Polyglutamylation of Tubulin as a Progressive Regulator of *in Vitro* Interactions between the Microtubule-Associated Protein Tau and Tubulin†

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ABSTRACT: The multiple functions of microtubules are mediated by various structural and motor microtubule-associated proteins (MAPs). To harmonize these functions in different places of a single cell, the key problem is to regulate the interactions of these proteins with microtubules. The chemical diversity of tubulin isoforms, which constitute the microtubule wall, could represent a molecular basis for this control. Using an *in vitro* assay of ligand blotting, we found that the microtubule-associated protein Tau interacts differentially with the diverse posttranslationally-modified isotubulins: its binding is mainly restricted to moderately-modified α- and β-tubulin isoforms. We obtained evidence that the recently-discovered polyglutamylation, which consists of the sequential, posttranslational addition of one to six glutamyl units to both α- and β-tubulin subunits, regulates the binding of Tau as a function of its chain length. The relative affinity of Tau, very low for unmodified tubulin, increases progressively for isotubulins carrying from one to three glutamyl units, reaches an optimal value, and then decreases progressively when the polygutamyl chain lengthens up to six residues. Our results suggest that the unmodified C-terminus of tubulin exerts a constitutive inhibition on Tau binding, probably by locking the MAP-binding site, and that this inhibition could be first released and then restored as the polyglutamyl chain grows. As the posttranslational chain does not appear to interact directly with Tau, it is thought that the growth of this chain from one to six glutamyl units causes a progressive, conformational shift in the structure of the C-terminal domain of tubulin, thus leading to the observed modulation of affinity.

In eucaryotes, microtubules are involved in many diverse cellular functions such as chromosome segregation in mitosis and meiosis, cilia and flagella-based motility, and intracellular traffic of organelles and macromolecules. In neurons, the dense microtubular network supports the outgrowth and maintenance of axonal and dendritic processes as well as the long-distance axonal transport. These functions are mediated by numerous structural and motor microtubule-associated proteins (MAPs)[1] (Tucker, 1990; Vallee & Shpetner, 1990) which interact with the microtubule surface. In a single cell, to coordinate these different functions in time and space, the binding of MAPs onto the different microtubules must be under a strong and sophisticated regulation. One possible, attractive molecular basis for this regulation is the heterogeneous surface of microtubules, a patchwork of a very high number of different α- and β-tubulin isoforms. The tubulin diversity is due not only to the expression of several α- and β-tubulin isogenes (Villasante et al., 1986; Wang et al., 1986; Sullivan, 1988) but especially to various posttranslational modifications (PTMs) affecting both subunits: reversible removal of the C-terminal Tyr^{451} (Raybin & Flavin, 1975; Barra et al., 1988), acetylation of Lys^{40} (L'Hernault & Rosenbaum, 1985; Le Dizet & Piperno, 1987), and excision of Glu^{450} in nontyrosinatable tubulin (Paturle-Lafanechère et al., 1991) for the α-tubulin subunit; phosphorylation of Ser^{444} for the class III β-tubulin (Gard & Kirschner, 1985; Alexander et al., 1991); polyglutamylation of Glu^{445} (Eddé et al., 1990), Glu^{438} (Alexander et al., 1991), and Glu^{435} (Rüdiger et al., 1992; Redeker et al., 1992) for the $m\alpha_1$, class III, and class II β-tubulin isotypes, respectively. It is striking to note that the main amino acid sequence divergence of the diverse tubulin isotypes and almost all of the reversible PTMs both take place within the C-terminal regions of α- and β-tubulin subunits (Sullivan, 1988; Joshi & Cleveland, 1990), surface domains also reported to bind MAPs (Serrano et al., 1984; Littauer et al., 1986; Paschal et al., 1989). These observations strongly suggest that the chemical diversity of the tubulin C-terminal domains, and especially that conferred by the reversible PTMs, could regulate the interactions between microtubules and MAPs.

To work out this hypothesis, it is impossible to test the binding of MAPs onto very homogeneous microtubule populations assembled from a unique set of tubulin isoforms all carrying only one posttranslational moiety, or, in the case of polyglutamylation, the same number of glutamyl residues. To overcome this difficulty, we used a ligand blotting assay to test the binding of MAPs onto tubulin isoforms separated by high-resolution two-dimensional (2-D) PAGE and immobilized onto a nitrocellulose membrane.

In this paper, we show that Tau protein does not bind equally to the diverse tubulin isoforms. Rather, the binding appears to be strongly affected by the level of modification in both tubulin subunits. Among the different PTMs of tubulin, the recently-discovered polyglutamylation appears to modulate the affinity of tubulin for Tau as a function of the length of the polyglutamylated chain (i.e., the number of glutamyl units present in the posttranslationally-added chain) which then

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[1] Abbreviations: MAP, microtubule-associated protein; PTM, posttranslational modification; MES, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

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acts like a rheostat. The affinity between tubulin and Tau progressively increases as the polyglutamyl chain grows, reaches a maximum value for glutamylated tubulin isoforms carrying around three glutamyl units, and then progressively decreases as the chain gets longer.

EXPERIMENTAL PROCEDURES

Purification of Tubulin and MAP Fractions. Tubulin was purified from a 100000*g* supernatant of adult mouse brain after two cycles of assembly–disassembly (Shelanski et al., 1975) followed by phosphocellulose chromatography (Weingarten et al., 1975). Heat-stable MAP was purified from adult mouse brain as described previously (Larcher et al., 1992). Briefly, twice-cycled microtubules were depolymerized at 4 °C in MEM buffer (100 mM MES, pH 6.8, 2 mM EGTA, and 2 mM $MgCl_2$), brought to 500 mM NaCl, and boiled for 5 min. After centrifugation, the heat-stable supernatant proteins comprised essentially Tau and MAP2, as judged by SDS–PAGE. Scanning densitometry of Coomassie-stained gels indicated a proportion of 3 molecules of Tau per 1 molecule of MAP2.

