Volume 320, number 1, 79-82 FEBS 12267 March 1993

Distribution of non-class-III β-tubulin isoforms in neuronal and non-neuronal cells

Irena Linhartová, Eduarda Dráberová, Vladimír Viklický and Pavel Dráber

Institute of Molecular Genetics, Czech Academy of Sciences, 142 20 Praha 4, Czech Republic

Received 7 February 1993

β-Tubulin isoforms in brain tissues and in cell lines were analyzed by high-resolution isoelectric focusing in combination with monoclonal antibodies. Post-translational modifications of brain non-class-III β-tubulin isoforms were found in phylogenetically distant species ranging from pig to carp. Less extensive modifications were also observed in Neuro-2a, HeLa and 3T3 cells, where most acidic isoforms were glutamylated, while the basic, most abundant isoforms were not. The data suggest post-translational modification of non-class-III β-tubulin isoforms in neuronal as well as in non-neuronal cells. Such modification might modulate interaction of tubulin isoforms with microtubule-associated proteins.

Isoelectric focusing; Post-translational modification; Subtilisin; Tubulin isotype

1. INTRODUCTION

In higher vertebrates the α- and β-tubulin subunits, major components of microtubules, exhibit a high level of charge heterogenity [1]. The heterogeneity of β-tubulin isoforms results from the tissue-specific expression of multiple β-tubulin isogenes [2] and from post-translational modifications. Up to now phosphorylation of Ser^{444} [3,4] and polyglutamylation of Glu^{438} [4] was described for the neuron specific class III isotype, and polyglutamylation of Glu^{435} for the most abundant brain class II isotype [5]. These post-translational modifications occur in the acidic isotype-defining domain that can be removed by subtilisin [6,7]. Developmentally regulated post-translational modification of the class III β-tubulin isotype was demonstrated in rat brain by immunodetection of tubulin isoforms separated by two-dimensional gel analysis [8]. Data on distribution and post-translational modifications of other β-tubulin isotypes are limited. In this report we have used HRIF, in combination with subtilisin digestion and mAbs against structural domains of tubulin, to characterize β-tubulin isoforms in brains of various species and in cell lines.

Correspondence address: I. Linhartová, Institute of Molecular Genetics, Czech Academy of Sciences, Vídeňská 1083, 142 20 Praha 4, Czech Republic. Fax: (42) (2) 4716629.

Abbreviations: HRIF, high-resolution isoelectric focusing; mAb(s), monoclonal antibody(ies); MTP(s), microtubule protein(s); SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis.

2. MATERIALS AND METHODS

2.1. *Protein preparation*

MTPs were isolated from porcine, rabbit, hen and carp adult brains as well as from cultured human epitheloid carcinoma cell line HeLa, mouse embryonic fibroblast cell line 3T3 and mouse neuroblastoma cell line Neuro-2a, by taxol-driven polymerization [9] with added proteinase inhibitors in a modification described [10]. The preparations of MTPs contained more than 80% of tubulin as assessed by SDS-PAGE and densitometric scanning of the gel. Protein concentration was determined [11] using bovine serum albumin as a standard. MTPs were carboxyamidomethylated [12], dialysed against distilled water and stored in small aliquots in liquid nitrogen.

2.2. *Proteolytic digestion*

Tubulin (1 mg/ml in 100 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM GTP adjusted to pH 6.8 with KOH) was treated at 25°C with subtilisin (Sigma, cat. no. P 8038) at 0.1% by weight. Aliquots removed after 0, 40 and 240 min were made 2 mM in PMSF and immediately boiled in SDS sample buffer.

2.3. *Antibodies*

Mouse mAb TU-01 against the α-subunit of tubulin, and mAbs TU-06, TU-11, TU-14 against the β-tubulin have been previously described [13,14]. The mAbs TU-01 and TU-06 react with the N-terminal structural domain of α- and β-tubulin, respectively, whereas the TU-11 and TU-14 react with the C-terminal structural domain of β-tubulin [14]. The mAb VI-01 against vimentin [15] was used as a negative control. Except for the TU-01, all antibodies belong to the IgM class. The mAbs were purified from ascitic fluid [14] and their purity was higher than 80% as determined by SDS-PAGE. The mAb GT335 against glutamylated tubulin was kindly provided by Dr. A. Wolff.

