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Differential Binding Regulation of Microtubule-associated Proteins MAP1A, MAP1B, and MAP2 by Tubulin Polyglutamylation*

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The major neuronal post-translational modification of tubulin, polyglutamylation, can act as a molecular potentiometer to modulate microtubule-associated proteins (MAPs) binding as a function of the polyglutamyl chain length. The relative affinity of Tau, MAP2, and kinesin has been shown to be optimal for tubulin modified by ~3 glutamyl units. Using blot overlay assays, we have tested the ability of polyglutamylation to modulate the interaction of two other structural MAPs, MAP1A and MAP1B, with tubulin. MAP1A and MAP2 display distinct behavior in terms of tubulin binding; they do not compete with each other, even when the polyglutamyl chains of tubulin are removed, indicating that they have distinct binding sites on tubulin. Binding of MAP1A and MAP1B to tubulin is also controlled by polyglutamylation and, although the modulation of MAP1B binding resembles that of MAP2, we found that polyglutamylation can exert a different mode of regulation toward MAP1A. Interestingly, although the affinity of the other MAPs tested so far decreases sharply for tubulins carrying long polyglutamyl chains, the affinity of MAP1A for these tubulins is maintained at a significant level. This differential regulation exerted by polyglutamylation toward different MAPs might facilitate their selective recruitment into distinct microtubule populations, hence modulating their functional properties.

changes in dynamic, structural, and functional properties of spindle MTs, as compared with interphasic MTs. Additional differences can also be observed in the binding of structural or motor MAPs to the various MT subpopulations—astral, polar, and kinetochore MTs—of the mitotic spindle (for review, see Refs. 3 and 4).

Interactions between MTs and structural as well as motor MAPs are thus of fundamental interest and must be tightly regulated by cells, both locally and temporally, to ensure proper MT functions. MAPs generally regulate their binding affinity for MTs by phosphorylation (see for instance Refs. 5–8). However, observations that one motor binds to only one MT in *Chlamydomonas* axoneme (9) or to only one MT subset in lobster axon (10) or in the mitotic spindle (4) clearly imply that MTs must also control their interactions with MAPs and somehow deliver information to permit or restrict MAPs from binding. The molecular basis for such control might be found in the tubulin polymorphism. Indeed, tubulin diversity can generate MT diversity by conferring to MTs heterogeneous interacting surfaces instead of a monotonous succession of identical subunits all along the polymers. In this context, post-translational modifications of tubulin represent interesting potential means to play such a role: They can be easily added to or removed from the MT surface through the intervention of specific modifying enzymes that exhibit net substrate preferences for either free or polymerized tubulin (for review see Refs. 1, 2). Dealing with the genetic diversity of the diverse tubulin isotypes would require active, time-consuming transcription and translation and would not be as efficient as the rapid post-translational events, in particular in the axonal compartment, where no protein synthesis occurs, or in mitotic cells where transcription and translation are turned off.

Among the multiple post-translational modifications of tubulin, polyglutamylation was the first oligomeric modification discovered (11). Functionally, and in a close parallel with its oligomeric structure, polyglutamylation was further shown to behave as a molecular potentiometer that modulates the binding of MAPs as a function of the polyglutamyl chain length (12, 13). For instance, Tau, MAP2, and kinesin motors have been shown to undergo the same mode of binding regulation by polyglutamylation: The relative affinity of these proteins first increases progressively for tubulin modified by 1 to 3 glutamyl units then progressively decreases when the chain lengthens further, up to 6 units. This effect is likely to be achieved by conformational changes of the C-terminal domain of tubulin, driven by the growth of the polyglutamyl chain (12, 13).

Thus, polyglutamylation could control the targeted binding of a particular MAP to MTs at a given level of glutamylation, without altering the binding of other MAPs. However, if polyglutamylation acts as a general regulator of MAP binding, it is

Microtubules (MTs)¹ are dynamic polymers, which are essential for a large variety of cellular functions such as cell morphology and polarity, cell motility, intracellular trafficking, and cell division. They are made up of α - and β -tubulin heterodimers, the two related subunits displaying a large isoform polymorphism due to the expression of multiple genes whose products are substrates for several post-translational modifications (for review, see Refs. 1 and 2). MTs are polymerized under the control of MT-associated proteins (MAPs), which also shape the MT networks and confer on them distinct functional properties. For instance, changes in MAP expression and/or activity when cells enter mitosis is accompanied by drastic

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¹ The abbreviations used are: MT(s), microtubule(s); PAGE, polyacrylamide gel electrophoresis; MAP(s), microtubule-associated protein(s); MES, 2-(*N*-morpholino)ethanesulfonic acid; OV buffer, overlay buffer; PIPES, 1,4-piperazinediethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

difficult to imagine the manner in which it could distinguish between the different MAPs. Consequently, we have sought to determine the binding behavior of other MAPs, namely MAP1A and MAP1B, toward glutamylated tubulin and compared it to that of another high molecular weight MAP, MAP2. We report that polyglutamylolation regulates the binding of MAP1A and MAP1B in a chain length-dependent manner, MAP1B behaving like MAP2. On the other hand, we found that the binding of MAP1A was differentially regulated by polyglutamylolation. In contrast to the other MAPs tested so far, which all display an optimal affinity for tubulins modified by around 3 glutamyl units, MAP1A has the selective property to maintain a high affinity for highly glutamylated α - and β -tubulins. A model for a transition between MAPs along axonal processes is presented.

EXPERIMENTAL PROCEDURES

Antibodies—The general anti- α -tubulin and anti- β -tubulin monoclonal antibodies DM1A (working dilution, 1:1000) and DM1B (1:500) were purchased from Amersham Pharmacia Biotech, and monoclonal anti-MAP1B (anti-MAP5, 1:3000) and monoclonal anti-MAP2 (1:2000) were purchased from Sigma Chemical Co. The anti-MAP1A (1A-1, 14) monoclonal antibody (1:20,000) was a generous gift of Dr. R. B. Vallee (University of Massachusetts Medical School, Worcester, MA). Peroxidase-conjugated anti-mouse IgG serum (1:10,000) was from Byosys (France).

Purification of Microtubule Proteins—Tubulin was prepared from a 150,000 \times *g* supernatant of adult mouse brain by one cycle of assembly-disassembly (15) in MEM buffer (50 mM MES, pH 6.7, 2 mM EGTA, 1 mM MgCl₂) containing a mixture of protease inhibitors (10 μ g.ml⁻¹ aprotinin, 10 μ g.ml⁻¹ leupeptin, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride). Tubulin was further purified by phosphocellulose cation-exchange chromatography (P11, Whatman) in PEM buffer (75 mM PIPES, pH 6.8, 2 mM EGTA, 1 mM MgCl₂). Tubulin aliquots were stored in PEM buffer containing 1 mM Mg-GTP in liquid nitrogen.

MAP1A was purified from twice-cycled MTs prepared from whole bovine brain according to Pedrotti and Islam (16). The protocol is based on a differential binding of MAPs to MTs depending on the sulfonate buffer used (17). Briefly, twice-cycled MT proteins were assembled in MEM buffer in the presence of 20 μ M taxol. After centrifugation, the pellet was resuspended in warm PEM buffer containing 20 μ M taxol and incubated 20 min at 37 °C prior to centrifugation. Most of MAP1A was released into the supernatant, with some other components, and the purification was achieved by a Mono-Q anion-exchange chromatography.

