described in Materials and Methods. Samples of the various homogenates containing 5 μg of protein were run into 8% SDS-polyacrylamide gels, transferred onto nitrocellulose sheets and further analysed for total α-tubulin content (a) and Δ2-tubulin content (b) using, respectively, monoclonal anti-α-tubulin antibody (DM1A) and polyclonal anti-Δ2-tubulin antibody as primary antibodies. The secondary antibodies were conjugated to alkaline phosphatase for enzyme visualization.

stained with the anti-Tyr-tubulin antibody. The anti-Glu-tubulin antibody brightly stained neuronal cells and yielded a weak signal in most glial cells (Figs 6e and 7e). Examination of such glial cells at a higher magnification than the one used in Figs 6 and 7, showed that this weak signal resulted from the staining of subsets of curly microtubules ressembling the Glu-microtubule subpopulations normally observed in non-neuronal cell types (not shown). Such Glu-microtubule networks were clearly visible in some glial cells (Fig. 6e). The anti-Δ2-tubulin antibody only reacted with neuronal cells (Figs 6h and 7h). Glial cells were not stained and careful examination of cells at higher magnification always failed to show any image of Δ2-tubulin microtubules in GFAP-positive cells (not shown). These results show strong neuronal specificity of Δ2-tubulin and suggest that it is absent from the curly Glu-microtubules normaly found in non-neuronal cells. Such a conclusion was further supported by study of other cell types (see below).

We also examined cerebellar cells in primary cultures, one day after seeding. Such cultures mainly contain neuronal cells showing growth cones. Previous work has shown that growth cones contain Tyr-tubulin but that, remarkably, Glu-tubulin is excluded from such structures (Robson and Burgoyne, 1989; Arregui et al., 1991). We wondered whether Δ2-tubulin was present in growth cones or was excluded from these structures as the Glu-tubulin isoform. Cerebellar cells were double labeled with phalloidin (Cambray-Deakin, 1991; Bamburg and Berstein, 1991) and with either anti-Tyr-tubulin antibody, anti-Glu-tubulin antibody or anti-Δ2-tubulin antibody. Results confirmed previous observations that growth cones contain Tyr-tubulin but no detectable amounts of Glu-tubulin (Fig. 8b and e). The bodies of the growth cones were stained by the anti-Δ2-tubulin antibody. As observed in the case of the tyrosinated isotype, many microspikes were not stained (Fig. 8h). The area of the cell preparation shown in Fig. 8h contained glial cells, brightly stained with phalloidin (Fig. 8g). Such cells provided an internal control for background staining with the anti-Δ2-tubulin antibody (see preceding section). The absence of such background staining showed that growth cones staining with the anti-Δ2-tubulin antibody was specific.