2.4. *Gel electrophoresis and immunodetection*

Tubulin subunits were separated by SDS-PAGE [16] and corresponding gel slices were electroeluted in Bio-Rad Model 422 Electro-Eluter according to the manufacturer's directions. Proteins separated by SDS-PAGE were electroblotted [17] to nitrocellulose membranes as described [18]. HRIF under denaturing conditions [19] was performed on slab gels according to [10]. Briefly, gels contained 4% (w/v)

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acrylamide, 0.1% (w/v) *N,N'*-methylenebisacrylamide, 9.16 M urea, 1% (v/v) Nonidet P-40 and 2.5% (w/v) Bio-Lyte 4/6. 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. Samples contained approximately 70–100 μg protein in sample buffer, HRIF was run at 2,500 V for 11 h at 15°C, and gels were stained by Coomassie brilliant blue R-250 or alternatively capillary blotted to nitrocellulose membranes [10]. The apparent gradients of gels were estimated by cofocusing carbamylated carbonic anhydrase IEF standards (Pharmacia AB, Uppsala, Sweden). The dried gels were evaluated on an LKB 2202 Ultroscan Laser Densitometer. To visualize protein bands, portions of the blots were stained by a modified colloidal silver staining method [20]. Details of immunostaining using secondary antibodies conjugated with alkaline phosphatase were given previously [18]. The mAbs were used at a concentration of 1–5 μg/ml. Neither the control mAb VI-01 nor the secondary conjugates alone stained the blots.

3. RESULTS

Separation of tubulin subunits by SDS-PAGE followed by their electroelution from the gel and subsequent HRIF on horizontal slab gels made it possible to unambiguously classify individual tubulin isoforms into α- or β-tubulin subunits. Altogether 23 isoforms were detectable in adult porcine brain tubulin; 10 α-tubulin isoforms and 13 β-tubulin isoforms. The isoforms were separated in apparent pH range 5.3–5.7 (Fig. 1). In order to determine to what extent the charge heterogeneity of β-tubulin isoforms originated in the C-terminal region of the subunit, the tubulin was treated with subtilisin and subjected thereafter to HRIF and to immunoblotting with mAbs against different epitopes on β-tubulin (Fig. 2). Subtilisin preferentially cleaves a 1–2 kDa peptide from the C-terminal end of β-tubulin [6,7] and removes the isotype-defining region. Tubulin samples containing no undigested β-tubulin but just β-subunits lacking the C-termini (Fig. 2a, lane 3) were separated by HRIF into 5 isoforms, as detected by Coomassie blue staining (Fig. 2b, lane 2). The removing of

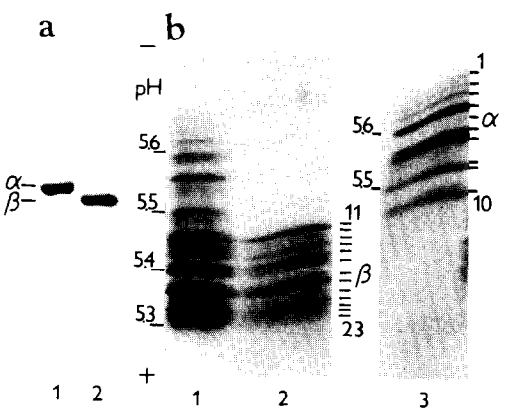

Fig. 1. Identification by HRIF of porcine brain tubulin isoforms in isolated α- and β-subunits. (a) Coomassie blue staining of electroeluted α- (lane 1) and β-subunits (lane 2) separated by 10% SDS-PAGE. (b) Coomassie blue staining of isoforms in whole tubulin (lane 1), β- (lane 2) and α-subunits (lane 3) separated by HRIF. Individual tubulin isoforms are denoted by bars between 1 and 10 for the α-subunit, and between 11 and 23 for the β-subunit.