MAP1B was prepared from whole calf brain by two ion-exchange chromatographies (18). After pelleting assembled MTs, the supernatant was submitted to a Mono-Q anion-exchange chromatography in MEM buffer containing 0.2 M NaCl. Fractions eluted at 0.4 M NaCl were pooled, dialyzed against MEM buffer, and loaded on a Mono-S anion-exchange chromatographic column. MAP1B, eluted at 0.33 M NaCl, was finally dialyzed against MEM buffer, rapidly frozen in liquid nitrogen, and stored at -80 °C.

MAP2 was purified by the heat treatment method according to Herzog and Weber (19). Briefly, MAP2 was prepared from two-cycled calf brain MTs, which were depolymerized at 4 °C, cleared by centrifugation, brought to 0.75 M NaCl and boiled for 5 min. The supernatant was collected and submitted to a 50% (w/v) ammonium sulfate precipitation. MAP2 and Tau were then separated by phosphocellulose cation-exchange chromatography followed by a Sepharose 4B exclusion chromatography.

Protein concentrations were determined using the bicinchoninic acid method (Micro BCA protein assay reagent kit, Pierce), with bovine serum albumin being used as standard.

Limited Proteolysis of Tubulin by Subtilisin—Phosphocellulose-purified tubulin from mouse brain was digested by subtilisin (Sigma) in a molar ratio of 240/1 at room temperature for increasing times (0–150 min) in 10 mM MES, pH 6.7, 0.1 mM MgCl₂, 0.1 mM EGTA, 2 mM GTP, 10 μ M EDTA and 50 μ M dithiothreitol were added to the digestion buffer to inhibit contaminating proteases. Aliquots were drawn at increasing times and analyzed by one-dimensional PAGE to follow the kinetics of proteolysis.

One- and Two-dimensional PAGE—Protein separation by one-dimensional (20) or two-dimensional PAGE (21) was carried out as described previously (13). Transfer of proteins onto nitrocellulose mem-

branes (Hybond C, Amersham Pharmacia Biotech) was performed essentially as described (22), and the blots were saturated in TBS-T (20 mM Tris, pH 7.5, 136.8 mM NaCl, 0.1% v/v Tween 20) containing 2% (w/v) low fat milk. Antibodies were incubated in TBS-T overnight at room temperature, and their binding was revealed by the chemiluminescence system ECL (Amersham Pharmacia Biotech). Signals were quantified by densitometric scanning using an integrating densitometer (Vernon) and the MultiAnalyst System (Bio-Rad).

Blot Overlay Assay—Binding of MAPs to high speed supernatant proteins or tubulin, separated by one-dimensional or two-dimensional PAGE and transferred onto nitrocellulose membranes (Hybond C, Amersham Pharmacia Biotech) was performed essentially as described previously (12). Briefly, precise locations of blotted tubulin subunits after one-dimensional PAGE or blotted tubulin isoforms after two-dimensional PAGE were registered with pencil marks on the membranes after Ponceau red staining following electrotransfer. Strips corresponding to lanes of one-dimensional gels, or rectangles corresponding to tubulin regions of the two-dimensional gels, were cut from the blots and placed into the grooves of a hand-made, Plexiglas incubation device adjusted to the size of the nitrocellulose pieces. Membranes were blocked overnight in overlay (OV) buffer (MEM buffer containing 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 (1 ml) then washed 5 \times 5 min with OV buffer. Protein interactions were stabilized with 0.5% (v/v) formaldehyde in OV buffer. Blots were then equilibrated in TBS-T buffer and processed for MAP immunodetection as described. Signals were scanned and processed by the MultiAnalyst System (Bio-Rad).

Statistical Processing of Two-dimensional Overlay Data—Two-dimensional overlay experiments with the different MAPs were done independently several times (6 with MAP1A, 2 with MAP1B, and 5 with MAP2). After MAP overlays, each blot was reincubated with anti- α - and anti- β -tubulin antibodies (post-control immunoblots) to ensure a perfect positioning of the MAP signals onto the different tubulins immobilized on the membrane. For each overlay experiment and for each α - and β -tubulin subunit, signals from overlays and from control immunoblots were scanned (MultiAnalyst System, Bio-Rad), giving one value for each 40 μ m. Data were exported and processed using Kaleidagraph (Abelbeck Software). Using marks prepositioned on the membranes and on the autoradiographic films, overlay signals were carefully aligned with those from the corresponding control immunoblots. Control immunoblots were then aligned together. Background was subtracted, and data were expressed as fractions of the maximal value taken as 1.0. Aligned scans were then divided into 12 sections, each one encompassing 75- to 90-point values and representing 3–3.6 mm of a tubulin two-dimensional spot in the pI dimension. For each section, the 75–90 values were added and the sums were exported into Excel files. Files corresponding to repeated experiments with a given MAP were used to calculate mean values and standard deviations (per tubulin subunit and per overlaid MAP for each of the 12 sections). These final values were used to create the graphics in Fig. 4 (*curves* and *histograms*). The mean values were also used for a statistical comparative test (Student's *t* test) between MAP1A and MAP1B, MAP1A and MAP2, and MAP1B and MAP2. From this test, *p* values were calculated for each section.

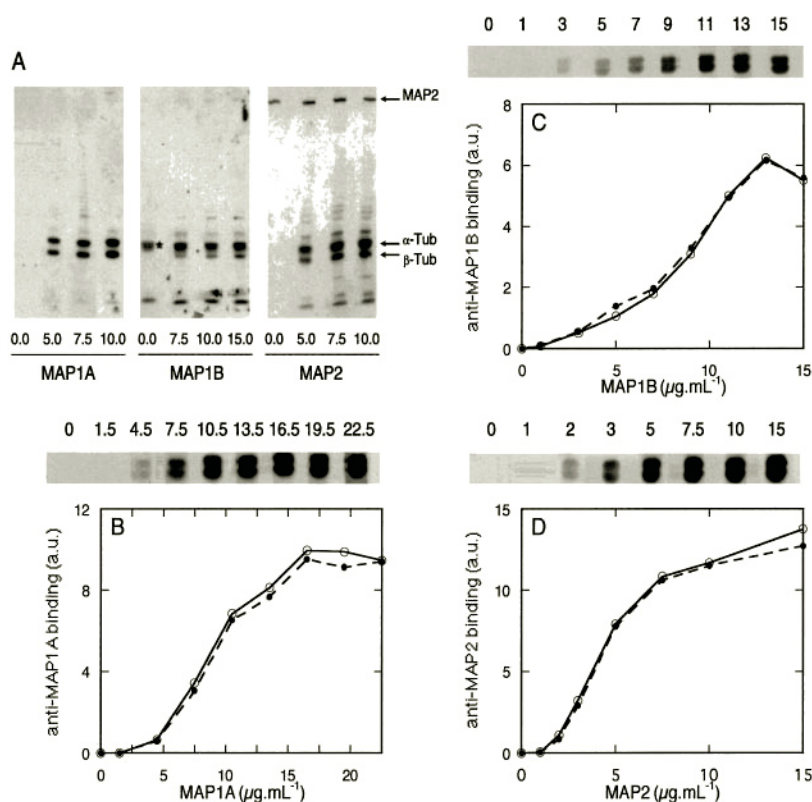
RESULTS

Specificity of MAP Binding by Blot Overlay Experiments—The specificity of the interactions between purified MAP1A, MAP1B, or MAP2 and tubulin was tested by blot overlay experiments. In a first step, proteins from whole mouse brain, high speed supernatant fraction (So) were separated by one-dimensional PAGE and transferred onto nitrocellulose. Increasing concentrations of each MAP were separately overlaid onto identical duplicate membranes, and MAPs bound to their protein targets were detected using specific antibodies. Fig. 1A shows that, under the experimental conditions used and among the large number of soluble brain proteins present on the membrane, α - and β -tubulin represent by far the main protein targets for each of the three MAP tested. Few other unidentified proteins that bound MAPs were also detected albeit at much lower level.

