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Immunological discrimination of β-tubulin isoforms in developing mouse brain

Post-translational modification of non-class-III β-tubulins

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Individual β-tubulin isoforms in developing mouse brain were characterized using immunoblotting, after preceding high-resolution isoelectric focusing, with monoclonal antibodies against different structural regions of β-tubulin. Some of the antibodies reacted with a limited number of tubulin isoforms in all stages of brain development and in HeLa cells. The epitope for the TU-14 antibody was located in the isotype-defining domain and was present on the β-tubulin isotypes of classes I, II and IV, but absent on the neuron-specific class-III isotype. The data suggest that non-class-III β-tubulins in mouse brain are substrates for developmentally regulated post-translational modifications and that β-tubulins of non-neuronal cells are also post-translationally modified.

INTRODUCTION

Tubulin, the αβ heterodimer subunit protein of microtubules, exists in cells as a mixture of polypeptides differing in their isoelectric points (Dustin, 1984). The tubulin charge heterogeneity is strikingly high in the brain, where more than 20 tubulin isoforms can be resolved by high-resolution isoelectric focusing (Wolff *et al.*, 1982; Field *et al.*, 1984; Field & Lee, 1988). Some of the tubulin isoforms in vertebrates result from the expression of multiple tubulin genes. Both tubulin subunits are encoded by a small family of genes that produce polypeptides differing primarily in a *C*-terminal variable domain consisting of 15 amino acids (Sullivan, 1988). On the basis of highly conserved variable domains and characteristic cell type distribution, vertebrate β-tubulins were classed into six β-tubulin isotypes [for a review, see Joshi & Cleveland (1990)]. The remaining isoforms are assumed to result from post-translational modifications. Class-III β-tubulin undergoes phosphorylation (Ludueña *et al.*, 1988; Díaz-Nido *et al.*, 1990; Alexander *et al.*, 1991) and glutamylation (Alexander *et al.*, 1991). For α-tubulin, acetylation (L'Hernault & Rosenbaum, 1985; Piperno & Fuller, 1985), detyrosination–tyrosination (Barra *et al.*, 1974; Raybin & Flavin, 1975) and glutamylation (Eddé *et al.*, 1990) have been reported.

The number of β-tubulin isoforms increases during mouse brain ontogenesis with the appearance and accumulation of acidic isoforms throughout the development from embryonic to adult stages (Wolff *et al.*, 1982; Denoulet *et al.*, 1982). Developmental stage-dependent increase in β-tubulin isoforms indicates extensive and regulated post-translational modifications. A correlation between brain β-tubulin isoforms and expression of neuron-specific class-III β-tubulin was derived from studies using a specific monoclonal antibody (Lee *et al.*, 1990*a*). The post-translational modifications of class-III β-tubulin are developmentally regulated and occur within the extreme *C*-terminal domain (Lee *et al.*, 1990*b*). The charge variants representing the other classes of β-tubulin present in brain have not been conclusively identified.

In the present study we used a panel of monoclonal antibodies directed against different antigenic determinants on the *C*- and *N*-terminal structural domains of β-tubulin for immunological discrimination of individual β-tubulin isoforms in developing mouse brain. Our results demonstrate differential distribution of corresponding epitopes on β-tubulin isoforms throughout the brain development. The epitope for one of the antibodies (TU-14) is located in the isotype-defining domain and is present on the β-tubulin isotypes I, II and IV, but is not detectable on the isotype III. The results presented further indicate that non-class-III β-tubulins are substrates for developmentally regulated post-translational modifications in mouse brain and that β-tubulin of HeLa cells is also post-translationally modified.

MATERIALS AND METHODS

Materials

Carrier ampholytes Bio-Lyte 4/6 and Ampholine pH 3.5–10 were purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.) and LKB Produkter AB (Bromma, Sweden) respectively. Nitro Blue Tetrazolium, 5-bromo-4-chloro-indol-3-yl phosphate, leupeptin, aprotinin, pepstatin A and phenylmethanesulphonyl fluoride (PMSF) were supplied by Sigma (Diesenhoffen, Germany), Nonidet P40 and Pipes by Fluka Chemical AG (Buchs, Switzerland) and urea (ARISTAR grade) by BDH Chemicals Ltd. (Poole, Dorset, U.K.). Phosphocellulose (P-11) was from Whatman Biochemicals (Maidstone, Kent, U.K.) and MOWIOL 4-88 was from Calbiochem AG (Lucerne, Switzerland). All other biochemicals were of analytical grade and were obtained from Serva Feinbiochemica (Heidelberg, Germany). Nitrocellulose membranes (Synpor 6; 0.4 μm pore size) were from Synthesia (Prague, Czechoslovakia) and GelBond PAG films from LKB-Produkter AB. Taxol was kindly provided by Dr. N. Lomax from the Natural Product Branch Division of Cancer Treatment, Cancer Institute (Bethesda, MD, U.S.A.).