Limited Digestion of Tubulin with Subtilisin. Twenty-five micrograms of purified brain tubulin was submitted to a limited digestion by 5 μg/mL subtilisin (Carlsberg, Sigma) for 45 min at 30 °C in 0.1 M MES, pH 6.4, 0.5 mM $MgCl_2$, and 2 mM EGTA (Serrano et al., 1984; Redecker et al., 1992). Digestion was stopped by addition of 1 mM PMSF. The sample was brought to 9.5 M urea and submitted to 2-D PAGE.

Electrophoresis, Immunoblotting, and Antibodies. Isoelectric focusing and one-dimensional (1-D) and two-dimensional (2-D) SDS–PAGE (Laemmli, 1970; O'Farrell, 1975) were carried out as described previously (Larcher et al., 1992; Wolff et al., 1992). For SDS–PAGE, 24-cm-long slab gels [8% acrylamide–0.1% bis(acrylamide)] were used to increase the separation between the α- and β-tubulin subunits. Transfer of proteins onto nitrocellulose membranes was performed as described (Towbin et al., 1979). Monoclonal antibodies directed against total α-tubulin (DM1A, 10^{-4} dilution) and total β-tubulin (DM1B, 5×10^{-3} dilution) were purchased from Amersham. Tau-1 (Binder et al., 1985), an anti-Tau monoclonal antibody (5×10^{-3} dilution), was from Boehringer-Mannheim. GT335, a monoclonal antibody which reacts specially with polyglutamylated α- and β-tubulin (10^{-4} dilution), was produced in our laboratory (Wolff et al., 1992). AH-1, a polyclonal anti-Tau antibody, was kindly provided by Dr. A. Himmler and Dr. S. R. Williams and was used at 10^{-4} dilution. Peroxidase-conjugated anti-mouse and anti-rabbit IgG sera were from Byosis (France); they were used at 5×10^{-3} dilution and detected using the ECL system (Amersham). Autoradiographs were quantified by densitometric scanning using a Vernon integrating densitometer.

Ligand Blotting Experiments. After electrotransfer, nitrocellulose sheets were stained with Ponceau red to register the locations of bound proteins and then cut into strips (1-D) or rectangles (2-D) which were each placed into incubation plastic grooves. Nitrocellulose pieces were blocked overnight in MES buffer [50 mM MES, pH 6.8, 2 mM $MgCl_2$, 2 mM EGTA, 1 mM dithiothreitol, 0.1% (v/v) Tween 20, and 0.1% (w/v) gelatin], incubated 1 h at room temperature with the overlaying protein fraction (5 μg/mL tubulin or 2.5 μg/mL MAP) diluted in MES buffer, and then washed 5×10 min in MES buffer. Protein interactions were stabilized with 0.5% (v/v) formaldehyde for 30 min in MES buffer followed by incubation in 2% (w/v) glycine (30 min in MES buffer) according to Kremer et al. (1988). Nitrocellulose pieces were then equilibrated in Tris-buffered saline and processed for immunodetection.

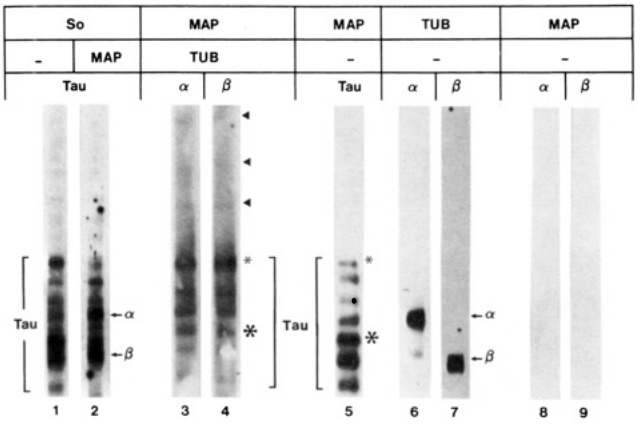

FIGURE 1: Ligand blotting experiments between brain tubulin and heat-stable MAP. Upper row: proteins blotted onto nitrocellulose (So, 100000*g* supernatant of adult mouse brain; MAP, heat-stable brain MAP; TUB, purified brain tubulin). Middle row: overlayed proteins. Lower row: monoclonal antibodies used for immunodetection (Tau, Tau-1; α and β, DM1A and DM1B, respectively). Lanes 1 and 2: So proteins directly immunoprobed with Tau-1 (lane 1) or overlayed first with heat-stable MAP before immunodetection with Tau-1 (lane 2). Lanes 3 and 4: heat-stable MAP overlayed with purified tubulin; tubulin, which had bound to the diverse Tau forms and to other very minor heat-stable MAPs present in the preparation (arrowheads), was detected with either DM1A (lane 3) or DM1B (lane 4). Lanes 5–7, controls: heat-stable MAP immunoprobed with Tau-1 (lane 5) and brain tubulin immunoprobed with DM1A (lane 6) or with DM1B (lane 7). Lanes 8–9: heat–stable MAP immunoprobed with DM1A (lane 8) or DM1B (lane 9), showing the absence of contaminating tubulin in this fraction. Asterisks on lanes 4 and 5 refer to major (large asterisks) and minor (small asterisks) Tau subspecies displaying respectively a low and a high binding capacity for tubulin.