In a second step, increasing concentrations of each MAP were overlaid onto a constant amount of phosphocellulose-purified tubulin separated by one-dimensional PAGE and immobilized onto nitrocellulose. Fig. 1 (B–D) shows that the sat-

FIG. 1. MAP1A, MAP1B, and MAP2 binding to α - and β -tubulin subunits.

A, increasing concentrations ($\mu\text{g/ml}$) of MAP1A, MAP1B, and MAP2 were overlaid onto whole supernatant proteins from mouse brain separated by one-dimensional PAGE and transferred onto nitrocellulose. Binding of MAPs, detected by specific anti-MAP antibodies, is mostly restricted to α - and β -tubulin. Endogenous MAPs present in brain samples are also revealed (*top* of autoradiographs, indicated only for MAP2 immunodetection, *right panel*). In the MAP1B experiment, the primary antibody recognizes in the tubulin region a protein (noted by an *asterisk*) that does not interact with MAP1B. **B**, **C**, and **D**, phosphocellulose-purified tubulin ($2\ \mu\text{g}$) was separated by one-dimensional PAGE, blotted onto nitrocellulose, and overlaid with increasing concentrations of MAP1A (**B**), MAP1B (**C**), or MAP2 (**D**). MAPs bound to separated α - and β -tubulin subunits were detected with specific anti-MAP antibodies, and the resulting signals (*upper panels*) were quantified by densitometric scanning and expressed in arbitrary units (*a.u.*). *Lower panels*: α -tubulin, *open circles* and *solid lines*; β -tubulin, *filled circles* and *dashed lines*.



uration curves of the α - and the β -tubulin subunits with the different MAPs are completely superimposed, indicating that MAP1A, MAP1B, and MAP2 each have the same intrinsic capacity to bind to either tubulin subunit. In these experimental overlay conditions, the half-saturation values were found to be similar (20–30 nM) for the different MAPs.

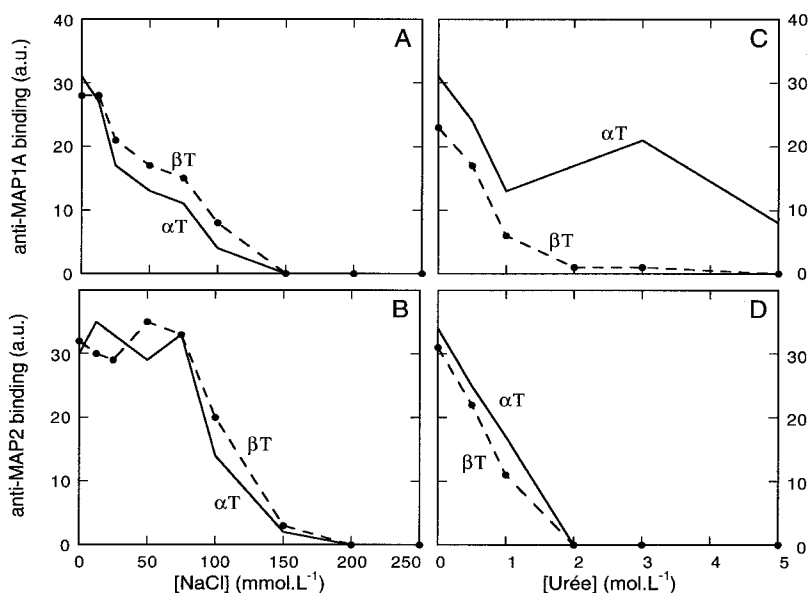
Physicochemical Parameters of MAP1A and MAP2 Binding to Tubulin—The protein-protein interactions between nitrocellulose-bound tubulin and soluble MAP1A or MAP2 in the presence of increasing concentrations of salt or urea were also tested by blot overlay. Binding of MAP1A and MAP2 to tubulin was differentially affected by these treatments (Fig. 2). Interaction of MAP1A appeared to be significantly more sensitive to salt when compared with MAP2 (Fig. 2, *A* and *B*). For example, addition of 50 mM NaCl resulted in a 50% decrease in MAP1A binding (Fig. 2*A*), whereas the presence of up to 75 mM NaCl did not affect the binding of MAP2 (Fig. 2*B*). However, neither MAP exhibited a detectable difference in its interaction with the α - or β -tubulin subunit.

In the presence of increasing concentrations of urea, the binding of MAP2 appeared to be highly sensitive to the presence of this chaotropic agent and decreased linearly as a function of urea concentration (Fig. 2*D*). A concentration of 1 M urea was sufficient to reduce MAP2 binding by 50%, and no interaction could be detected above 2 M urea. Once again, no difference could be observed between the two tubulin subunits. By contrast, MAP1A in the presence of urea exhibited important differences in binding to the α - and β -tubulin subunit (Fig. 2*C*). As noted above with MAP2, a 50% decrease in the binding of MAP1A to tubulin was observed in the presence of 1 M urea. However, as the urea concentration was raised further, binding of MAP1A to β -tubulin decreased rapidly but the binding to α -tubulin appeared to remain largely and reproducibly unaffected. These results suggest that interaction of MAP1A with tubulin mainly depends on ionic bonds, to a larger extent than

for MAP2, and that binding of MAP2 depends, to a larger extent than for MAP1A, on the protein conformation through the implication of critical hydrogen bonds. Additionally, MAP1A appears to interact differently with the two tubulin subunits implying that, although MAP1A binds to both subunits, the binding occurs at nonhomologous domains. Taken together, these results suggest that different amino acid residues of tubulin are involved in the interaction with MAP1A and MAP2. A possible effect of calcium ions (from 1 to 10 mM) on the binding of MAP1A was also investigated, but no qualitative or quantitative modification in the binding of MAP was observed (data not shown).

Tubulin Polyglutamylation Modulates Differentially the Binding of the Various MAPs—We previously reported that polyglutamylation of tubulin can regulate the binding of Tau, MAP2, and different kinesin motors as a function of the polyglutamyl chain length through progressive conformational changes of the C-terminal domain of tubulin, the post-translational polyglutamyl chain acting as a molecular potentiometer (12, 13). Because Tau and MAP2, on one hand, and kinesin motors, on the other hand, are thought to interact at separate but close sites within the C-terminal domain of tubulin (13), we observed logically a similar effect of polyglutamylation on the modulation of binding of these three different proteins. In view of the observations that the putative tubulin binding sites present on MAP1A and MAP2 are quite different from those described for Tau or MAP2 (see “Discussion”), we asked whether MAP1A and MAP1B binding could also be affected by tubulin polyglutamylation. Consequently, we tested their binding to tubulins carrying glutamyl chains of various lengths (from 0 to 6 or 7 glutamyl units). Because polyglutamylation is, by far, the major post-translational modification of both α - and β -tubulin in adult brain (11, 23, 24), an electrophoretic separation of brain tubulin by two-dimensional PAGE allowed us to differentiate between the various glutamylated forms of both