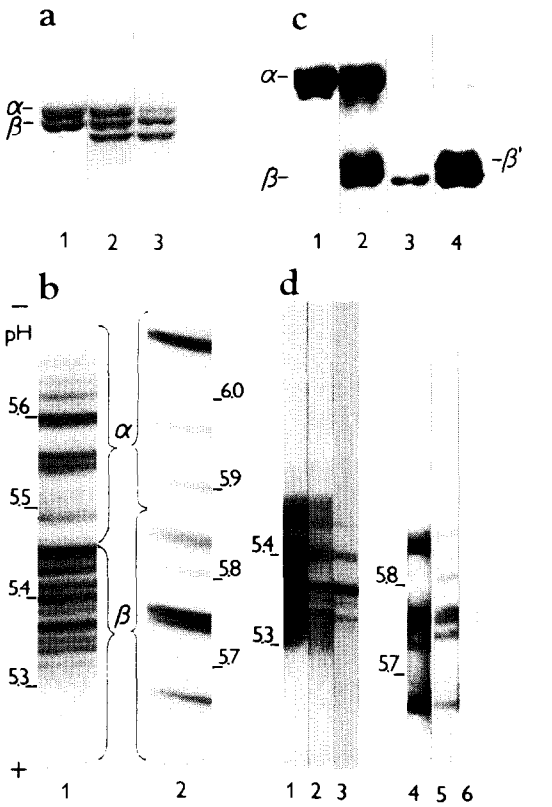

Fig. 2. Reduction in the number of β-tubulin isoforms after subtilisin digestion as detected by HRIF and immunoblotting. (a) 10% SDS-PAGE of untreated tubulin (lane 1) or after subtilisin digestion for 40 (lane 2) and 240 min (lane 3). Bars on the right margin denote positions of tubulin subunits lacking the C-termini. (b) Coomassie blue staining of HRIF of untreated tubulin (lane 1) or after subtilisin digestion for 240 min (lane 2). Positions of α- and β-tubulin isoforms were identified on parallel lanes immunoblotted with mAbs against α-tubulin (TU-01) and β-tubulin (TU-06). (c) Immunoblot of carboxyamidomethylated tubulin with mAbs TU-01 (lane 1), TU-14 (lane 3), TU-06 (lane 4). Lane 2, colloidal silver staining of blotted proteins. 6% SDS-PAGE. β', position of class-III β-tubulins. (d) Immunoblot of tubulin isoforms in untreated tubulin (lanes 1–3) or after subtilisin digestion for 240 min (lanes 4–6) with mAbs TU-06 (lanes 1 and 4), TU-11 (lanes 2 and 5), TU-14 (lanes 3 and 6).

acidic C-termini resulted in the generation of more basic isoforms in the apparent pH range 5.65–6.05. A reduced number of isoforms was also confirmed by immunoblotting with mAbs TU-06 and TU-11, whereas TU-14 gave no staining (Fig. 2d). The epitope for TU-14, located in the isotype-defining region [14], was detected on the cloned fusion proteins of isotype classes I, II and IV, but not on neuron-specific isotype class-III [10]. Non-reactivity of TU-14 with class-III isotype was further confirmed by immunoblotting of carboxyamidomethylated tubulin (Fig. 2c), where the mAb did not react with β' tubulin species corresponding to class-III β-tubulin [21]. HRIF of porcine, rabbit and hen brain tubulins revealed similar overall distribution of β-tubulin isoforms with differences just in relative intensities of corresponding isotubulins. In carp, however, there were only 9 β-tubulin isoforms detected. In con-