Immunofluorescence localization of Δ2-tubulin in other mammalian cells

A variety of cell types were stained with the anti-Δ2-tubulin antibody (Figs 9 and 10). In most cell types, results showed a distinct staining of centrosomes (Figs 9d and 10g). However, in HeLa cells no staining could be detected (not shown). Centrosomes comprise very stable microtubule assemblies. We wondered whether other structures of the same type, as primary cilia, would also react with the anti-Δ2-tubulin antibody. Growth of primary cilia was induced in various cell types either by serum deprivation or, in the case of bovine adrenal cortex cells, by ACTH treatment. Results were similar in all cases showing progressive appearance of Δ2-tubulin in the proximal part of the primary cilia. A complete analysis, using antibodies directed against total α-tubulin or against the Tyr-, Glu- and Δ2-isoforms, is shown in Fig. 9 in the case of ACTH-treated bovine adrenal cortex cells. As expected, the total α-tubulin antibody and the anti-Tyr-tubulin antibody stained a dense microtubule network (Fig. 9a,b). The anti-Glu-tubulin antibody stained a network of curly microtubules, centrosomes and primary cilia
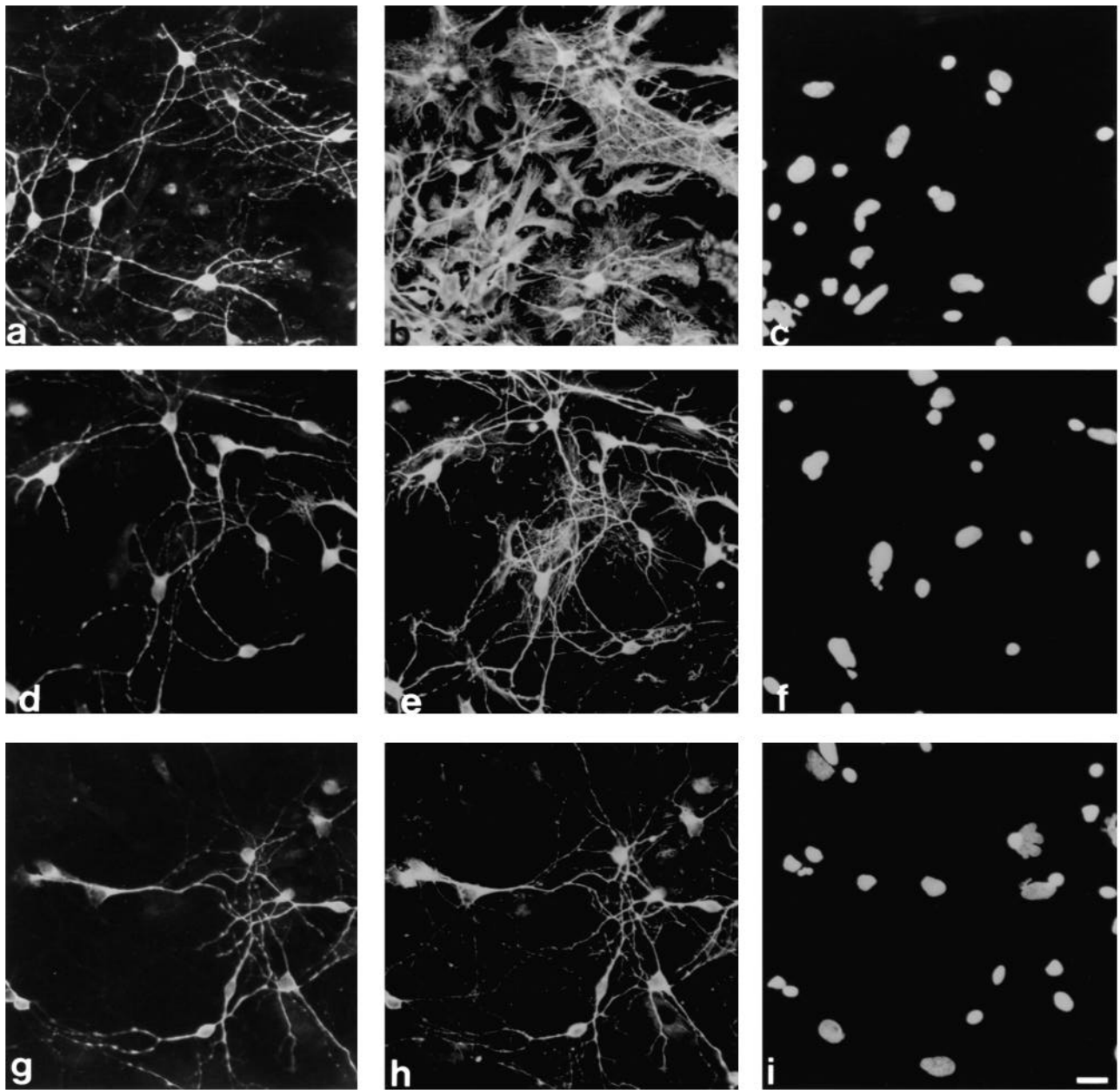
Fig. 6. Double immunostaining of cerebellar cells in primary cultures with anti-MAP2 and with anti-tubulin isoforms antibodies, eight days after seeding. Cells were grown and further processed for immunofluorescence analysis, as described in Materials and Methods. (a,d,g) Cells stained with monoclonal anti-MAP2 antibody. (b,e,h) Corresponding staining patterns observed with anti-Tyr-tubulin (YL$\frac{1}{2}$, b), anti-Glu-tubulin antibody (e) and anti-Δ2-tubulin antibody (h). (c,f,i) DNA staining with Hoechst. Bar, 20 μm.

(Fig. 9c). Δ2-Tubulin antibodies only stained centrosomes and the proximal part of the primary cilium (Fig. 9d).

Thus, Δ2-tubulin showed a specific distribution in cells, which differs from other known carboxy-terminal isoforms and is restricted to very stable microtubule assemblies.

Drug effects

We wondered whether the introduction of stable microtubule assemblies by drugs would promote the appearance of Δ2-tubulin in these structures.

Taxol treatment using concentrations of 5 to 10 μM gave negative results, despite the fact that microtubule bundles were

brightly stained by the anti-Glu-tubulin antibody (data not shown).

However, at higher drug concentrations (50 μM), Δ2-tubulin appeared in microtubule bundles in many cells (Fig. 10).