FIG. 2. MAP1A and MAP2 binding to α - and β -tubulin subunits in the presence of NaCl or urea. For each experiment, 2 μ g of tubulin separated by one-dimensional PAGE and blotted onto nitrocellulose were overlaid with 7 μ g of MAP1A (A and C) or MAP2 (B and D) in 1 ml of OV buffer containing 0–250 mM NaCl (A and B) or 0–5 M urea (C and D). MAPs bound to separated α -tubulin (α -T, solid lines) and β -tubulin (β -T, broken lines) subunits were detected with specific anti-MAP antibodies. The resulting signals were quantified by densitometric scanning and expressed in arbitrary units (a.u.).



subunits toward the acidic portion of the gel, proportionally to the number of the additional acidic charges of the glutamyl units, in other words, proportionally to the polyglutamyl chain length. After two-dimensional PAGE, the separated glutamylated isoforms of tubulin were immobilized by electrotransfer onto a nitrocellulose membrane where they could be easily located and probed for their capacity to interact with a given MAP. Fig. 3A shows a typical two-dimensional pattern of adult brain tubulin after immunodetection with anti- α - and anti- β -tubulin antibodies. The unmodified primary translation products are present at the extreme basic side of the different tubulin spots (25, 26), and the glutamylated derivatives are spread toward the acidic sides of the spots (24, 27), as a function of the number of glutamyl units oligomerized into the post-translational chains. The extreme acidic sides of the spots correspond to α - or β -tubulins carrying polyglutamylated chains 6–7 units in length. Nitrocellulose membranes, identical to those shown in Fig. 3A, were overlaid with purified MAP1A, MAP1B, or MAP2. Fig. 3 (B–D) shows the immunodetection of MAP1A, MAP1B, and MAP2, respectively, that bound to α - and β -tubulin isoforms. Compared with Fig. 3A, which displays the whole repertoire of tubulin isoforms present on the membrane and available for MAP interaction, MAP1A, MAP1B, and MAP2 did not obviously bind to all of the tubulins with the same intensity but rather bound to a restricted subpopulation of mid-glutamylated tubulin isoforms. This observation strongly suggests that, as already reported for Tau, MAP2, or kinesin (12, 13), the length of the polyglutamyl chains linked to α - and β -tubulin can also regulate the binding of MAP1A and MAP1B.

A closer examination of the positions of the MAPs bound onto tubulin in Fig. 3 (B–D) shows that each MAP displays a distinct tubulin pattern of preferential binding. Compared with MAP2 (Fig. 3D), MAP1B appears to bind roughly to the same moderately glutamylated tubulin isoforms (Fig. 3C). MAP1A, however, seems to bind to additional more acidic α - and β -tubulin isoforms, *i.e.* those carrying longer polyglutamyl chains (Fig. 3B).

To analyze more precisely the binding preference of the diverse MAPs, signals obtained from control immunoblots and from overlays (such as those shown in Fig. 3, A–D) were integrated by densitometric scanning. Several independent experiments with different MAPs were processed and submitted to statistical analysis to confirm or invalidate the differences ob-

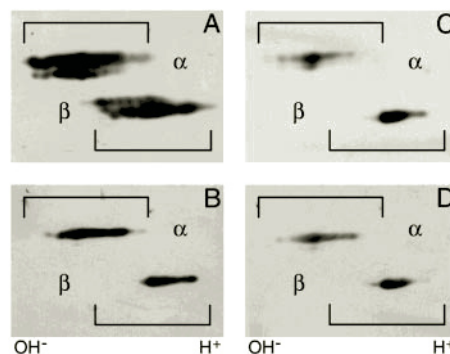
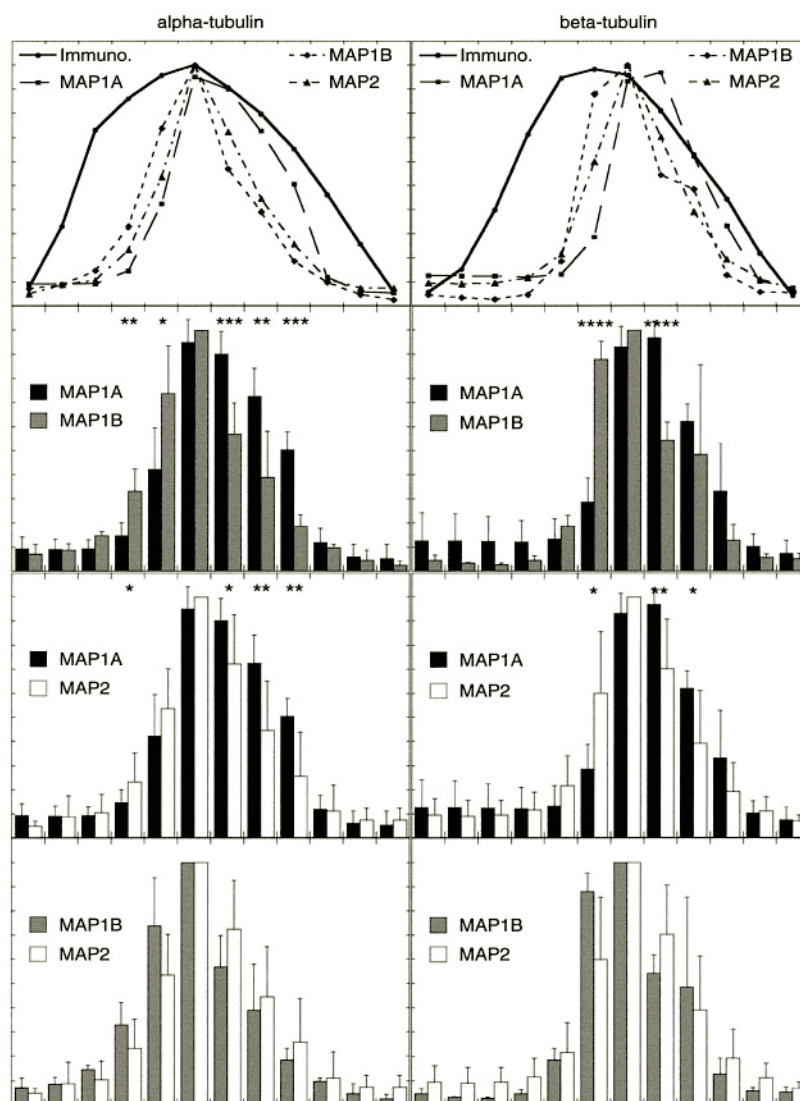


FIG. 3. MAP1A, MAP1B, and MAP2 binding to α - and β -isotubulins separated by two-dimensional PAGE. Tubulin (20 μ g) was separated by high resolution two-dimensional PAGE, transferred onto nitrocellulose, and stained with Ponceau red. Positions of tubulin isoforms were carefully registered with pencil marks on the nitrocellulose membranes for accurate comparisons between the different membranes (brackets). Tubulin was either immunodetected with general anti-tubulin antibodies (A) or overlaid with 16 μ g/ml MAP1A (B), 12 μ g/ml MAP1B (C), and 10 μ g/ml MAP2 (D). A, control: the different isotubulins were immunodetected first with DM1B (anti- α -tubulin) then with DM1B (anti- β -tubulin). B, C, and D, immunodetection of tubulin-bound MAP1A, MAP1B, and MAP2, respectively, after overlay with the corresponding purified proteins. Brackets are localized precisely in the same coordinates on the four panels and indicate the positions of the whole range of α - and β -tubulin isoforms.