Inhibition of Tubulin–Tau Interactions with Anti-Tubulin Antibodies. Purified tubulin was loaded into a 10-cm-wide well of a SDS–polyacrylamide slab gel, electrophoresed, and blotted onto nitrocellulose. Adjacent 5-mm strips, with an identical amount of immobilized α- and β-tubulin, were cut from the membrane and preincubated without or with increasing concentrations (10^{-4}, 5×10^{-3}, 10^{-3}, 5×10^{-2}, and 10^{-2} dilutions) of either DM1A, DM1B, or GT335 antibodies. After extensive washing, the nitrocellulose strips were overlayed with 2.5 μg/mL heat-stable MAP for 1 h, and tubulin-bound Tau was detected with AH-1, a polyclonal antibody directed against the fourth tubulin-binding repeat of bovine Tau (Himmler, 1989). Anti-Tau antibody was revealed with a secondary anti-rabbit, peroxidase-labeled antibody by the ECL system. The fixation of the preincubated monoclonal antibodies was checked by using a secondary anti-mouse antibody (not shown). It was also checked that the signal obtained with the polyclonal AH-1 was identical to that obtained with the monoclonal Tau-1 (Larcher et al., 1992).

RESULTS

In a first step, the validity of the ligand blotting technique was checked by assaying the qualitative and quantitative binding of Tau on tubulin and, conversely, that of tubulin on Tau (Figure 1). Under the conditions used, the binding was efficient in both directions within 1 h incubation. Figure 1 shows that the monoclonal antibody Tau-1 detected the endogenous Tau proteins present in a high-speed supernatant from brain (Figure 1, lanes 1 and 2) and, by superposition, the overlayed Tau proteins bound onto endogenous α- and β-tubulin (lane 2). While the binding of Tau to α-tubulin is

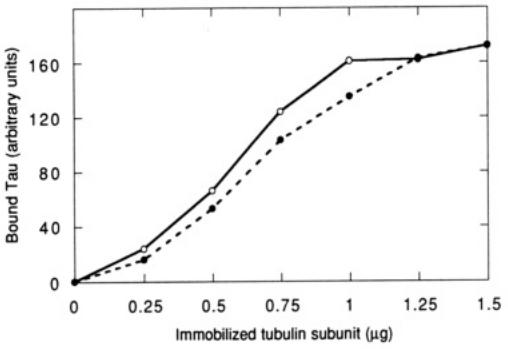

FIGURE 2: Increasing amounts (0.5, 1, 1.5, 2, 2.5, and 3 μg) of tubulin, corresponding to 0.25, 0.5, 0.75, 1, 1.25, and 1.5 μg of each α- or β-tubulin subunit, were separated by 1-D SDS–PAGE, blotted onto nitrocellulose, and overlayed with MAP (2.5 μg/mL). Tau bound to tubulin was detected with Tau-1, quantified by scanning densitometry, and plotted against the amount of immobilized α-tubulin (open circles) or β-tubulin (black circles).

obvious, the signal obtained over the β-tubulin band is partly hidden by major endogenous Tau bands of the same apparent molecular weight. Although the proteins were denatured and adsorbed onto the membrane, Tau does bind to both tubulin subunits under these *in vitro* conditions (see also Figures 2–5). The absence of other additional bands in lane 2 *vs* lane 1 (Figure 1) indicates that the binding of Tau was specific to tubulin. Likewise, when tubulin was overlayed onto blotted heat-stable MAPs, comprising mostly Tau and MAP2 (Figure 1, lanes 3 and 4), both anti-α- and anti-β-tubulin antibodies detected the corresponding tubulin subunits bound to the multiple Tau bands and to other minor heat-stable MAPs of higher molecular weight (arrowheads).

The linearity of the response (amount of bound Tau as a function of the amount of blotted tubulin) was checked by overlaying a MAP solution (2.5 μg/mL) onto increasing amounts of α- and β-tubulin separated by 1-D SDS–PAGE and transferred onto nitrocellulose (Figure 2). Under these conditions, it appears that Tau binding is proportional to the amount of tubulin immobilized onto the membrane, the linearity being maintained up to ~1 μg of each blotted tubulin subunit. For higher values (>1–1.25 μg), and given the surface of blotted tubulin bands (~3.5 × 1.5 mm), the amount of tubulin available for interaction does not increase further, and the reaction reaches a plateau. All of the subsequent experiments were then carried out within this linear range of response, corresponding to tubulin "concentrations" below 0.25 μg/mm² (expressed as amount of immobilized tubulin per surface unit of nitrocellulose).

Tau Binds Preferentially to Moderately-Modified Tubulin Isoforms. In a second step, brain tubulin was separated by high-resolution 2-D PAGE and immunoblotted with general anti-α (DM1A) and anti-β (DM1B) tubulin antibodies (Figure 3a). The different α- and β-isotubulins were numbered according to their p*I*, from OH^- to H^+. It has been previously reported (Denoulet et al., 1986, 1988) that all of the major α-tubulin primary translation products (mα$_1$, mα$_2$, and mα$_4$) migrate in 2-D gels at the basic side of the spot, at the α$_1$ position. Similarly, the brain-specific class III β-tubulin isotype (mβ$_6$, denoted here β′) migrates at the basic side of a satellite spot (β′$_1$ position) slightly above the main β-tubulin spot. The major class II β-tubulin (mβ$_2$) migrates also at the basic side of the main β-spot, at the β$_3$ position. Additional minor β-tubulin isotypes expressed in brain (mβ$_4$-class IVa and trace amounts of mβ$_5$-class I) migrate also close to the basic side of the β-spot at the β$_4$ position (Denoulet et al., 1986). The diverse posttranslational derivatives of all of these