We then examined the staining of paracrystalline bundles of protofilamentous tubulin induced by vinblastine treatment. After 2 hours of treatment, paracrystals were brightly stained by the anti-total α-tubulin antibody and by the anti-Tyr-tubulin antibody, faintly stained by the anti-Glu-tubulin antibody and unstained by the anti-Δ2-tubulin antibody. After 14 hours of drug treatment, the paracrystals were stained by all antibodies (Fig. 11).
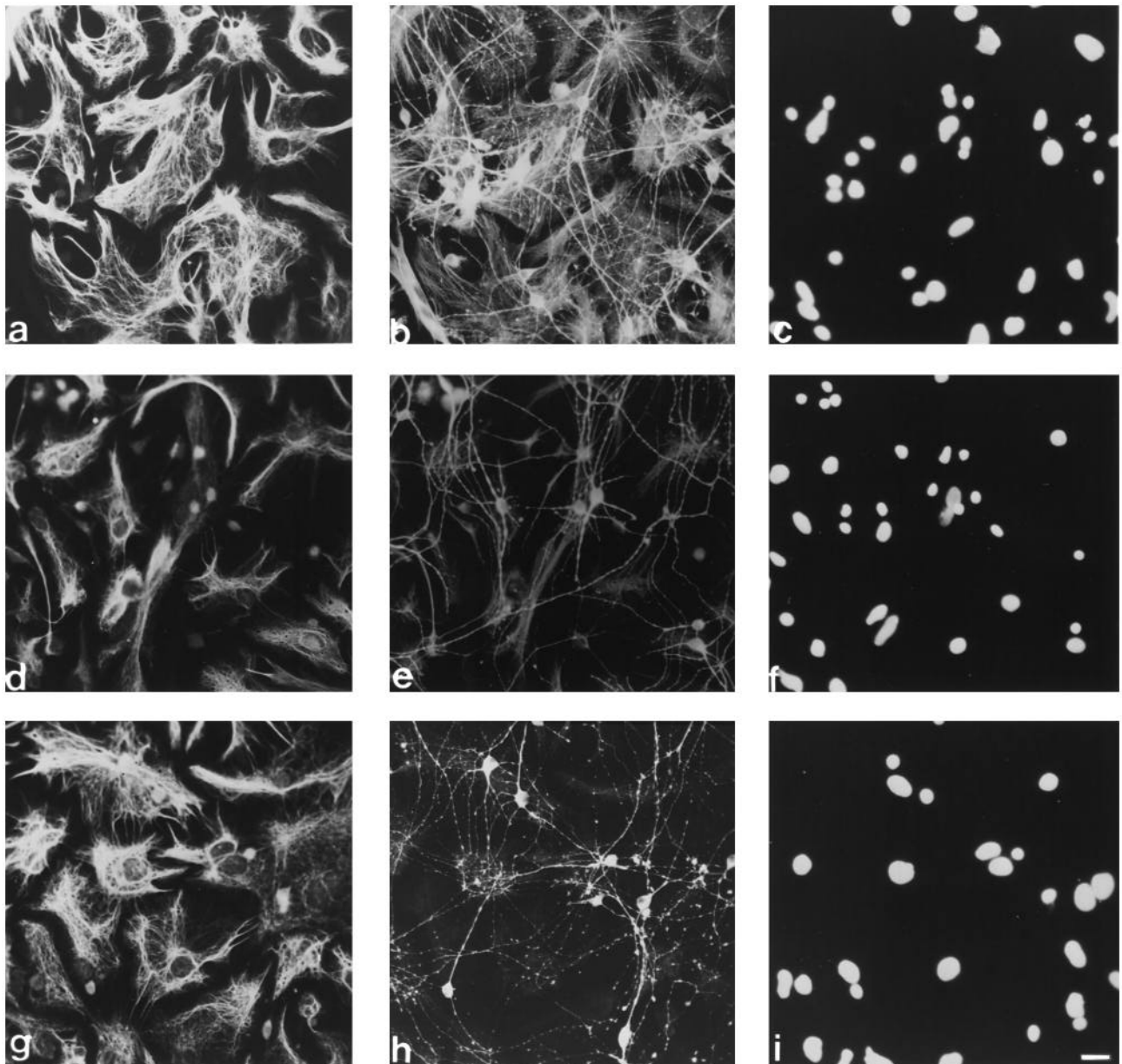
Fig. 7. Double immunostaining of cerebellar cells in primary cultures with anti-GFAP-antibody and with anti-tubulin isoforms antibodies, eight days after seeding. Cells were grown and further processed for immunofluorescence analysis as described in Materials and Methods. (a,d,g) Cells staining with anti-GFAP antibody. (b,e,h) Corresponding staining patterns observed with anti-Tyr-tubulin antibody (YL$\frac{1}{2}$, b), anti-Glu-tubulin antibody (e), anti-Δ2-tubulin antibody (h). (c,f,i) DNA staining with Hoechst. Note that some non-specific staining of the rounded neuronal cell bodies is observed in d and g. Such staining also occurs in controls. In the neuronal processes, intense varicose staining is visible with anti-tubulin antibodies. In contrast, GFAP-positive cells are brightly stained with anti-Tyr-tubulin antibody, faintly stained with anti-Glu-tubulin antibody and apparently unstained with anti-Δ2-tubulin antibody. Bar, 20 μm.

These results strongly suggested that Δ2-tubulin is slowly generated in very long-lived microtubule structures whether naturally occurring or induced by drug treatments.

Δ2-Tubulin in sea urchin flagella and in embryo cilia

To discover if Δ2-tubulin was confined to mammalian cells or if it was widespread, we looked for its presence in sea urchin flagella and in sea urchin embryo cilia (Fig. 12). Available sequence data show that sea urchin α-tubulin has a carboxy-terminal part homologous to that found in mammalia (Little and Seehaus, 1988). Furthermore, it is known that sea urchin

tubulin can undergo tyrosination and detyrosination (Kobayashi and Flavin, 1981). These considerations do not totally eliminate the possibility that an unsequenced sea urchin α-tubulin isotype has a carboxy terminus analogous to that of Δ2-tubulin, independently of post-translational modifications. However, this possibility is remote, since such a carboxy-terminal sequence has never been observed in any species (Little and Seehaus, 1988).