served in the binding of distinct MAPs to a given subset of tubulin isoforms. To compare the relative affinities of the three MAPs for tubulins at different levels of glutamylation, scans were divided into 12 regions of identical size along the pI dimension from the basic to the acidic side of the tubulin two-dimensional spots, that is, from unglutamylated to highly glutamylated tubulin isoforms, respectively. Results for both tubulin subunits are shown in Fig. 4. In the *upper panels* are represented the mean distribution of the different α - and β -tubulin isoforms present on the membrane and the mean relative amounts of MAP1A, MAP1B, and MAP2 bound to the tubulin isoforms as a function of pI. These mean scans from control immunoblots and overlay experiments were accurately aligned one to the other relative to the pI on the x axis (see “Experimental Procedures” for details). The *lower panels* show the comparison of the binding capacities of MAP1A *versus* MAP1B, MAP1A *versus* MAP2, and MAP1B *versus* MAP2 toward the

FIG. 4. Preferential binding of MAP1A, MAP1B, and MAP2 to the different α - and β -tubulin isoforms. Signals detected over the α - and the β -tubulin two-dimensional spots from control tubulin immunoblots and from MAP immunodetections after overlay (such as those shown in Fig. 3) were scanned and integrated. Several independent experiments were carried out (6 for MAP1A, 3 for MAP1B, and 5 for MAP2). Data were processed as indicated under "Experimental Procedures" after dividing the tubulin spots into 12 equal sections in the pI dimension. Curves shown in the upper panels represent the preferential binding of MAP1A, MAP1B, and MAP2 to α - (left panel) and β -tubulin (right panel) isoforms as a function of their pI (in the different sections, from basic, left, to acidic, right), that is, of the length of their polyglutamyl chains (from 0 to 6 or 7 units). Solid lines represent the distribution of total α - and β -tubulins present on the membranes and detected by general anti-tubulin antibodies (control immunoblots). Histograms of the lower panels represent the mean values (\pm S.D.) of the amounts of MAP1A, MAP1B, and MAP2 (expressed in arbitrary units) bound to the different subsets of isotubulins present in the 12 sections. For each section, data from couples of MAPs were submitted to Student's *t* test. When mean values representing the binding of two different MAPs to a given subset of tubulins within a given section were assessed as "statistically different values" (confidence test), the corresponding sections were labeled with one, two, three, or four asterisks, representing *p* values < 0.05, 0.02, 0.01, or 0.001, respectively.



different tubulin isoforms as a function of their degree of glutamylation. Asterisks positioned in sections indicate that the differences observed in the binding behavior of two MAPs toward a given isotubulin subset can be considered as significant according to Student *t* test calculations (see "Experimental Procedures"). Fig. 4 shows that the relative affinity of MAP2 for α - and β -tubulin increases with the polyglutamyl chain length, as reported previously (12), reaching an optimum for a length of about 3 units then gradually decreasing for longer chains. Similarly, the tubulin isoform binding range of MAP1B is also centered around triglutamylated tubulins and does not differ significantly from that of MAP2. By contrast, the tubulin isoform binding range of MAP1A is much broader and clearly comprises more acidic isoforms from both tubulin subunits. Compared with MAP2 or MAP1B, the optimal preference of MAP1A is shifted toward tubulins with longer polyglutamyl chains. All of these differences were confirmed to be statistically significant.

From data shown in Fig. 4, it can be calculated that tubulins with short (<3 units) polyglutamyl chains can bind MAP1B or MAP2 ~2 times more efficiently than MAP1A. This ratio is reversed for tubulins carrying long polyglutamyl chains (>4 units). Although the relative affinity of MAP1B or MAP2 for tubulins comprising more than four glutamates shows a drastic 70% decrease, MAP1A shows a unique capacity to significantly

maintain a high affinity for these highly glutamylated tubulins (80–90% of the optimal value for α -tubulins; 75–120% for β -tubulins). In conclusion, it appears that polyglutamylation of tubulin has the property to regulate differentially the binding of distinct structural MAPs.

MAP1A and MAP2 Do Not Compete Each Other for Binding to Tubulin—As shown above, MAP1A and MAP2 display distinct affinities toward the different polyglutamylated tubulins. However, it is important to mention that mid-glutamylated tubulin isoforms are efficient targets for both MAPs. In this context, MAP1A and MAP2 could compete for binding to the same site on tubulin. In the hypothesis of a single common site, a high level of polyglutamylation could well alter the binding of MAP2 but not that of MAP1A, suggesting erroneously the existence of two distinct sites. For a mid level of glutamylation, however, competition could be more apparent. To answer this question, we performed competition binding experiments by overlay assay onto tubulin separated by two-dimensional PAGE (Fig. 5) and one-dimensional PAGE (Fig. 6).

MAP1A and MAP2 were mixed and overlaid together onto tubulin separated by two-dimensional PAGE and immobilized onto nitrocellulose (Fig. 5). The experiments were performed in duplicate, and the binding of each MAP was detected using specific anti-MAP antibodies on separate membranes (Fig. 5, upper panels). A control membrane with no MAP added was

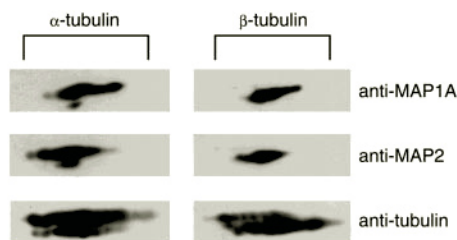


FIG. 5. Simultaneous overlay of MAP1A and MAP2 on tubulin separated by high resolution two-dimensional PAGE. Tubulin (20 μ g) was separated by high resolution two-dimensional PAGE and transferred onto nitrocellulose membrane. Positions of α - and β -tubulin isoforms were carefully registered with pencil marks after Ponceau red staining (brackets above the upper panels). This experiment was done in triplicate. Two blots were overlaid simultaneously with MAP1A (8 μ g/ml) and MAP2 (4 μ g/ml). MAP binding was detected using specific anti-MAP antibodies (MAP1A on the first blot, upper panels; MAP2 on the second blot, middle panels). Tubulin isoforms were immunodetected on the third blot (lower panels) with DM1A (general anti- α -tubulin) then with DM1B (general anti- β -tubulin). To facilitate the comparison between the resulting signals, the α - and β -tubulin regions of the blots were cut and precisely aligned vertically.

used to precisely locate the different tubulin isoforms (Fig. 5, lower panels). The results confirm the data from Figs. 3 and 4 and show that, besides the moderately glutamylated α - and β -tubulins that share the property to bind both MAP1A and MAP2, the α - and β -tubulin species carrying longer polyglutamyl chains are efficient and specific targets for MAP1A.

To test the possible competition between MAP1A and MAP2 for the same tubulin binding sites, we then performed two complementary competition overlay protocols with tubulin separated by one-dimensional PAGE (Fig. 6). In a first approach, MAP1A and MAP2 were mixed and overlaid simultaneously onto immobilized tubulin, MAP2 at a constant concentration and MAP1A at increasing concentrations (Fig. 6A) and, conversely, MAP1A at a constant concentration and MAP2 at increasing concentrations (Fig. 6B). In the second approach, increasing concentrations of one MAP were added first to progressively saturate the binding sites on the tubulin molecules, then the other MAP was added at a constant concentration (Fig. 6, C and D). In this latter series of experiments, interactions established between tubulin and the first MAP were stabilized with formaldehyde prior to the addition of the other one to avoid any displacement of the initially bound MAP by the other one. The binding of each MAP to tubulin was then detected using specific anti-MAP antibodies. The binding curves in Fig. 6 show that, independently of the protocol used, when the two MAPs are added simultaneously or successively the binding of a given MAP is always representative of its respective concentration and is not affected at all by that of the other one. These data strongly argue that MAP1A and MAP2 bind to distinct and independent sites on the tubulin molecules.