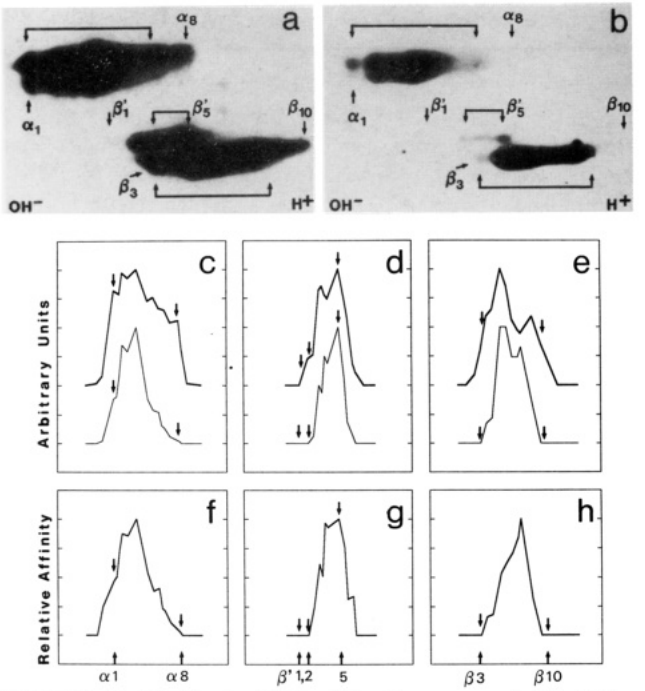

FIGURE 3: Purified brain tubulin (20 μg) was separated by high-resolution 2-D PAGE and blotted onto nitrocellulose. (a) Immunoblotting with DM1A and DM1B. The different isotubulins are numbered following their positions of migration in 2-D gels, from OH^- to H^+ (α$_{1-8}$, β′$_{1-5}$, and β$_{3-10}$). α$_1$, β′$_1$, and β$_3$ indicate the positions of migration of the major translation products of mα$_{1-2}$ and mβ$_6$ (class III), and mβ$_2$ {class II) tubulin, respectively. The minor β$_4$ (class IVa) and β$_5$ (class I) β-tubulin isotopes migrate also close to the basic side of the spot, at position β$_4$ (Denoulet et al., 1986). The elongated shape of the spots is due to the progressive acidification of the diverse isotypes following posttranslational modifications (Denoulet et al., 1988). (b) Tau-1 immunodetection of tubulin-bound Tau after overlay with heat-stable MAP. The precise superposition of the signals on both blots was carried out by aligning the pencil marks done after Ponceau red staining of tubulin after electrotransfer and by reprobing the blot after the overlay reaction with general anti-tubulin antibodies. Brackets, which indicate the extent of Tau binding onto α-, β′-, and β-tubulin species, are placed in the same coordinates in both blots. The signals obtained in both blots were specific: over- or underexposure of the blots did not change the signals qualitatively. (c–e) Signals from immunoblot (upper curves, thick lines) and overlay (lower curves, thin lines) were scanned in the p*I* dimension and aligned for α- (c), β′- (d), and β-tubulin (e). (f–h) The ratios of overlay *vs* immunoblot scans represent the relative affinity of Tau for the diverse α- (f), β′- (g), or β-tubulin (h) isoforms. If Tau bound equally to all of the diverse tubulin isoforms, the resulting curve should be a horizontal line denoting a uniform affinity.

isotypes spread in 2-D gels toward more acidic p*I*'s (up to positions α$_8$, β′$_5$, and β$_{10}$, respectively), depending on their level of modification (or their combination of modifications): acetylation of α-tubulin (Eddé et al., 1987, 1991), phosphorylation of β′-tubulin (Eddé et al., 1987, 1989), and polyglutamylation of α-, β′-, and β-tubulin (Eddé et al., 1990; Wolff et al., 1992). The reversible removal of the C-terminal tyrosine does not affect the p*I* of α-tubulin but can occur in combination with the other modifications (Eddé et al., 1992). In a parallel blot (Figure 3b), tubulin was overlayed with heat-stable MAP, and tubulin-bound Tau was detected with Tau-1. Figure 3b–e shows that Tau binds very weakly (for α), if any (for β′ and β), to unmodified primary translation isotypes. Significant binding of Tau starts with the first modified α- and β-tubulin isoforms, increases with the degree of modification of tubulin up to the middle of the spots, and then progressively decreases as the degree of modification continues to increase. The most acidic, fully-modified α- and β-tubulin isoforms do not interact with Tau anymore. The

plots of the ratios of overlay *vs* immunoblot scans (Figure 3f–h) show the changes in the relative affinity of Tau for the diverse isotubulins as a function of their acidification.