Sea urchin sperm flagella gave a positive signal when stained with the anti-Δ2-tubulin antibody (Fig. 12a). Immunoblot analysis of solubilized axonemal protein fractions
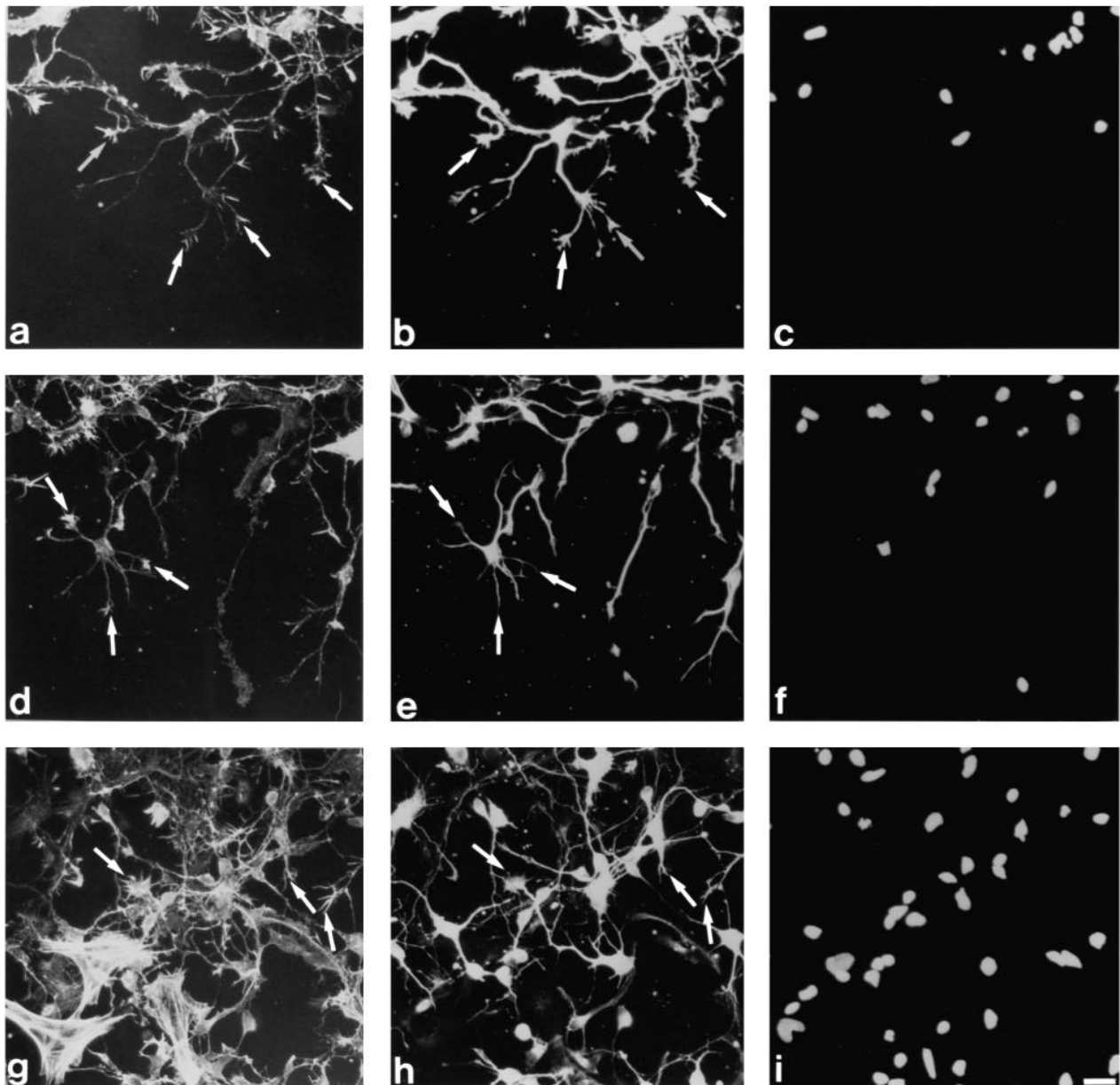
Fig. 8. Immunofluorescence analysis of cerebellar cells in primary cultures, one day after seeding. Cells were isolated from foetal rat cerebella, grown, and further processed for double actin/anti-tubulin isoform staining as described in Materials and Methods. (a,d,g) Cells stained with fluorescein-conjugated phalloidin. (b,e,h) Corresponding staining pattern observed using anti-Tyr-tubulin antibody (YL$\frac{1}{2}$, b), anti-Glu-tubulin antibody (e) and anti-Δ2-tubulin antibody (h) as primary antibodies. (c,f,i) DNA stained with Hoechst. Arrows, neuronal growth cones. Bar, 20 μm.

showed that the antibody specifically reacted with α-tubulin (Fig. 12a, insert).

In developing sea urchin embryos, cilia staining was negative at the blastula stage (data not shown), but was clearly positive at the gastrula stage (Fig. 12b). These results strongly suggest that Δ2-tubulin is a developmentally regulated, widespread component of stable tubulin assemblies.