Finally, an additional experiment was performed to rule out the possibility of a competition between MAP1A and MAP2 for a single binding site on tubulin that would not have been detectable in the experimental conditions of Figs. 5 and 6. Because polyglutamylation is thought to play a central role by differentially modulating MAP binding, we tested the binding of MAP1A and MAP2 to tubulin cleaved with subtilisin (Fig. 7). After protease treatment, the distal C-terminal sequences of α - and β -tubulins carrying the polyglutamyl chains are cut off and hence polyglutamylation cannot interfere anymore. Purified tubulin was submitted to limited proteolysis with subtilisin, and aliquots were drawn at various times of digestion. After one-dimensional PAGE analysis, we chose a time point corresponding to a partially digested sample containing both subtilisin-cleaved tubulins (named α S and β S) and uncleaved tu-

bulin subunits to serve as internal binding controls (Fig. 7, lanes 1 and 2). Binding of MAP1A (lanes 3–5) and MAP2 (lanes 6–8) was then probed on a duplicate series of nitrocellulose strips where both MAPs had been overlaid in the following orders: MAP1A then MAP2 (lanes 4 and 7) and MAP2 then MAP1A (lanes 5 and 8). Removal of the C-terminal sequences of tubulin by subtilisin does not prevent MAP1A and MAP2 from binding to α S- and β S-tubulins, although the binding of both MAPs appears significantly reduced on α S-tubulin. When compared with the overlay controls (MAP1A alone, lane 3; MAP2 alone, lane 6), it is clear that the prior binding of one MAP does not affect at all the subsequent binding of the other, which strongly suggests, again, that MAP1A and MAP2 do bind tubulin at different sites.

In summary, polyglutamylation of tubulin, in addition to Tau, MAP2, and kinesin motors, can also regulate the binding of the high molecular weight MAP1A and MAP1B. Interestingly, we observed that polyglutamylation can exert a differential control on the binding of these MAPs. Changes in the relative affinity of MAPs for tubulins as a function of the length of the polyglutamyl chains do not follow a unique pattern for all of the MAPs. On the contrary, polyglutamylation appears to act as a differential rather than a general regulator of tubulin-MAP interactions. Subtilisin experiments indicate that the polyglutamyl chains do not represent the direct physical target for these MAPs (Refs. 12 and 13 and Fig. 7 in this paper). But as the binding sites for most of these MAPs are located within the C-terminal domain of tubulin, these sites likely undergo conformational changes driven and modulated by the length of the polyglutamyl chain, the optimal binding of a given MAP to tubulin, or the optimal accessibility of this MAP for tubulin, being achieved for a defined polyglutamyl chain length. In the case of MAP1A and MAP2, the establishment and/or the stability of MAP1A-tubulin complexes tends to be retained for tubulin carrying long polyglutamyl chains, lengths for which MAP2-tubulin interactions are largely destabilized.

DISCUSSION

Blot Overlay Approach to Study Tubulin-MAP Interactions— Blot overlay assay represents a rapid and useful means to study protein-protein interactions. This is particularly useful in the case of tubulin that comprises a very large number of isoforms arising from different gene products and multiple post-translational modifications. These isoforms cannot be readily purified for use in classical interaction experiments. Separation of such isoforms by two-dimensional PAGE and their subsequent immobilization onto the surface of a nitrocellulose membrane, however, provides the advantageous opportunity for analyzing their respective binding capacities toward a given MAP added in solution as a potential interaction partner.

Accurate positioning of tubulin and MAP to engage in their mutual interaction depends on an active protein conformation involving the establishment of critical bonds. In overlay experiments, in which tubulin is first submitted to SDS-PAGE and transferred onto nitrocellulose, the recovery of a functional conformation is therefore essential. First, SDS is released from the protein during its transfer onto the membrane, the transfer buffer containing 20% methanol (22). Second, renaturation in an appropriate sulfonate buffer (or at least the remodeling of an active local conformations) is largely time-dependent. After transfer, nitrocellulose membranes need to be incubated at least overnight in OV buffer to observe a subsequent efficient protein binding capacity of the membrane-bound tubulin. The same is also true for reverse overlay, where soluble tubulin interacts with membrane-bound MAPs (12).

By this technique, the specificity of the interactions can also

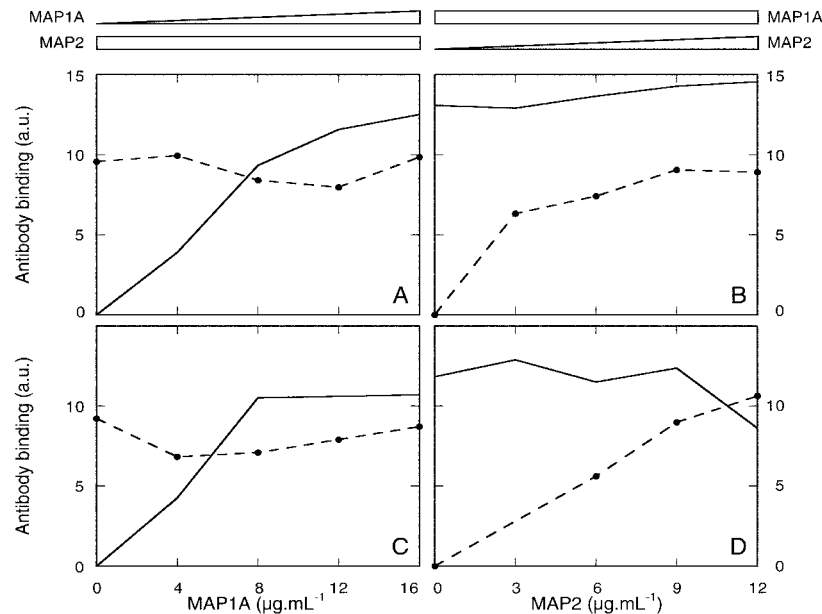


FIG. 6. Competition binding experiments with MAP1A and MAP2 on tubulin separated by one-dimensional PAGE. Tubulin (2 µg per lane) was separated by one-dimensional PAGE, transferred onto nitrocellulose, and overlaid simultaneously (A, B) or successively (C, D) with MAP1A and MAP2 at increasing or constant concentration (symbolized by triangles or rectangles, respectively, at the top of the figure). A, co-overlay with increasing concentrations of MAP1A (0–16 µg/ml) and a constant concentration of MAP2 (12 µg/ml); B, co-overlay with increasing concentrations of MAP2 (0–12 µg/ml) and a constant concentration of MAP1A (16 µg/ml); C, first overlay with increasing concentrations of MAP1A (0–16 µg/ml) then second overlay with a constant concentration of MAP2 (12 µg/ml); D, first overlay with increasing concentrations of MAP2 (0–12 µg/ml) then second overlay with a constant concentration of MAP1A (16 µg/ml). Tubulin-bound MAP1A (solid lines) and MAP2 (broken lines) were detected with specific anti-MAP antibodies and signals were quantified by densitometric scanning (expressed in arbitrary units, a.u.). When MAPs were overlaid one after the other, interactions between tubulin and the first MAP were stabilized with formaldehyde prior to the second overlay to avoid any displacement of the first MAP by the second one.