The Polyglutamyl Chain of Tubulin Is Involved in Tau Binding via Progressive Conformational Changes. As Tau only binds significantly to modified tubulin isoforms, the PTMs of tubulin thus appear to be implicated in the control of the binding between tubulin and MAPs. Among the diverse PTMs, which one(s) is (are) concerned? For several reasons, polyglutamylation represents an excellent candidate for regulating the tubulin–Tau interactions following the pattern observed in Figure 3: (i) polyglutamylation is the main PTM of neuronal tubulin (Eddé et al., 1990, 1991; Audebert et al., 1993); (ii) it is the only PTM present in the three α-, β'-, and β-tubulin subfamilies (Eddé et al., 1990; Alexander et al., 1991; Rüdiger et al., 1992); and (iii), by opposition to the other PTMs which represent only binary events, *i.e.*, presence or absence of the posttranslational moiety, polyglutamylation displays an oligomeric structure which could fit well with the potentiometric effect observed in the binding of Tau as a function of tubulin acidification, that is, the number of glutamyl units added in the posttranslational chain. To verify that polyglutamylation of tubulin is an actual regulatory element in the interaction with Tau, we used the monoclonal antibody GT335, which specifically recognizes glutamylated α-, β'-, and β-tubulin (Wolff et al., 1992), to mask the polyglutamyl chain on both subunits. Brain tubulin, separated by 1-D SDS–PAGE and blotted onto nitrocellulose, was first incubated with increasing concentrations of either DM1A, DM1B, or GT335 and then overlayed with MAP (Figure 4). In these conditions, the binding of Tau onto α- and β-tubulin was not affected at all by the presence of DM1A or DM1B (α-tubulin with DM1B and β-tubulin with DM1A serving as internal control) but was drastically inhibited on both tubulin subunits after preincubation with GT335. This inhibition can be explained by the fact that GT335, bound to the polyglutamyl chain of tubulin, either prevents Tau from binding directly to this chain or prevents the polyglutamyl chain from exerting in the C-terminal region of tubulin a progressive, chain length-dependent structural change which could modulate the affinity for Tau. Directly or not, however, the polyglutamyl chain clearly appears to be involved in the binding of Tau.

Finally, brain tubulin was submitted to a mild digestion with subtilisin which has been reported to cut off the C-terminal regions of α- and β-tubulin (Serrano et al., 1984; Redecker et al., 1992) as indicated in Figure 5a. Upon subtilisin treatment, most of the genetic heterogeneity as well as all of the known PTMs, except α-tubulin acetylation, are removed from α- and β-tubulin. When analyzed by 2-D PAGE (Figure 5b), subtilisin-cleaved α- and β-tubulin (termed α_s and β_s, respectively) migrated with a slightly lower apparent molecular weight and a more basic p*I* than undigested tubulin due to the loss of very acidic sequences present at the end of both subunits. Figure 5b shows that α_s- and β_s-tubulin were still detected by DM1A and DM1B, but no more by GT335 (not shown; Wolff et al., 1994), confirming that the C-terminal part was well deleted and that the cleavage occurred between the epitopes of DM1A (or DM1B) and GT335 (see Figure 5a). On a parallel blot (Figure 5c), overlayed Tau bound, as expected, to undigested moderately-modified α- and β-tubulin and not with the unmodified isotypes or with the fully-modified isoforms (see Figure 3). Surprisingly, Tau also bound to the main species of α_s- and β_s-tubulin. This result suggests that, in intact tubulin, the unmodified C-terminal domains must behave as constitutive inhibitors of Tau binding. This result

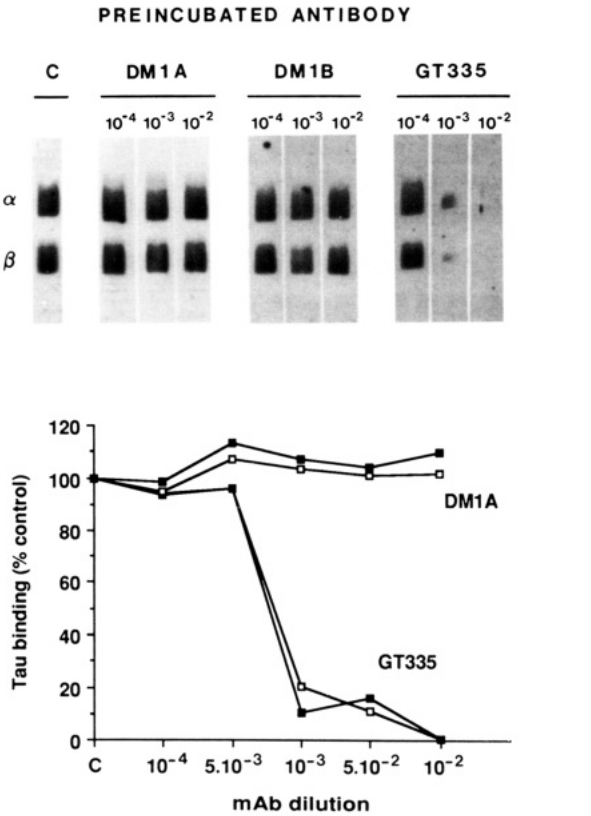

FIGURE 4: Inhibition of tubulin–Tau interaction by GT335. Upper panel: Effects of preincubation of increasing concentrations of DM1A, DM1B, or GT335 on the binding of Tau onto α- or β-tubulin separated by 1-D SDS–PAGE and transferred onto nitrocellulose. Antibody dilutions are indicated above each lane. C: binding control in the absence of preincubated antibody. Lower panel: Tau binding onto either α-tubulin (open squares) or β-tubulin (black squares) was plotted against increasing concentrations of DM1A or GT335 (expressed as antibody dilutions of stock solutions). The plot with DM1B (not shown) was similar to that obtained with DM1A. 100% refers to the binding control without preincubated antibody (see lane C, upper panel).

also indicates that Tau can bind tubulin upstream from the subtilisin cleavage sites, Asp^{438} for α-tubulin and Gln^{433} for β-tubulin (Redecker et al., 1992; and see Discussion), leading to the suggestion that the modulating effect exerted by the polyglutamyl chain on Tau binding is then indirect via progressive structural changes.