DISCUSSION

Our results show that Δ2-tubulin is a widespread physiological isoform of tubulin. In mammalian cells and tissues, the distribution of Δ2-tubulin ressembles that of Glu- and of

polyglutamylated tubulins. Δ2-Tubulin is abundant in neurons. In non-neuronal cells, Δ2-tubulin is present in long-lived microtubules. As previously shown in the case of Glu-tubulin (Gundersen et al., 1987; Wehland and Weber, 1987), Δ2-tubulin accumulates in taxol-stabilized cellular microtubules and in the paracrystalline bundles of protofilamentous tubulin formed after vinblastine treatment of cells. Such observations strongly suggest that, like Glu-tubulin, Δ2-tubulin accumulates in long-lived polymers during the course of a maturation process caused by long periods of exposure of polymers to the action of tubulin carboxypeptidase(s) (Wehland and Weber, 1987; Khawaja et al., 1988). However, the results of the present study suggest that, as compared to
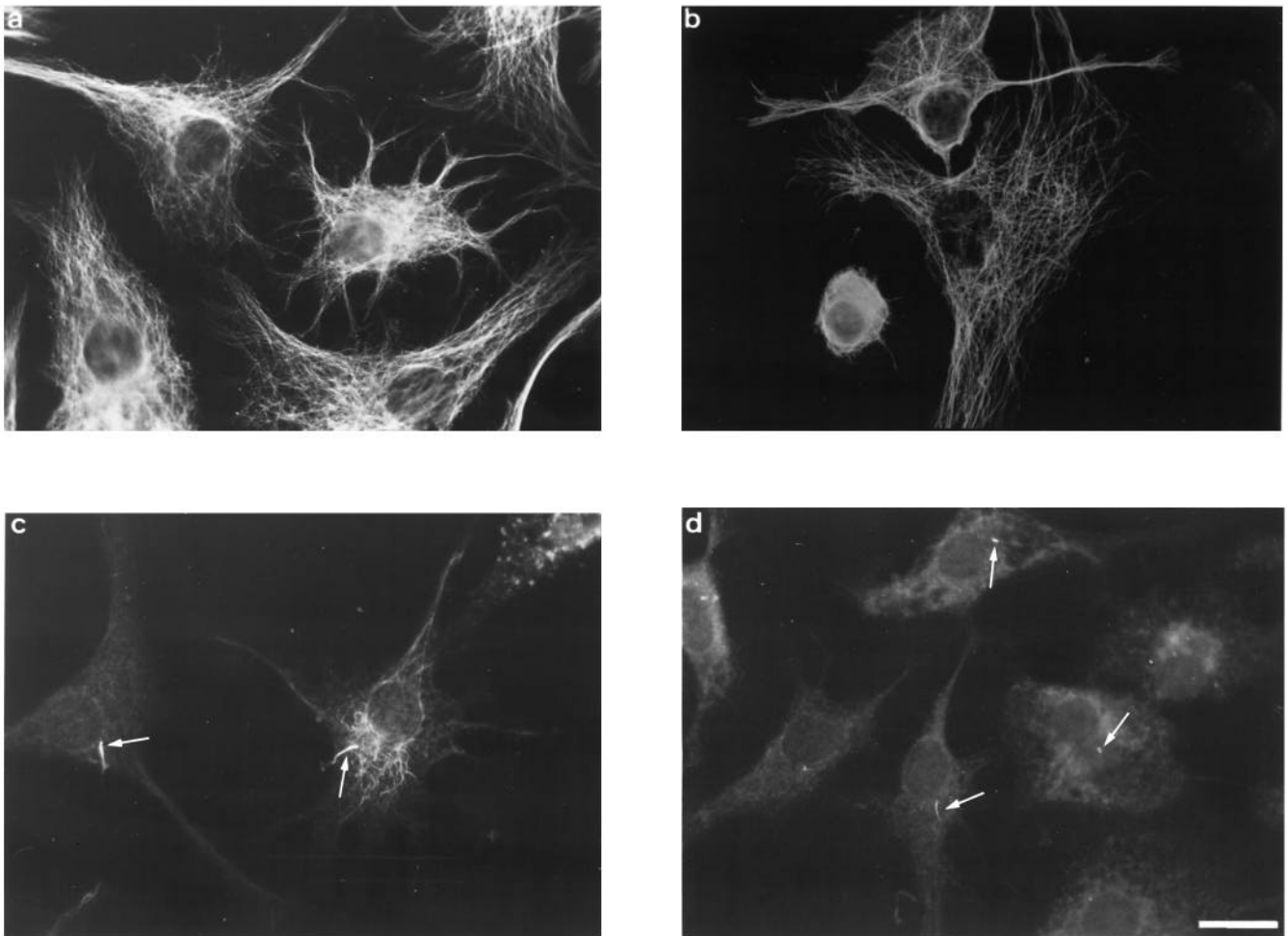
Fig. 9. Immunofluorescence staining of bovine adrenal cells treated for 24 hours with 0.1 μM ACTH. (a) Anti-total α-tubulin antibody (DM1A); (b) anti-Tyr-tubulin antibody (YL$\frac{1}{2}$); (c) anti-Glu-tubulin antibody; (d) anti-Δ2-tubulin antibody. Centrosomes and primary cilia are indicated by arrows. Bar, 20 μm.

Glu-tubulin, Δ2-tubulin accumulation occurs at a later stage of such maturation process. In non-neuronal cells, the distribution of Δ2-tubulin is more restricted than that of Glu-tubulin. Δ2-Tubulin is confined to the very long-lived microtubules found in centrioles and primary cilia. Δ2-Tubulin is absent from the curly detyrosinated microtubules normally observed in cycling cells. Such polymers are known to have a very slow turnover (Kreis, 1987; Khawaja et al., 1988). Our results suggest that such subunit turnover is nevertheless still too fast to allow significant generation of Δ2-tubulin. In taxol-stabilized cellular microtubules and in vinblastine paracrytals, Δ2-tubulin is generated over longer periods of time than Glu-tubulin, again suggesting that Δ2-tubulin generation requires polymer exposure to carboxypeptidase(s) for a longer duration of time than is necessary for the generation of Glu-tubulin.