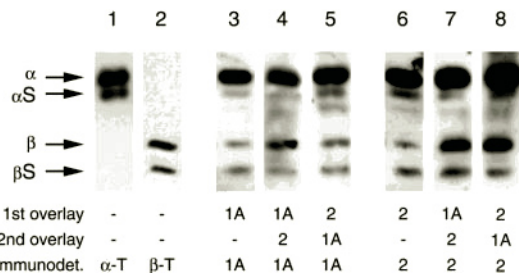


FIG. 7. Binding of MAP1A and MAP2 to subtilisin-cleaved tubulin. Purified mouse brain tubulin was submitted to limited proteolysis with subtilisin. Time of digestion was chosen to obtain both cleaved and uncleaved tubulin for each subunit. Digested samples were subjected to one-dimensional PAGE (4 µg per lane), transferred onto nitrocellulose, and either immunoprobed with DM1A (lane 1) and DM1B (lane 2) to localize the cleaved (αS and βS) and uncleaved (α and β) tubulin subunits or overlaid with MAP1A or/and MAP2 (lanes 3–8). Competition overlays with MAP1A and MAP2 were performed in duplicate as indicated: first overlay with MAP1A then second overlay with MAP2 (lanes 4, 7), first overlay with MAP2 then second overlay with MAP1A (lanes 5, 8), followed by the immunodetection of MAP1A (lanes 4, 5) or MAP2 (lanes 7, 8). Lanes 3 and 6 show the binding of MAP1A and MAP2, respectively, in the absence of the other MAP.

be easily tested. When soluble proteins from a whole mouse brain supernatant are presented on the membrane to the overlaying MAP, α- and β-tubulin subunits represent the main protein targets (see Fig. 1A), although other minor MAP-interacting proteins are also detected. For instance, in experiments performed with higher MAP concentrations, two other proteins were found to specifically bind MAP1A under these conditions (data not shown): Actin, according to the previously reported actin-binding activity of MAP1A (28), and an 80-kDa protein, which remains unidentified.

When the physicochemical conditions were altered by the addition of salt or urea, differences in the binding of MAP1A

and MAP2 were observed. Binding of MAP1A to tubulin is much more sensitive to salt than that of MAP2, indicating that ionic bonds are involved to a greater extent in tubulin-MAP1A than in tubulin-MAP2 interactions. Moreover, this differential sensitivity would suggest that the two MAPs bind to different sites on the tubulin molecule, and this is further confirmed by the competition binding experiments shown in Figs. 5–7. Differences were also observed between α- and β-tubulin subunits in the binding of MAP1A. In the presence of urea, MAP1A can still efficiently engage in an interaction with the α- but not with the β-tubulin subunit. The establishment of the complex between MAP1A and β-tubulin, as those between MAP2 and α- or β-tubulin, appears to be more dependent on precise hydrogen bonding between the two protein partners. These results suggest that, contrary to the other MAPs tested so far, MAP1A contacts α- and β-tubulin subunits at asymmetrical, nonrelated sequences but apparently with a similar efficiency. An alternative possibility, in view of the fact that multiple tubulin binding motifs differing in their physicochemical properties have been reported for MAP1A (29–31), is that the different binding conditions selectively affect one motif without affecting the functional state of others. In particular, the latter hypothesis may be more applicable to the α-tubulin subunit.

Differential Regulation of MAP Binding by Polyglutamylation—Polyglutamylation of tubulin (11) is an oligomeric post-translational modification comprising a secondary chain added close to the C terminus of α- and β-tubulin to the γ-carboxylic group of the lateral chain of a glutamate residue. This chain is made of 1 to 6 or 7 glutamyl units linked one to the other by peptidic and/or isopeptidic bonds (11, 27, 32–37). This oligomeric structure works as a molecular potentiometer to regulate in a length-dependent manner the affinity of the C-terminal domains of tubulins for structural and motor MAPs such as Tau, MAP2 (12), or kinesin motors (13). Masking the polyglutamyl chains with the specific monoclonal antibody GT335 (27)

leads to a strong inhibition of MAP binding *in vitro* (12, 13) as well as a strong inhibition of motility of axonemes of flagella or cilia (38–40). Microinjection or electroporation of GT335 into proliferating HeLa cells has been shown to be followed by a transient and reversible disappearance of centrioles (41). Microinjection of GT335 into Atlantic cod melanophores has also been shown to interfere with kinesin-based pigment granule dispersion but not with its cytoplasmic dynein-based aggregation, suggesting that polyglutamylation could also differentially regulate both motor proteins (42). A common feature of these results obtained *in vivo* might be that the antibody blocks the function of the polyglutamyl chains, thus preventing or drastically altering the interaction of structural and motor MAPs with tubulins, just as observed in the *in vitro* experiments cited above.

Polyglutamyl chains can be removed together with the extreme C-terminal peptides of α - and β -tubulin by enzymatic cleavage with subtilisin (33, 43). In this case, subtilisin-cleaved tubulin was shown to retain the ability, albeit to a lesser extent, to bind Tau, MAP2, or kinesin (12, 13). Similar results were obtained with MAP1A (see Fig. 7). These results indicate that the polyglutamyl chains are not the direct targets for MAP binding but, rather, could play an indirect role in modulating the overall conformation of the C-terminal domain of tubulin. Such conformational changes may fine-tune the opening or the closure of the MAP binding sites. Tau and kinesin are thought to bind tubulin at close but independent sites inside of a predicted two- α -helix motif closed by ionic bonds (12, 13) and located close to the C terminus, exposed at the outer surface of the MT, facing the incoming MAPs. This motif is now clearly visible in the electron crystallography structure of the tubulin heterodimer (α -helices H11 and H12 in Ref. 44). It is therefore possible that this two- α -helix motif may gradually open as a consequence of the incoming of the negative charges from the growing polyglutamyl chain when it lengthens up to 3 units and consequently increase the accessibility to the MAP binding sites (12, 13). Because Tau, MAP2, and kinesin all bind to this same structural motif, it is not surprising that they exhibit a similar mode of regulation by polyglutamylation. The location of the MAP binding site could then correlate with the mode of regulation driven by polyglutamylation. For instance, we observed that the binding of STOP proteins (55) was insensitive to polyglutamylation,² suggesting that the corresponding binding site should be out of the structural influence of polyglutamylation. On the other hand, MAP1A and MAP1B have tubulin-binding motifs (29–31, 45, 46) unrelated to those shared by Tau, MAP2, or MAP4 (47–49) and are thought to bind to tubulin at different sites. Although related, MAP1A and MAP1B display different affinities for tubulin and induce distinct MT shapes (17, 50). Furthermore, MAP2 can compete with MAP1B for binding to tubulin (51, 52) but not with MAP1A (16, 53, 54; see also this paper, Figs. 5–7). MAP1A and MAP1B therefore offer the possibility to test whether their binding sites could be under a different mode of regulation than that of MAP2.

The modulation of MAP1B binding to tubulin isoforms at different states of glutamylation resembles roughly that of MAP2. MAP1B preferentially interacts with moderately glutamylated α - and β -tubulin isoforms (see Figs. 3–5). This observation is in agreement with previous reports that MAP1B and MAP2 bind to similar sites on MTs (51, 52). Consequently, their binding is modulated in a similar manner. Interestingly, MAP1A displays a significantly distinct binding behavior to-

ward the various glutamylated tubulin isoforms (see Figs. 3–5). In contrast to all of the other MAPs tested so far, MAP1A can maintain a selective and high relative affinity for α - and β -tubulin isoforms carrying long polyglutamyl chains, from 3 to 5 or 6 units in length. MAP1A thus appears to be the only adult MAP capable of an efficient binding to highly glutamylated α - and β -tubulins; such isoforms are present in large amounts in neuronal (24, 27, 56) and axonemal (40, 57) MTs. The presence of MAP1A could then be particularly useful for stabilizing MTs made of these highly glutamylated tubulins for which other MAPs like Tau, MAP2, or MAP1B have but a weak affinity.