Antibodies

Mouse monoclonal antibodies TU-01, TU-06, TU-11 and TU-14 directed against tubulin were described previously. The TU-01 and TU-06 antibodies react with the *N*-terminal structural domain of, respectively, α- or β-tubulin, whereas the antibodies

Abbreviations used: FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline (0.15 M-NaCl/20 mM-phosphate buffer, pH 7.2); PMSF, phenylmethanesulphonyl fluoride.

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TU-11 and TU-14 react with the *C*-terminal structural domain of *β*-tubulin (Dráber *et al.*, 1989). The TEC-01 monoclonal antibody against the carbohydrate epitope of teratocarcinoma stem cells (Dráber & Pokorná, 1984) was used as a negative control. Except for the TU-01, all antibodies belonged to the IgM class. Antibodies of the IgG class were purified from ascitic fluid by DEAE-chromatography after precipitation with $(NH_4)_2SO_4$, IgM-class monoclonal antibodies were precipitated by poly(ethylene glycol) and purified by hydroxyapatite chromatography (Stanker *et al.*, 1985). The antibody purity attained was higher than 80% as assessed by SDS/PAGE and densitometric scanning of the gel. Preparation of rabbit polyclonal affinity-purified anti-tubulin antibody was described previously (Dráber *et al.*, 1991). Secondary anti-mouse antibodies conjugated with alkaline phosphatase were purchased from Promega Biotec (Madison, WI, U.S.A.), fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin (Ig) antibody was from Sevac (Prague, Czechoslovakia), Texas Red-conjugated anti-mouse Ig antibody was from Dianova (Hamburg, Germany).

Preparation of microtubule protein

Female BALB/c mice were obtained from the Institute's breeding colony and were killed by cervical dislocation. The brains from mice of different developmental stages were washed in cold PBS [phosphate-buffered saline (0.15 M-NaCl/20 mM-phosphate buffer, pH 7.2)] and homogenized with a Teflon-on-glass homogenizer (ten passes of the pestle) in the same volume of cold PDEM buffer (100 mM-Pipes/1 mM-dithiothreitol/1 mM-EGTA/1 mM-$MgSO_4$, adjusted to pH 6.8 with KOH) containing proteinase inhibitors (1 μg each of aprotinin, leupeptin, pepstatin·ml^{-1} and 1 mM-PMSF). The homogenate was sonicated for 30 s (setting on 50%) with an Ultrasonic dismembrator, model 150 (Artek System Corp., Farmingdale, NY, U.S.A.) and centrifuged at 40000 ***g*** for 10 min (4 °C). The supernatant was removed and centrifuged at 150000 ***g*** for 90 min (4 °C). The high-speed supernatant extract was polymerized in the presence of 20 μM-taxol and 1 mM-GTP and spun down through a 10% (w/v)-sucrose cushion as described by Vallee (1982). HeLa S3 cells (kindly provided by Dr. J. C. Bulinski, Columbia University, New York, NY, U.S.A.) were grown in suspension in RPMI medium (SEVAC, Prague, Czechoslovakia) supplemented with 3 mM-L-glutamine, 1 mM-sodium pyruvate, penicillin (100 units/ml), streptomycin (100 μg/ml), gentamycin (20 μg/ml) and 10% (v/v) heat-inactivated bovine serum (Bioveta, Opava, Czechoslovakia). Cells were harvested into RPMI medium, washed twice in PBS and twice in PDEM at 4 °C. The cell pellet was left to swell in 5 vol. of ice-cold 1 mM-EGTA/1 mM-$MgSO_4$, pH 6.8, for 4 min (4 °C) and then pelleted at 500 ***g*** for 10 min (4 °C). The supernatant was aspirated and the cells were resuspended in PDEM buffer with inhibitors (2 vol. of the original cell pellet). Sonication for 20 s (setting on 50%) resulted in more than 70% cell breakage. The preparation of high-speed lysate and taxol-driven polymerization of tubulin was performed as described above for mouse brain microtubule preparation. The microtubule protein from porcine brain was purified by taxol-driven polymerization or through two cycles of assembly and disassembly (Shelanski *et al.*, 1973). In this case the reassembly PDEM buffer was supplemented with 1 mM-GTP. Tubulin depleted of microtubule-associated proteins was obtained by phosphocellulose chromatography (Weingarten *et al.*, 1975). The preparations of microtubule proteins contained more than 80% tubulin as assessed by densitometric scanning of SDS/polyacrylamide gels. Carboxyamidomethylation of microtubule protein or tubulin was carried out as described by George *et al.* (1981); samples were dialysed against distilled water and portions were stored in liquid N_2. Protein concentration was determined by the method of Lowry *et al.* (1951), with BSA as standard.