The enzyme(s) that promotes the conversion of α-tubulin to the Δ2-isoform is not known. It is not known whether the reaction involves either a dipeptylcarboxypeptidase acting on the Tyr-tubulin isotype or a Glu-tubulin intermediate.

Our results suggest that the formation of Δ2-tubulin involves the sequence Tyr-tubulin → Glu-tubulin → Δ2-tubulin. This is the order in which the various isotypes appear in naturally occurring or drug-induced long-lived tubulin structures. Convincing evidence that this order really reflects the substrate specificity of the enzyme(s) that generates Δ2-tubulin will necessitate the use of engineered cells containing only the Glu isoform in basal conditions. Assuming that the above proposed sequence of reactions is correct, it is possible that the same carboxypeptidase transforms Tyr-tubulin into Glu-tubulin and then slowly into Δ2-tubulin. The test of this hypothesis will require the purification of tubulin carboxypeptidase and controlled modifications of its activity in cells.

The high abundance of Δ2-tubulin in neurons fits with the well-documented presence of stable microtubules in these cells (Black et al., 1984; Binet et al., 1987; Sahenk and Brady, 1988; Baas and Black, 1990) and with the considerably longer half-life of tubulin in neuronal tissues as compared to non-neuronal tissues (Caron et al., 1985; Garner, 1988; Mitchell et al., 1990; Safaei and Fisher, 1990). Such high abundance of Δ2-tubulin suggests that the dynamics of neuronal microtubules are more different from those of non-neuronal polymers than previously indicated by the relative proportions of Glu and Tyr-tubulin in neuronal and non-neuronal cell types.

The coexistence of Δ2- and Tyr-microtubules in neurons points to a remarkable heterogeneity of neuronal microtubule

subpopulations with respect to dynamic behaviour within these cells.

Recent work on microtubule behaviour in axons has shown the coexistence of highly persistant polymers that migrate all the way from cell bodies towards axonal terminations and of dynamic polymers (Baas and Black, 1990; Reinsch et al., 1991). In the present study, it was not demonstrated that Δ2-tubulin co-localizes with stable microtubules. However, previous work has shown that individual microtubules in the axon are composed of stable and labile domains, and that the stable domains contain α-tubulin devoid of carboxy-terminal tyrosine (Baas and Black, 1990). A reasonable assumption is
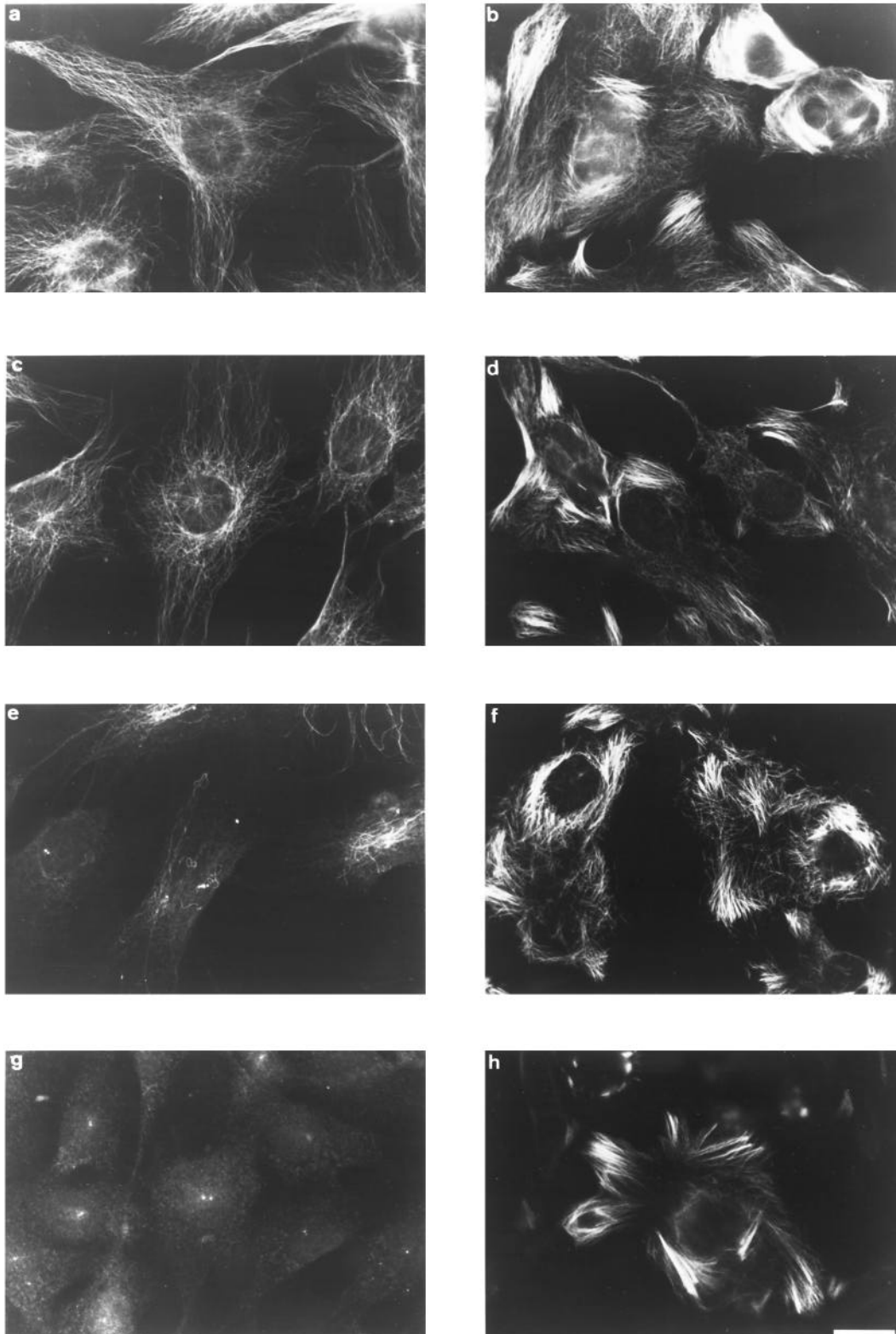