A Model for MAP Transition Driven by Polyglutamylation—The original property of polyglutamylation to act as a differential regulator of MAP binding could fulfill an interesting function in neurons concerning MAP transitions during development or along axonal and dendritic processes. During development, polyglutamylation is regulated differently on α - and β -tubulin, both qualitatively and quantitatively. At birth, most of neuronal α -tubulin is already glutamylated, with chain lengths covering the whole range from 1 to 6 or 7 units. By contrast, β -tubulin is quantitatively far less modified and in particular the glutamyl chains progressively increase in size throughout postnatal development (56). During the same period, in developing neurons, the juvenile MAP1B is progressively replaced by MAP1A (58, 59). It is striking to note that MAP1B is expressed in these neurons up to a time when tubulin is glutamylated at a maximal level of 2 or 3 units (at least in the case of the β -subunit), which corresponds to the optimal affinity for this MAP. As development proceeds, the length of glutamyl chains continues to increase while MAP1A progressively replaces MAP1B. In other words, MAP1B is substituted by MAP1A, which is much better adapted to interact with the β -tubulin isoforms equipped with longer polyglutamyl chains.

In adult neurons, several MAPs are expressed simultaneously and could compete for binding to MTs. On the other hand, for noncompetitive MAPs, the question of their specific recruitment to different MTs, to give them distinct structural and/or functional properties, is of importance and should require specific mechanisms. Tau and MAP2 share nearly identical tubulin binding motifs and are under the same mode of regulation by polyglutamylation. The problem of competition between them is topologically resolved, because Tau is predominantly found in axons (60) and MAP2 in dendrites (61, 62). Because MAP1A is present in both types of processes (14), the problem of its differential recruitment by MTs arises with MAP2 in dendrites and with Tau in axons. Polyglutamylation of tubulin could potentially solve this problem. In our previous studies on the axonal transport of tubulin and Tau, we observed that Tau proteins were found associated with axonal MTs in the very proximal segments of the sciatic motor axons but apparently detach from them rapidly and become undetectable in the following, more distal axonal segments.³ Similar evidences of asymmetrical distribution have been reported for different MAPs, including Tau, MAP1B, and MT motors (63–68). In light of our results on the differential regulation of MAP binding by polyglutamylation, we propose that this modification could control a transition between Tau and MAP1A on the axonal MTs along neuronal processes (Fig. 8). Indeed, when one considers tubulins with polyglutamyl chains beyond 3 units, MAP1A can maintain a strong binding to MTs, whereas at this level of modification both Tau and MAP2 tend to leave or are destabilized from MTs. Fig. 8 shows that, in the neuronal cell body, tubulins are synthesized and assembled with Tau

² C. Bonnet, C. Bosc, E. Denarier, and J.-C. Larcher, personal communication.

³ J. C. Larcher, D. Boucher, and P. Denoulet, unpublished results.

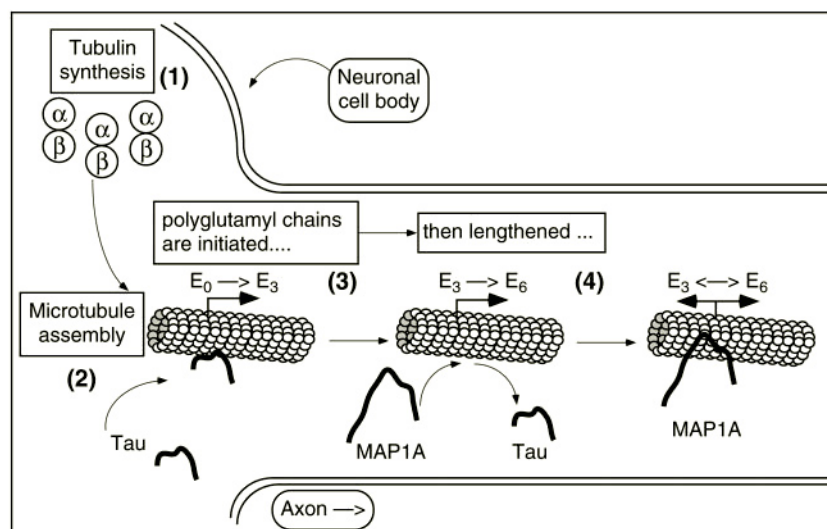


FIG. 8. **Model for a Tau-MAP1A transition on axonal microtubules driven by tubulin polyglutamylation.** Schematic representation of a neuronal cell showing part of the cell body and the beginning of the axon. After synthesis of tubulin heterodimers (step 1) and their assembly with Tau proteins (step 2), MTs are conveyed into the axon. Because MTs are formed and as they move down the axon, assembled tubulins are substrates to the enzyme tubulin-polyglutamylase that gradually lengthens the glutamyl chains (E_n) (steps 3 and 4). When the length of the glutamyl chains increases beyond 3 units, and up to 6 or 7 units (step 4), the relative affinity of Tau for these MTs decreases drastically and Tau can but detach from them. At the same time Tau is destabilized, and given the affinity of MAP1A for highly glutamylated tubulins, MAP1A can bind and/or stay efficiently bound to these axonal MTs to maintain their stability and confer new structural, functional, and interacting properties. According to the role of polyglutamylation as a differential regulator of MAP interactions, this post-translational modification could drive automatic MAP transitions (Tau-MAP1A in axons, MAP2-MAP1A in dendrites, MAP1B-MAP1A during development, etc.) and, more generally, could possibly control the recruitment of specific MAPs or motor proteins onto specialized subsets of MTs such as axonemes or mitotic spindles in non-neuronal cells.

into MTs that are then sent into the axons (for a review, see Ref. 69). Tubulin polyglutamylase proceeds on these newly assembled MTs (70) and lengthens the glutamyl chains as MTs move into the axons (26). When the length of polyglutamyl chains exceeds 3 units, and given the relative affinities of Tau and MAP1A, Tau would be destabilized and detached from the MTs and would be progressively replaced by MAP1A, which can interact with a broader range of polyglutamylated tubulins. This MAP replacement could consequently confer new structural and functional properties to the MTs (e.g. the ability to interact with another set of axonal proteins, change the spacing between MTs or the parameters of their movement down the axons, etc.).

This model could be generalized to other biological situations; polyglutamylation of MTs or of specific MT subsets could offer the capacity to sort the different MAPs to be recruited on these MTs to modulate their properties. In non-neuronal cells, if centrioles are constantly and strongly polyglutamylated, the polyglutamylation level of cytoplasmic MTs is highly variable from one cell type to another and generally lower than that found in neurons or in axonemal structures. However, even a low level of glutamylation could give a substantial advantage to the modified MTs to recruit a given MAP or a specific motor needed for a given function. Such a situation is typically illustrated by the mitotic spindle. The different subpopulations of astral, polar, and kinetochore MTs are each characterized by specific dynamic and functional properties and particularly by selective interactions with motor proteins. Moreover, glutamylation of tubulin increases during mitosis, and polar/kinetochore but not astral MTs appear more glutamylated than interphase MTs (71, 72), in good agreement with the proposed role for glutamylation as a differential regulator of MAP interactions.

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