Isoelectric focusing and immunoblotting

Isoelectric focusing under denaturing conditions was performed by the method of O'Farrell (1975) on slab gels (230 mm × 110 mm × 0.5 mm) containing 4% (w/v) acrylamide, 0.1% (w/v) *NN'*-methylenebisacrylamide, 9.16 M-urea, 1% (v/v) Nonidet P40 and 2.5% (w/v) Bio-Lyte 4/6. Gels were cast on GelBond PAG films, wrapped in plastic film, stored at 15 °C overnight, and used the following day. The catholyte was 0.5 M-histidine in 1 M-NaOH, the anolyte was 0.3 M-glutamic acid in 1 M-H_3PO_4. The gels were pre-run at 8.0 W for 30 min at 15 °C on LKB 2117 Multiphor II apparatus. Samples (25 μl) contained approx. 70–100 μg of protein in sample buffer consisting of 9.16 M-urea, 2.5% (v/v) Nonidet P40, 2.5% (w/v) Ampholine 3.5–10 and 5% (v/v) 2-mercaptoethanol. Isoelectric focusing was done at 2500 V for 11 h at 15 °C. The gels were fixed for 10 min in 10% (w/v) trichloroacetic acid, placed in destaining solution (ethanol/acetic acid/water, 4:1:5, by vol.) for 45 min, stained in 0.05% (w/v) Coomassie Brilliant Blue R-250 in destaining solution for 20 min and transferred to destaining solution for two 30 min washes. The dried gels were evaluated on an LKB 2202 Ultroscan laser densitometer. Alternatively, the gels were washed, after isoelectric focusing, for 30 min in 0.7% (v/v) acetic acid and proteins were transferred to nitrocellulose membranes by capillary blotting for 40 min (Albaugh *et al.*, 1987). To reveal protein bands, portions of the blots were stained by the colloidal-silver staining method (Moeremans *et al.*, 1985). Details of immunostaining using secondary antibody conjugated with alkaline phosphatase and Nitro Blue Tetrazolium with 5-bromo-4-chloro-indol-3-yl phosphate as chromogene are described elsewhere (Dráber *et al.*, 1988). Monoclonal antibodies were used at a concentration of 1–5 μg·ml^{-1}; the secondary antibody was diluted 1:6000.

SDS/PAGE

SDS/PAGE was performed by the method of Laemmli (1970) and separated proteins were transferred on to nitrocellulose sheets by electroblotting (Towbin *et al.*, 1979). The preparation of SDS samples, i.e. bacterial lysates with induced, cloned fusion proteins containing the *C*-terminal peptides of *β*-tubulin sequences of various isotypic classes was described by Lopata & Cleveland (1987). Immunostaining with monoclonal antibodies was the same as that described for blots prepared from isoelectric-focusing gels.

Immunofluorescence

Cells grown on coverslips were extracted with 0.2% Triton X-100 and fixed in 3% formaldehyde as described by Dráber *et al.* (1989). For double-label fluorescence staining, the mouse and rabbit primary antibodies were added simultaneously and the coverslips incubated for 30 min at room temperature. All antibody dilutions were made in 2% BSA in PBS. Monoclonal antibodies were used at a concentration of 20 μg·ml^{-1}; the polyclonal antibody was diluted 1:20. After being washed the slides were incubated simultaneously with FITC-conjugated anti-(rabbit Ig) antibody (1:20 dilution) and Texas Red-conjugated anti-mouse Ig antibody (1:50 dilution) for 30 min at room temperature. Washed slides were then mounted in MOWIOL 4-88 and examined with a Leitz Orthoplan microscope. Control experiments have shown that conjugated anti-rabbit antibodies and conjugated anti-mouse antibodies did not cross-react with the respective primary antibodies. Neither the control antibody TEC-01 nor the conjugates alone gave any detectable staining.