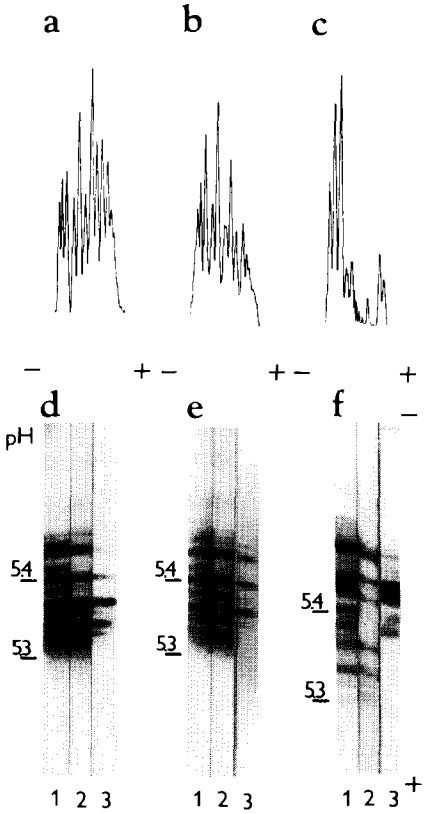

Fig. 3. HRIF pattern and immunoreactivity of β-tubulin isoforms in rabbit (a,d), hen (b,e) and carp (c,f) brain. (a,b,c) Densitometric scans of β-tubulin isoforms. (d,e,f) Immunoblots of β-tubulin isoforms with mAbs TU-06 (lanes 1), TU-11 (lanes 2), TU-14 (lanes 3).

trast to TU-06, mAbs TU-11 and TU-14 reacted in all tested samples only with some isoforms. The non-class-III β-tubulins, identified by TU-14, resolved to 6 charge variants (Fig. 3). To compare the isoforms of β-tubulin from brain tissues with those from neuronal and non-neuronal cell lines, MTPs were prepared from HeLa, Neuro-2a and 3T3 cells. The preparations contained a lower number of β-tubulin isoforms; 7 in Neuro-2a cells, 6 in both HeLa and 3T3 cells. HRIF of porcine brain and HeLa tubulins on adjacent lines (Fig. 4) showed that the doublet of dominant HeLa β-tubulin isoforms was in a position that corresponded to that of β12 and β13 in basic region of porcine brain isotubulins (see Fig. 1 for comparison). In approximately the same positions were located the dominant β-tubulin isoforms of 3T3 and Neuro-2a cells (Fig. 5b,c). Also in the cell lines tested, TU-06 stained all β-tubulin isoforms while mAbs TU-11 and TU-14 reacted only with a subset of isoforms. The mAb TU-14 stained 2–3 β-tubulin isoforms in all cell lines, including the neuronal line Neuro-2a. The mAb GT335 against glutamylated tubulin reacted only with the two acidic isoforms and did not stain the dominant isoforms (Fig. 5d,e,f).

4. DISCUSSION

A lower number of β-tubulin isoforms found in porcine brain tubulin after subtilisin cleavage, in comparison with undigested tubulin, clearly demonstrate that some charge variants are generated in the C-terminal region. Non-reactivity with cleaved tubulin of TU-14, which recognizes the epitope within the region β(431–444) in classes I, II and IV [10], proved that the isotype-defining domain of β-tubulin was completely removed. However, the mAbs TU-06 and TU-11, recognizing epitopes located outside this domain, reacted with 8 and 6 isoforms, respectively. As the mammalian brain β-tubulin appears to consist of 5 isotypes [2], reactivity with a higher number of isoforms could reflect hitherto unknown post-translational modifications outside the isotype-defining domain. Previously we have shown that non-class-III β-tubulin isoforms recognized by TU-14 are post-translationally modified in developing mouse brain [10]. Here we show that such modifications are evolutionary conserved and occur in brain β-tubulins in species ranging from pig to carp. As the identification of tubulin isoforms in brain tissue is complicated by the presence of various cell types, we have analysed MTPs from established cell lines of neuronal and non-neuronal origin. The most dominant isoforms of Neuro-2a, HeLa and 3T3 cells were located in the same positions as those corresponding to the most basic β-tubulin isoforms of porcine brain. Compared to brain, all tested cell lines contained a lower number of β-tubulin isoforms. Their number was, however, greater than actual number of tubulin genes [2,22]. Post-translational modifications of β-tubulins are therefore not limited to neuronal cells. The mAb TU-11, recognizing the epitope within the region β(345–430) in classes I, II, III and IV [10], reacted in all tested samples with a limited number of isoforms. If the corresponding epitope were localized more precisely, it could be a useful