Unfortunately, no high-resolution 3-D structure of the tubulin molecule on an atomic scale is available [see Wade and Chrétien (1993)]. However, in light of our results, and based on a predicted structure of the carboxy terminus of α-tubulin (Ponstingl et al., 1979), the regulation of Tau interaction with tubulin and microtubules could proceed as follows (Figure 6): the primary translation products of tubulin do not bind Tau because of the presence of their unmodified C-termini which probably lock the two 383–403 (cationic) and 413–435 (anionic) α-helices together and thus prevent Tau from reaching its binding site. As glutamyl units are sequentially added onto tubulin through the polyglutamylation reaction (black arrow in Figure 6), this inhibition is progressively released, allowing the affinity of tubulin for Tau to increase gradually. The optimal affinity is reached when the polyglutamyl chain is around three units long [a mean value which can be estimated from the gels in Figure 3; see also Wolff et al. (1992) and Audebert et al. (1993)]. As the polyglutamyl chain lengthens further, the binding inhibition is progressively restored, and tubulin carrying chains of six glutamyl units cannot bind Tau anymore. The growing

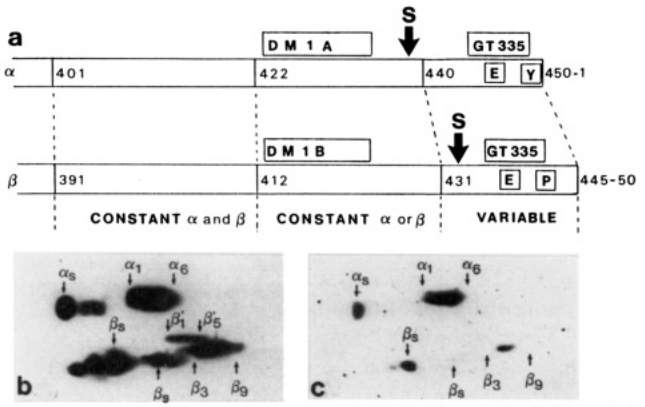

FIGURE 5: Tau interaction with subtilisin-digested tubulin. (a) Structure of the C-termini of α- and β-tubulin showing the major sites of subtilisin cleavage (S): Asp^{438} for mα_1-tubulin and Gln^{433} for class II β-tubulin [taken from Redecker et al. (1992)]. Positions of amino acid residues are indicated by numbers. The left part corresponds to highly conserved sequences between both α- and β-tubulin, the central part of conserved sequences in either α- or β-tubulin, and the right part to highly variable sequences. Boxed letters: posttranslational modifications: Y, tyrosination cycle (no. 451); P, phosphorylation at Ser^{444} for the class III β-tubulin; E, polyglutamylation at Glu^{445} for m$\alpha_{1,2}$-tubulin and at Glu^{435} and Glu^{438} for class II and class III β-tubulin, respectively. Epitope locations for DM1A, DM1B (Breitling & Little, 1986; Andreu & de Pereda, 1993), and GT335 (Wolff et al., 1992) are also noted in frames above the sequences. (b) Subtilisin-digested tubulin was separated by 2-D PAGE, blotted, and immunoprobed with DM1A and then with DM1B. The heterogeneity of the digestion products suggests that minor subtilisin sites were also cleaved (Redecker et al., 1992). (c) A parallel blot was overlayed with heat-stable MAP, and tubulin-bound Tau was detected using Tau-1.

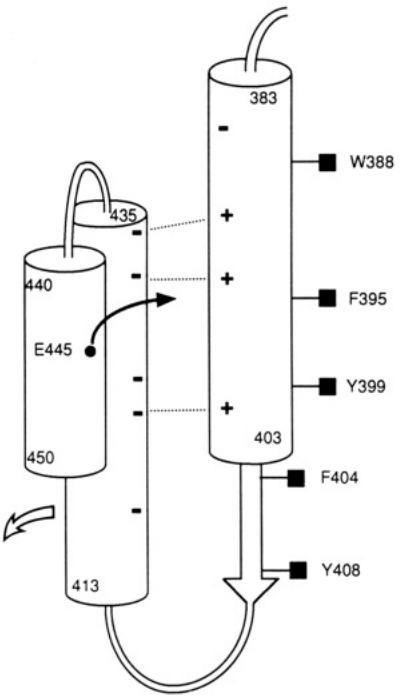

FIGURE 6: Schematic representation of the putative structure of the C-terminal domain of mα_1-tubulin [after Ponstingl et al. (1979)]. α-Helices are represented by cylinders, the β-strand by a large arrow, and turns by loops. Amino numbers are indicated. At the right of the amphipathic 383–403 α-helix, black squares symbolized aromatic residues aligned along one side of the structure. It is likely that these residues hold firmly the subterminal structure with upstream regions of the α-tubulin molecule. Symbols – and + represent positive and negative charges distributed onto facing surfaces of the two long helices and correspond, from the amino to the carboxylic end, to Glu^{386}, Arg^{390}, Lys^{394}, Lys^{401}, Glu^{420}, Glu^{423}, Asp^{424}, Asp^{431}, and Glu^{434}. Black spot: Glu^{445} polyglutamylation site. Black arrow: growth of the polyglutamyl chain. Dashed lines: ionic bonds holding the two helices together. Open arrow: putative shift of the 413–435 helix displaced by the growing polyglutamyl chain.

polyglutamyl chain could, by its serial negatively-charged carboxylic groups, progressively displace the ionic bonds required to keep the two adjacent subterminal α-helices into a closed conformation (dashed lines in Figure 6). This progressive displacement would gradually open the Tau-binding surface (open arrow) and modulate at first upwards and then dowards the affinity for Tau.