RESULTS

In order to improve the accuracy when evaluating tubulin heterogeneity we have modified the high-resolution isoelectric-focusing technique on horizontal-slab polyacrylamide gels; the modification enables a distinct separation of tubulin isoforms and their subsequent transfer to nitrocellulose membranes. It allows us to examine the distribution of antigenic determinants on tubulin isoforms using well-characterized antibodies. Examples of the separation of tubulin isoforms of porcine and mouse adult brain tubulin are shown in Figs. 1(*a*) and 1(*c*). No qualitative or quantitative differences in tubulin isoforms were observed in preparations of porcine brain tubulin prepared either by taxol-driven polymerization or by two cycles of polymerization–depolymerization followed by phosphocellulose chromatography. Typically, 22 tubulin isoforms were detectable in adult brain tissues. Though their overall distribution was similar, the porcine and mouse tubulins differed in relative intensities of corresponding isotubulins. The blotting procedure

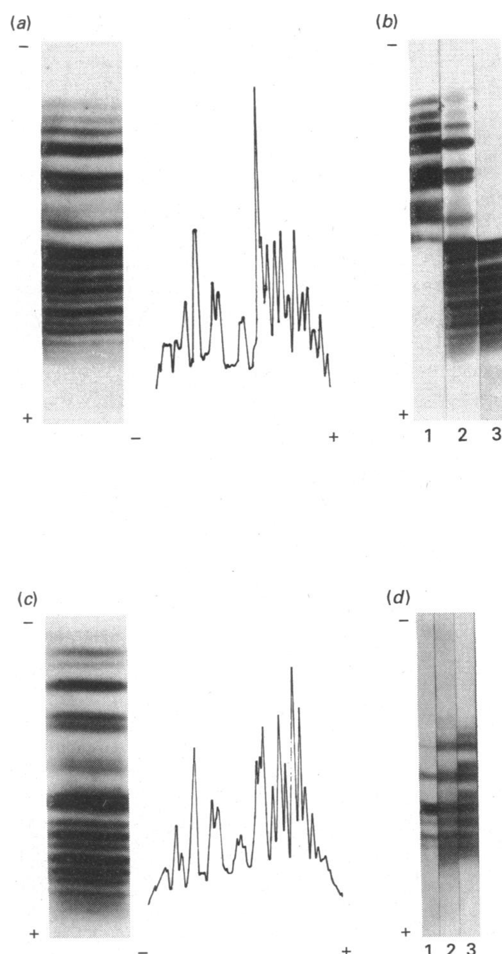

Fig. 1. Isoelectric-focusing patterns and immunoreactivity of porcine (*a*, *b*) and mouse (*c*, *d*) tubulin isoforms

(*a*) Coomassie Blue staining of porcine tubulin isoforms and corresponding densitometric scan. (*b*) Immunoblot of porcine tubulin isoforms with TU-01 (lane 1) and TU-06 (lane 3) antibodies to identify, respectively, the α- and β- isotubulins. Proteins transferred on to nitrocellulose were stained with colloidal silver (lane 2). (*c*) Coomassie Blue staining of tubulin isoforms isolated from a 3-month-old mouse and corresponding densitometric scan. (*d*) Parallel lanes were immunoblotted with antibodies TU-14 (lane 1), TU-11 (lane 2) and TU-06 (lane 3) against β-tubulin.

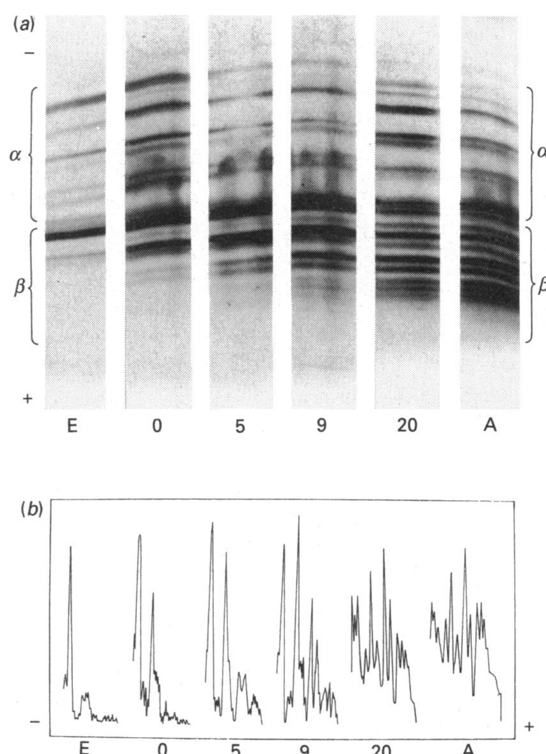

Fig. 2. Isoelectric-focusing patterns of brain tubulin at different stages of mouse development