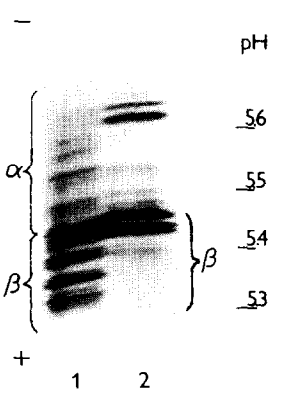

Fig. 4. Comparison of HRIF patterns of porcine brain and HeLa tubulins. Coomassie blue staining of brain tubulin (lane 1) and HeLa tubulin (lane 2). Positions of α- and β-tubulin isoforms were identified on parallel lanes immunoblotted with mAbs against α-tubulin (TU-01) and β-tubulin (TU-06).

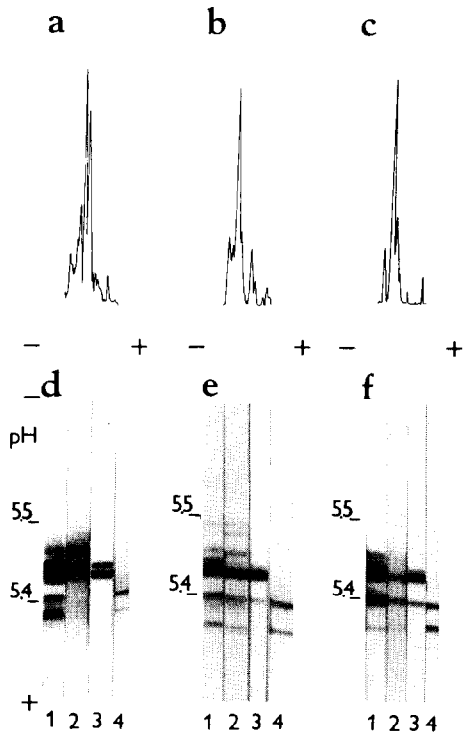

Fig. 5. HRIF pattern and immunoreactivity of β-tubulin isoforms in HeLa (a,d), Neuro-2a (b,e) and 3T3 (c,f) cell lines. (a,b,c) Densitometric scans of β-tubulin isoforms. (d,e,f) Immunoblots of β-tubulin isoforms with mAbs TU-06 (lanes 1), TU-11 (lanes 2), TU-14 (lanes 3), GT335 (lanes 4).

isoform marker outside the isotype-defining domain. Charge variants of non-class-III β-tubulin isoforms discriminated by TU-14 in brains could be the result of recently described post-translational polyglutamylation of Glu^{435} in the most abundant brain class II isotype [5]. Glutamylated β-tubulin was also detected in mouse non-nervous tissues by means of mAb GT335 against glutamylated tubulin (Dr. A. Wolff, personal communication). Glutamylation on the most acidic isoforms in tested cell lines, as detected by GT335, demonstrated that β-tubulin isotypes in homogeneous cell populations of various types were likewise substrates for this modification. The basic, most abundant isoforms were not glutamylated. As the C-terminal domain of β-tubulin is thought to be involved in the interaction of tubulin with microtubule associated proteins [23], post-translational modifications in this region might modulate such interactions in neuronal as well as non-neuronal cells.

Acknowledgements: We are indebted to the Natural Products Branch of the National Cancer Institute for providing the taxol used in this study. We thank Dr. A. Wolff for the kind gift of antibody GT335.

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