DISCUSSION

To analyze the influence of tubulin heterogeneity, and primarily that of PTMs, on the interaction with MAPs, we adapted a technique of ligand blotting to take advantage of the separation of the different tubulin isoforms obtained after resolutive 2-D PAGE. In our hands, a 1-h incubation time was found suitable and gave optimal binding and quantitative signals. The reciprocal Tau–tubulin interactions in overlay experiments were found specific, as judged by the absence of non-sense binding of either protein over whole supernatant proteins from mouse brain. Moreover, tubulin did not bind proportionally to the different Tau isoforms expressed in brain: major Tau species of low molecular weight (large asterisks on lanes 3–4 and 5 in Figure 1) were bound weakly while minor Tau species of higher molecular weight (small asterisks) were bound strongly by tubulin, indicating a nonuniform affinity between the diverse Tau isoforms and tubulin, taken as a whole, as already reported (Francon et al., 1982; Lee et al., 1989; Goedert et al., 1990).

Likewise, it appears from the blot overlay experiments that Tau binds also differentially to the diverse tubulin isoforms carrying different levels of posttranslational modification. The identification of the polyglutamyl chain of tubulin as the modulating element comes from several observations. First, the unique oligomeric structure of polyglutamylation fits well with the potentiometrical effect observed in the binding of Tau. Second, polyglutamylation is the only known PTM occurring on α-, β'-(class III), and β-tubulin, which all display the same profile of Tau binding as a function of acidification. Finally, the monoclonal antibody GT335, which binds to and hence masks the glutamylated motif present in α-, β'-, and β-tubulin (Wolff et al., 1992), prevents Tau from interacting with α-, β'-, and β-tubulin isoforms. It is not known whether this inhibition is due directly to the masking of the chain or indirectly to a steric effect of the bound antibody. In the same conditions, however, the monoclonal antibodies DM1A and DM1B show no inhibitory effect, although they bind the C-terminal domain of tubulin only few amino acids upstream from the GT335 binding site (see Figure 5). In the case of DM1A, at least, the antibody binds on the external side of the 413–435 α-helix (see Figures 5 and 6) and does not interfere with the negatively-charged amino acids located on the internal surface of this helix nor with the polyglutamyl chain, thus allowing the posttranslational chain to exert its effect.

Regarding the β-tubulin subunit, many important charged amino acid residues are substituted as compared with the α-tubulin sequence. Most of the amino acids thought to be engaged in ionic bonds in the α-tubulin molecule, *i.e.*, Lys^{394}, Glu^{423}, Asp^{424}, and Glu^{434} (see Figure 6), are replaced in the β-tubulin sequence at homologous positions, by Gln^{384}, Ser^{413}, Asn^{414}, and Gln^{424}, respectively. The decrease in charged residues in β-tubulin could lead to a more relaxed structure of the C-terminal domain of this subunit, as reported by Reed et al. (1992), which would then open more readily and thus bind MAPs more easily. It appears, nonetheless, that the

overall profile of Tau binding onto polyglutamylated tubulin is the same for both subunits.

Interaction of Tau with Class III β-Tubulin. The increase of affinity associated with the growth of the polyglutamyl chain from one to three glutamyl units is particularly obvious for the β′-tubulin. In our 2-D gel system, β′-tubulin is resolved into five isoforms: β'_1 is the primary translation isotype (Denoulet et al., 1986; Burgoyne et al., 1988), β'_2 is its phosphorylated derivative (Eddé et al., 1987), and β'_{3-5} correspond to the mono-, di-, and triglutamylated derivatives, respectively, of β'_2 (Audebert et al., 1993). As the β'_2 species is never stained by the GT335 antibody (Audebert et al., 1993), we conclude that the isotype cannot be glutamylated directly unless it has been previously phosphorylated. This agrees with the observation of Alexander et al. (1991) that the monoglutamylated form β'_3 was always found phosphorylated. It can be seen from Figure 3 that Tau binds neither to β'_1 nor to β'_2 and that the binding starts and progressively increases with the glutamylated species, from β'_3 to β'_5. These observations suggest that phosphorylation of Ser^{444} in β′-tubulin has no effect on the interaction with Tau. Rather, phosphorylation could play its role in allowing the β′-tubulin to be glutamylated, at the neighboring position Glu^{438}, possibly via the neutralization of the positive charges of Arg^{446} and Lys^{450} present in this particular mouse isotype (Burgoyne et al., 1988). Indeed, the glutamylation sites of α- and β-tubulin described so far (Eddé et al., 1990; Alexander et al., 1991; Rüdiger et al., 1992), although not identical, share an acidic environment. The positive carboxy-terminal charges carried specifically by the class III β′-tubulin could prevent the glutamylation enzyme from binding efficiently to this divergent β-tubulin substrate. Moreover, as the class III β′-isoforms carrying several glutamyl units are not all phosphorylated (Alexander et al., 1991), the phosphorylation event could only be required for priming the reaction of polyglutamylation.