(*a*) Coomassie Blue staining of tubulin isoforms. (*b*) Corresponding densitometric scans of β-tubulin isoforms. Developmental stages: E, 15-day-old embryo; 0, newborn mouse; 5, 9 and 20 indicate 5-, 9- and 20-day-old mice; A, 6-month-old mouse (adult). Positions of α- and β-tubulin isoforms were identified on parallel lanes immunoblotted with antibodies against α- and β-tubulin (TU-01, TU-06 respectively).

preserved the separation of isotubulins observed in polyacrylamide gels, as shown on blots stained with colloidal silver (Fig. 1*b*, lane 2). Monoclonal antibodies TU-01 and TU-06 against conserved epitopes located on *N*-terminal domains of α- or β-tubulin respectively, were used for identification of isoforms belonging to α- or β-tubulin subunits (Fig. 1*b*, lane 1 and lane 3). As expected, more acidic isotubulin variants belonged to β-tubulin; similarly the heterogeneity of β-tubulin was higher in comparison with α-tubulin. A closer inspection of immunoblots suggested that the very acid α-tubulin isoforms and the very basic β-tubulin isoforms co-migrated in two bands, approximately in the middle of the isoelectric-focusing pattern. By means of monoclonal antibodies TU-01 and TU-06 we detected nine α-tubulin isoforms and 13 β-tubulin isoforms in porcine brain tubulin. The polyclonal affinity-purified anti-tubulin antibody reacted with all isoforms, whereas the control TEC-01 antibody did not stain the blots. In contrast with the TU-06, antibodies TU-11 and TU-14 directed against different antigenic determinants on the *C*-terminal domain of β-tubulin (Dráber *et al.*, 1989) reacted only with a limited number of β-tubulin isoforms, both in porcine and mouse microtubule preparations. The staining pattern obtained with TU-11 and TU-14 antibodies was not identical; the TU-14 reacted with only some of the β-tubulin isoforms recognized by TU-11. The reactivity of antibodies with adult mouse tubulin isoforms is demonstrated in Fig. 1(*d*).

To determine whether or not the antibodies can discriminate between β-tubulin isoforms at various stages of brain devel-

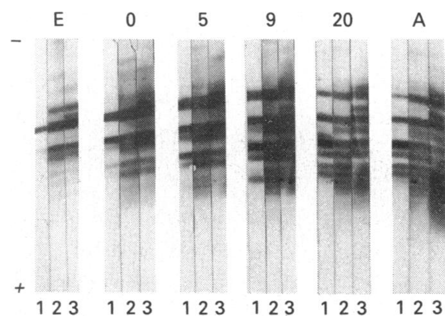

Fig. 3. Immunoblotting analysis of brain tubulin isoforms at different stages of mouse development with antibodies against β-tubulin

Lane 1, immunoreactivity with TU-14; lane 2, immunoreactivity with TU-11; lane 3, immunoreactivity with TU-06. Developmental stages: E, 15-day-old embryo; 0, newborn mouse; 5, 9 and 20 indicate 5-, 9- and 20-day-old mice; A, 6-month-old mouse (adult).

Type I	E	E	E	E	D	F	G	E	E	A	E	E	E	A						
Type II	D	E	Q	G	E	F	E	E	E	E	G	E	D	E	A					
Type III	E	E	E	G	E	M	Y	E	D	D	E	E	E	S	E	S	Q	G	P	K
Type IV	E	E	E	G	E	F	E	E	E	A	E	E	E	V	A					

Fig. 4. *C*-Termini of vertebrate β-tubulin isotypes

The *C*-terminal sequences of the fusion proteins used with β-tubulin isotype-defining domains, beyond residue 430, are shown in the one-letter amino-acid code.