Fig. 10. Effect of taxol on the distribution of Tyr-, Glu- and Δ2-tubulins. NIH/3T3 cells were stained with: (a, b) anti-total α-tubulin antibody (DM1A); (c,d) anti-Tyr-tubulin antibody (YL$\frac{1}{2}$); (e,f) anti-Glu-tubulin antibody; or (g,h) anti-Δ2-tubulin antibody. (a,c,e,g) Controls; (b,d,f,h) after 20 hours of taxol treatment (50 μM). Bar, 20 μm.
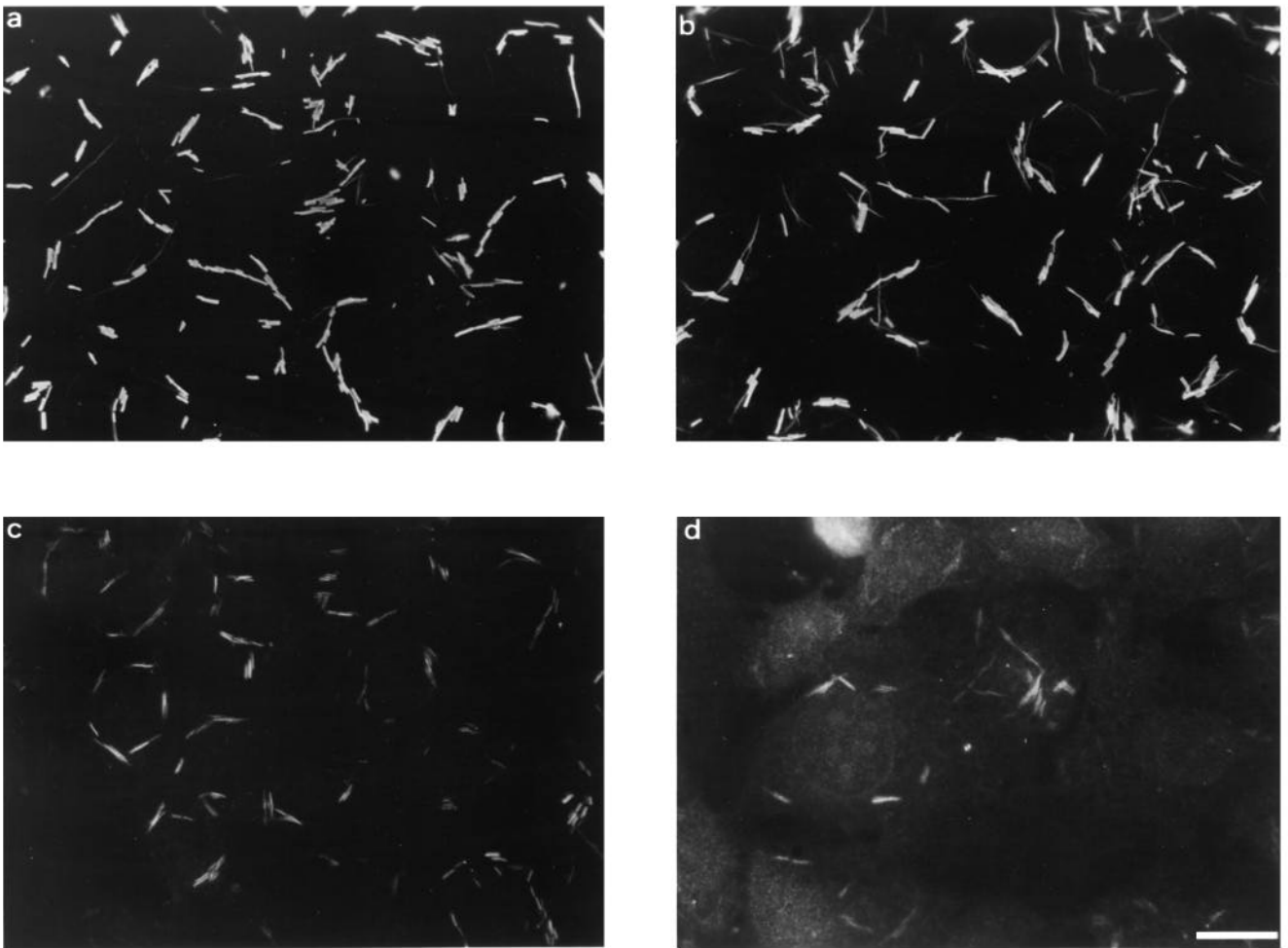
Fig. 11. Effect of vinblastine on the distribution of Tyr-, Glu- and Δ2-tubulins. NIH/3T3 cells were treated with 10 μM vinblastine for 14 hours, fixed and stained with: (a) anti-total α-tubulin antibody (DM1A); (b) anti-Tyr-tubulin antibody; (c) anti-Glu-tubulin antibody; or (d) anti-Δ2-tubulin antibody. Bar, 20 μm.
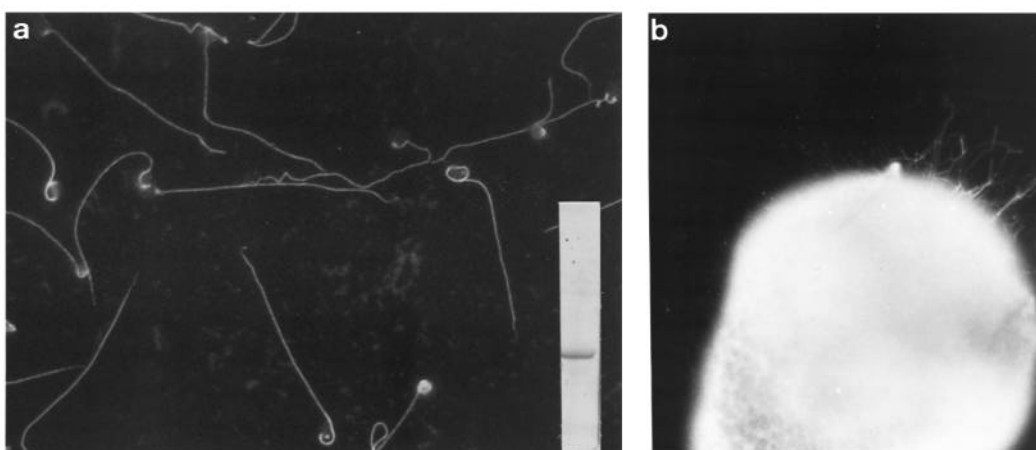