Role of the Other PTMs of Tubulin. Are the other PTMs of tubulin engaged in distinct or similar functions? The C-terminal detyrosination cycle (Barra et al., 1988) and the related excision of the subterminal Glu^{450} residue (Paturle-Lafanechère et al., 1991) occur only five amino acids downstream from the glutamylation site in α-tubulin. This close vicinity could suggest that all of these PTMs are involved in the same function. The detyrosination/retyrosination mechanism could interfere with or adjust the effects of the polyglutamyl chain, the excision of Glu^{450} blocking this adjustment by preventing any further readdition of tyrosine. In this case, it is notable that the β-tubulin subunit has no PTM counterpart to the C-terminal detyrosination of α-tubulin and could not regulate the mode of action of its polyglutamyl chain. Within a tubulin heterodimer, the two tubulin subunits could thus behave differently to bind Tau, maybe via the diverse repeated motifs of the Tau tubulin-binding domain which are thought to be 4 nm apart, that is, the distance separating two adjacent tubulin subunits along a microtubule protofilament (Lewis et al., 1988).

Polyglutamylation and Regulation of Microtubule Dynamics. It has been recently reported (Audebert et al., 1993) that polyglutamylation is a reversible PTM and that deglutamylation of α-tubulin in neurons operates following two distinct rates depending on the length of the polyglutamyl chain. The distal glutamyl units (fourth to sixth) are removed rapidly whereas the proximal ones (first to third) appear to be much more resistant to deglutamylation. It is striking to note that the limit between these two rates of deglutamylation is exactly the same value (around 3 glutamyl units) as that described here which confers to tubulin an optimal affinity of Tau. Thus, following microtubule disassembly and release of highly-glutamylated free tubulin, the slow rate component of deglutamylation could prevent the neuronal cell from complete deglutamylation of its soluble tubulin, with a very weak affinity for Tau, but, rather, could maintain a substantial level of triglutamylated tubulin at an optimal capacity to repolymerize rapidly with MAPs. This correlation might suggest that the regulation exerted by the polyglutamyl chain revealed here *in vitro* could also proceed likewise *in vivo*. In neurons, at least, where the level of tubulin is particularly high and the control of mirotubule polymerization is particularly critical, microtubule dynamics could be adjusted by the length of the polyglutamyl chains of polymerized tubulin molecules. A continuous glutamylation of tubulin would lead to metastable microtubules, first stabilized with short polyglutamyl chains and then disassembling rapidly as the chains lenghten, pass beyond the optimal limit, and release bound Tau. Another subset of microtubules could prevent their tubulins from an overglutamylation and thus would maintain as stable, MAP-bound polymers. Indeed, during the microtubule assembly procedure, nondepolymerizing microtubules left in the pellet fraction after cold-induced disassembly of one-cycled microtubules do not contain α_1- and very acidic α-tubulin isoforms and are enriched in Tau (our unpublished results). This reinforces the fact that the influence of the polyglutamyl chain on Tau binding observed *in vitro* on blots could be also effective *in vivo* with microtubules.

Does the Polyglutamyl Chain Work with Other MAPs? The question as to whether the polyglutamyl chain of tubulin acts as a general regulation element for all of the other MAPs is of importance. Parallel experiments indicate that the polyglutamyl chain can also regulate the interaction between tubulin and MAP2 in a similar manner as for Tau (data not shown). This result is not very surprising since, as Tau and MAP2 share very similar repeated tubulin-binding motifs (Lewis et al., 1988; Lee et al., 1988, 1989), as also does MAP4 (Chapin & Bulinski, 1991), one expects that their interaction and the regulation of this interaction with tubulin would proceed similarly. The situation could be different concerning other MAPs such as MAP1B, which exhibits a quite different tubulin-binding motif (Noble et al., 1989), or the mechano-chemical MAPs. However, it has been reported that MAP1B could bind to the carboxy-terminal region of β-tubulin through the interaction of α-helices carrying opposite charges (Avila, 1991). Furthermore, it has been shown that MAP1C, the cytoplasmic dynein heavy chain (Paschal et al., 1989), and MAP1 (Kuznetsov et al., 1984) can compete with MAP2 for binding to tubulin, suggesting that they intact with the same (or very close) tubulin domain and, thus, that they could also be regulated via polyglutamylation. Goldsmith et al. (1991) have reported that a motor binding site (for kinesin and flagellar and cytoplasmic dyneins) was located within the C-terminal domain (between aa 400 and 436) of β-tubulin and that there was a functionally-distinct but similarly-located domain on α-tubulin. As pointed out by these authors, the sequence and chemical diversity of the extreme C-terminus of tubulin seem not to be involved *per se* in the binding site. Our experiments with subtilisin lead to the same conclusion. In these experiments, subtilisin removed only a dozen amino acids from the C-termini of α- and β-tubulin, leaving sequences at the extremities of major α_s- and β_s-tubulin large fragments available for Tau binding, that is, sequences corresponding to the synthetic peptides (α430–441 and β422–434) previously reported to be involved in the Tau- and MAP2-binding site

(Maccioni et al., 1988). Although the extreme C-terminal sequence of tubulin is not directly involved in Tau binding (Vera et al., 1989), the results reported here show, however, that its chemical diversity could be essential in the control of MAP binding, without which, in turn, the microtubule functions should not be regulated. Anyhow, the tubulin–MAP interactions are regulated on both sides by both proteins. It has been widely reported that the level of phosphorylation of MAP2 or Tau also controls their interaction with tubulin (Lindwall & Cole, 1984; Burns et al., 1984; Brugg & Matus, 1991; Biernat et al., 1993). This clearly shows that the regulation of these protein–protein interactions does not proceed on a simple mode and is thoroughly controlled. Some subtle differences in binding features and/or in the regulation process between the various MAPs could help the microtubule to sort the different MAPs to be bound, leading to a given cytoskeletal structure or cellular function.

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