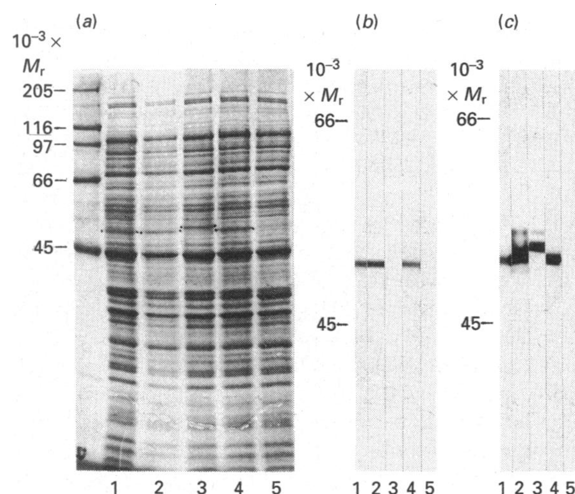

Fig. 5. Specificity of binding of antibodies to β-tubulin isotypes

(*a*) Coomassie Blue staining of bacterial lysates containing induced cloned fusion proteins separated on SDS/polyacrylamide gel. The positions of fusion proteins are indicated by dots. (*b*) Immunoreactivity of fusion proteins with antibody TU-14. (*c*) Immunoreactivity of fusion proteins with antibody TU-11. Lanes 1–4, bacterial lysates containing, respectively, fusion protein type I, II, III and IV. Lane 5, induced pATH vector alone. Positions of M_r markers are indicated on the left. Proteins were separated on 8.5%-(w/v)-polyacrylamide gel.

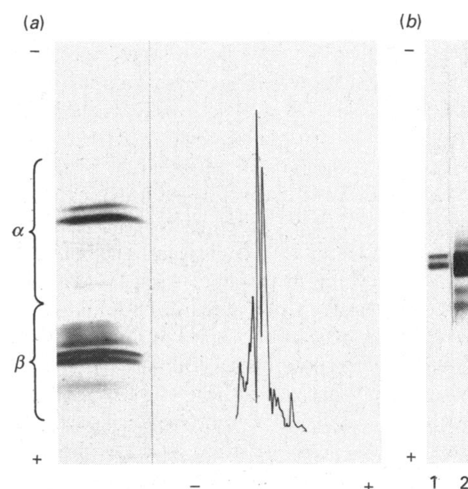

Fig. 6. Isoelectric-focusing pattern and immunoreactivity of tubulin isoforms of HeLa cells

(*a*) Coomassie Blue staining of HeLa tubulin isoforms and corresponding densitometric scan of β-tubulin isoforms. (*b*) Immunoblot of tubulin isoforms with antibodies TU-14 (lane 1) and TU-06 (lane 2). Positions of α- and β-tubulin isoforms were identified on parallel lanes immunoblotted with antibodies against α- and β-tubulin (TU-01 and TU-06 respectively).

opment, microtubules were isolated from 15-day-old-mouse embryo, newborn mouse and from mice that were 5, 9 and 20 days old. Substantial qualitative and quantitative changes in the expression of β-tubulin isoforms during mouse brain development are evident in Fig. 2(*a*) and in a corresponding densitometric scan (Fig. 2*b*). The applied high-resolution isoelectric focusing made it possible to identify five β-tubulin isoforms at embryonic day 15 and 12 β-tubulin isoforms at postnatal day 20; 12 β-tubulin isoforms were also found in 6-month-old mouse. The increase in the total number of tubulin isoforms and appearance of the more acidic β-tubulin isoforms is consistent with previous reports (Denoulet *et al.*, 1982; Wolff *et al.*, 1982). The immunoblotting experiments disclosed that the differential staining pattern of isotubulins with antibodies TU-11 and TU-14 was retained throughout all tested developmental stages (Fig. 3). At embryonic day 15 the TU-14 antibody reacted strongly only with the most prominent β-tubulin isoform and faintly with another one; at postnatal day 20 the same antibody reacted with six isoforms. The TU-11 antibody reacted with four isoforms in embryonic brain and with nine isoforms in the brain of adult animals.