Fig. 12. Immunofluorescence staining of sea urchin spermatozoa (a) and sea urchin embryo at the gastrula stage (b) with anti-Δ2-tubulin antibody. Inset in (a), immunoblot analysis of sea urchin axonemal protein (2 μg) with anti-Δ2-tubulin antibody.

that such detyrosinated domains are not solely composed of Glu-tubulin as naturally inferred by Baas and Black, but also contain substantial amounts of Δ2-tubulin.

Purkinje cells apparently only contain Tyr- and Δ2-microtubules, without detectable amounts of Glu-microtubules.

Purkinje cells are known to contain stable polymers (Faivre-Sarrailh and Rabié, 1988). Therefore, in previous work, in which only the Tyr- and Glu-tubulin isoforms were studied, the absence of Glu-microtubules was thought to be anomalous and possibly artefactual (Cambray-Deakin and Burgoyne, 1990).

Clearly, the presence of Δ2-microtubules in Purkinje cells solves the apparent paradox of stable microtubules composed uniquely of Tyr-tubulin. Such a presence of Δ2-tubulin suggests that in Purkinje cells, as in other cell types, stable microtubules have a modified α-tubulin carboxy terminus. The absence of the Glu-isoform implies that a subpopulation of Tyr-polymers in Purkinje cells turns over too rapidly to allow the occurrence of Glu-tubulin and that such subpopulation coexists with dynamically inactive Δ2-microtubules. In this view, Purkinje cells would represent an extreme example of heterogeneity with respect to the dynamics of coexisting microtubule subpopulations.

Our results indicate that neuronal growth cones also only contain the Tyr- and the Δ2-tubulin isoforms, being devoid of Glu-tubulin despite the presence of this isoform in the nearby axon. It is known that growth cones contain dynamic microtubules (Sabry et al., 1991; Tanaka and Kirschner, 1991). The absence of Glu-tubulin in growth cones implies that Glu-microtubules are destabilized in growth cones, so that Glu-tubulin is recycled and retyrosinated in its unpolymerized form. Since neuronal growth cones are apparently devoid of stable polymers (Lim et al., 1989; Tanaka and Kirschner, 1991), we believe that Δ2-microtubules are also destabilized in these structures. The persistance of Δ2-tubulin in growth cones might mean that Δ2-tubulin cannot go back to the Glu- or Tyr-isoforms, suggesting that the formation of Δ2-tubulin from normal tubulin is an irreversible process.

Despite its widespread occurrence in living organisms, the significance of the tyrosination cycle is not understood. One reason might be that the Tyr- to Glu-tubulin conversion is a reversible but intermediary step in a series of reactions whose end product is Δ2-tubulin.

If this view is correct, detailed studies of the behaviour and of the fate of Δ2-tubulin in cells could shed light on the significance of the tyrosination/detyrosination cycle.

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