As the epitopes recognized by the antibodies TU-11 and TU-14 are in both cases located within the *C*-terminal domain of β-tubulin (Dráber *et al.*, 1989), the limited reactivity of the antibodies could be attributed to the presence of corresponding epitopes just on some β-tubulin isotypes. For this reason bacterial lysates containing specific induced cloned fusion proteins were used for immunoblotting. The *N*-terminal region of each of the fusion proteins is formed by the bacterial protein trpE, and the *C*-terminal domains are formed by β-tubulin sequences beginning at β-tubulin residue 345 (Lopata & Cleveland, 1987). *C*-Termini sequences of the used fusion proteins with their β-tubulin isotype-defining regions are shown in Fig. 4. Immunoblotting on bacterial cell lysates containing fusion proteins (Fig. 5*a*) have shown that the TU-14 reacted with the fusion proteins containing sequences characteristic for isotypes I, II and IV, but not for isotype III (Fig. 5*b*). The antibody TU-11 reacted with all fusion proteins (Fig. 5*c*), and TU-06 gave no reaction (result not shown).

To examine the distribution of TU-14 epitope on β-tubulin isoforms of non-neuronal cells, microtubule protein was prepared from HeLa cell line. In comparison with adult brain tubulin, the preparation contained only six β-tubulin isoforms (Fig. 6*a*). The doublet of dominant isoforms was in a pI position that cor-

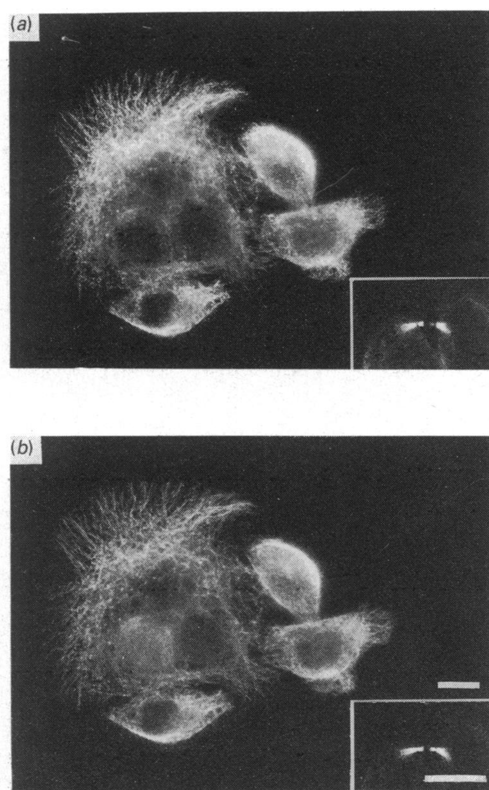

Fig. 7. Immunofluorescence staining of HeLa cells with TU-14 antibody

Cells were double-labelled with TU-14 antibody (*a*) and polyclonal anti-tubulin antibody (*b*). Interphase microtubules and midbodies (insets) are shown. The bar represents 10 μm.

responded to that of the most prominent isoform in embryonic brain (result not shown). Whereas the TU-06 antibody stained all isoforms on immunoblots, the TU-14 reacted with only two of them (Fig. 6*b*). Yet the limited reactivity of TU-14 with β-tubulin isoforms had no effect on decoration of microtubular structures of Hela cells. Double-label immunofluorescence with polyclonal anti-tubulin antibody revealed that TU-14 stained all microtubules in cytoplasmic arrays, mitotic spindles and midbodies (Figs. 7*a* and 7*b*).

DISCUSSION

The experiments described here demonstrate that a combination of high-resolution isoelectric focusing with immunoblotting enables antigenic characterization of individual charge variants of tubulin subunits. Although antibodies specific for different β-tubulin isotype classes had been prepared (Lopata & Cleveland, 1987; Lewis *et al.*, 1987; Banerjee *et al.*, 1988; Banerjee *et al.*, 1990; Asai & Remolona, 1989), the correlation between tubulin isoforms and tubulin isotypes during brain development has so far been experimentally verified only for extensively modified neuron-specific class-III β-isotype (Lee *et al.*, 1990*b*). Results presented here indicate that β-tubulin isotypes that are not neuron-specific are also post-translationally modified in cells during brain development as well as in non-neuronal cells.

In mammalian brain there appear to exist four classes of β-tubulin, designated I, II, III, IV, which are distinguished by their unique *C*-terminal sequences (Sullivan, 1988; Banerjee *et al.*, 1988). To discriminate other isotype classes from the β-tubulin isotype class III, we have used the TU-14 monoclonal antibody. Previously we have shown that this antibody does not react with the 48 kDa β-tubulin poplypeptide after partial proteolysis with subtilisin (Dráber *et al.*, 1989). As this proteinase preferentially cleaves a 1–2 kDa peptide from the *C*-terminal end of tubulin (Sackett *et al.*, 1985; de la Viña *et al.*, 1988), and consequently removes the isotype-defining domain (Lee *et al.*, 1990*b*), the epitope recognized by TU-14 is within the β-tubulin *C*-terminal isotype-defining domain. Immunoblotting with fusion proteins has shown that the epitope is located on the β-tubulin isotypes of classes I, II and IV, but not on the neuron-specific β-tubulin isotype of class III (Fig. 5). It is most likely, therefore, that the epitope is within the β region (431–444).

Non-class-III β-tubulins were resolved to six charge variants in a microtubule preparation isolated from adult brain. The gradual appearance of acidic isoforms at different stages of brain ontogenesis indicates that post-translational modification of non-class-III β-tubulin isotypes is developmentally regulated. This is in agreement with an observation of Lee *et al.* (1990*b*), who, by two-dimensional electrophoresis of rat brain tubulin, found more acidic β-tubulin variants unreactive with class-III-specific antibody. In their case, however, distinct isoforms were not detected, owing to a loss of resolution in the second dimension. Isotype-specific antibodies will be necessary to estimate more precisely the number of charge variants that represent each β-tubulin isotype in the brain, because some isoforms of individual β-tubulin classes can cofocus. As phosphorylation of brain tubulins is limited to Ser-444 of β-tubulin isotype class III (Díaz-Nido *et al.*, 1990; Alexander *et al.*, 1991), other post-translational modifications ought to be responsible for the multiplicity of isoforms of other β-tubulin isotype classes in the brain. It is possible that polyglutamylation, which was described on Glu-445 of α-tubulin (Eddé *et al.*, 1990) as well as on Glu-438 of the class-III β-tubulin isotype (Alexander *et al.*, 1991), might be involved in the generation of charge variants in other brain β-tubulin classes. If so, an increase in the acidity of the already highly negative *C*-terminal extreme of non-class-III β-tubulin isotypes could regulate interaction of these isotypes with microtubule-associated proteins that have their binding sites in this region (Littauer *et al.*, 1986; Paschal *et al.*, 1989).

The limited reactivity of TU-11 antibody with brain β-tubulin isoforms cannot be explained by the restriction of the corresponding epitope only to certain β-tubulin isotypes. The epitope is not located in the isotype-defining region (Dráber *et al.*, 1989); moreover, the antibody reacted with all tested fusion proteins (Fig. 5). Since the fusion proteins contained the *C*-terminal β-tubulin sequences beginning at residue 345, the epitope could be located in the region β (345–430). A very similar staining pattern as that characteristic for TU-11, may, however, be obtained with another antibody, TU-12, which recognizes a non-identical antigenic determinant in the *C*-terminal domain of β-tubulin outside of the isotype-defining region (Dráber *et al.*, 1991; P. Dráber & I. Linhartová, unpublished work). More precise location of corresponding epitopes is necessary before the reactivity of these antibodies with particular brain isoforms may be fully understood.

The existence of six β-tubulin isoforms in HeLa cells (Fig. 6), i.e. a number greater than the actual number of tubulin genes (Lewis *et al.*, 1987), indicates that isotypes of non-neuronal cells likewise are substrates for post-translational modifications. In comparison with adult mouse brain, however, a substantially lower number of isoforms was observed, and in this respect HeLa cells resembled embryonic brain. The restricted reactivity of TU-14, compared with TU-06, demonstrates that it is possible to discriminate immunologically tubulin isoforms also in a

tubulin preparation isolated from a homogeneous cell population of non-neuronal cells. Differential spatial distribution of β-tubulin isotypes was described on a single cell-level for pleochromocytoma PC-12 cells (Asai & Remolona, 1989; Joshi & Cleveland, 1989). In HeLa cells, differential distribution of β-tubulin isoforms recognized by TU-14 was not observed within the limits of immunofluorescence-microscopy resolution. The isoforms were involved in the formation of all microtubular structures, and the contribution of these isoforms to functional diversity of HeLa microtubules, is probably subtle.

The data indicate that non-class-III β-tubulins are substrates for developmentally regulated post-translational modifications in brain, and that β-tubulins in non-neuronal cells are post-translationally modified as well. Although the actual physiological significance of the modifications remains to be elucidated, we assume that post-translational modification of various classes of β-tubulin isotypes are important for regulation of tubulin interactions with various microtubule-associated proteins in neuronal as well as non-neuronal cells.

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