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Developmental regulation of polyglutamylated α- and β-tubulin in mouse brain neurons

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SUMMARY

Polyglutamylation is an important posttranslational modification of tubulin that is very active in nerve cells, where it accounts for the main factor responsible for tubulin heterogeneity. In the present work, we have analyzed quantitative and qualitative changes in glutamylated α- and β-tubulin occurring during neuronal differentiation in culture. Glutamylated α- and β-tubulin both markedly accumulate during this process with a time course remarkably similar to that observed in vivo during brain development. However, the characteristics of the glutamylation of the two subunits are not exactly the same. Glutamylated α-tubulin is already abundant in very young neurons and displays, at this stage, a wide range of its degree of glutamylation (1 to 6 glutamyl units present in the lateral polyglutamyl chain), which remains unchanged during the entire period of the culture. Glutamylated β-tubulin is present at very low levels in young neurons and its accumulation during differentiation is accompanied by a progressive increase in its degree of glutamylation from 2 to 6 glutamyl units. Posttranslational incorporation of [^{3}H]glutamate into α- and β-tubulin decreases during differentiation, as well as the rate of the reverse deglutamylation reaction, suggesting that accumulation of glutamylated tubulin is accompanied by a decrease in the turnover of glutamyl units onto tubulin.

Neuronal differentiation is also accompanied by an increase of other posttranslationally modified forms of tubulin, including acetylated and non-tyrosinatable α-tubulin, which can occur in combination with polyglutamylation and contributes to increase the complexity of tubulin in mature neurons.

Key words: neuron, neuronal differentiation, tubulin, posttranslational modification, glutamylation, acetylation, non-tyrosinatable tubulin

INTRODUCTION

Neuronal cells exhibit very asymmetrical morphologies that are characterized by the presence of very long axodendritic processes extending from the cell body. Growth and maintenance of these processes depend upon a complex cytoskeletal network. In particular, extensive arrays of microtubules and neurofilaments co-align with nerve extensions (for a recent review, see Solomon, 1992). Although neuronal microtubules exhibit the classical 13-protofilament structure, their isotubulin composition differs drastically from that of non-neuronal cells. Indeed, comparative analyses have shown that tubulin heterogeneity is much higher in brain and neurons than in any other tissue or cell type, thus revealing the existence of numerous isoforms that are present specifically or predominantly in nerve cells (George et al., 1981; Gozes et al., 1981; Denoulet et al., 1982; Wolff et al., 1982; Moura-Neto et al., 1983; Field et al., 1988; Eddé et al., 1989). Compelling evidence shows that tubulin heterogeneity results from both the expression of multiple isogenes and the occurrence of various posttranslational modifications (PTMs) (for recent reviews, see Joshi and Cleveland, 1990; Murphy, 1991; Luduena, 1993).

Differential expression of tubulin mRNA's among cell types and tissues and during development suggests that some tubulin isotypes could play distinct roles during neuronal differentiation (Lewis et al., 1985; Wang et al., 1986; Miller et al., 1987). Confirming this view, a selective accumulation of class II and class III β-tubulin isotypes was observed during NGF-induced differentiation of PC12 cells (Joshi and Cleveland, 1989). Class III β-tubulin, also designated β′, exhibits some specificity towards neuronal cell types where its presence is temporally correlated with neuronal commitment (Eddé et al., 1983, 1987).

On the other hand, PTMs of tubulin are among the most complex encountered in proteins up to today. These modifications include detyrosination and acetylation of α-tubulin and phosphorylation of class III β′-tubulin. In addition, two other major modifications of tubulin were recently characterized: (1) polyglutamylation, which consists of the addition of 1-6 glutamyl units on the side chain of an encoded glutamate near

the carboxy terminus of α- and β-tubulin (Eddé et al., 1990; Alexander et al., 1991; Redeker et al., 1992; Rüdiger et al., 1992); and (2) the formation of an α-tubulin variant, non-tyrosinatable tubulin (NTT), which lacks the carboxy-terminal glutamyl-tyrosine group and which cannot be tyrosinated (Paturle-Lafenechère et al., 1991). The precise role of tubulin PTMs remains to be determined. However some PTMs, such as phosphorylation of class III β′-tubulin and acetylation of α-tubulin, are correlated with morphological differentiation (Gard and Kirschner, 1985; Eddé et al., 1987; Black and Keyser, 1987; Black et al., 1989; Lim et al., 1989; Lee et al., 1990). Glutamylated tubulin is very abundant in nerve cells but little is known about its developmental pattern of expression.

In this report, we present an extensive analysis of the developmental expression of polyglutamylated α- and β-tubulin, as well as of acetylated α-tubulin and NTT, during long-term culture of embryonic mouse brain neurons. As shown previously (Berwald-Netter et al., 1981; Chelly et al., 1990), this model is particularly useful for studying late phases of neuronal differentiation. Marked changes in the abundance and the heterogeneity of polyglutamylated α- and β-tubulin are observed with a time course remarkably similar to that occurring in vivo during post-natal brain development. Our results indicate that tubulin PTMs play important roles in the formation of the neuronal cytoskeleton.

MATERIALS AND METHODS

Antibodies

Monoclonal antibodies (mAbs) directed against α- (DM1A) and β-tubulin (DM1B), respectively, were purchased from Amersham (N.356 and N.357); 6-11B-1 (Piperno and Fuller, 1985), a mAb specific for acetylated α-tubulin was a kind gift from Dr G. Piperno (Mount Sinai Medical Center, New York, USA); and purified antiserum directed against NTT (Paturle et al., 1989) was a generous gift from Dr D. Job (Centre d'Etudes Nucléaires, Grenoble, France). SDL.3D10 (Banerjee et al., 1990), a mAb specific for class III β′-tubulin was purchased from Sigma. GT335, a mAb specific for glutamylated tubulin was prepared in our laboratory (Wolff et al., 1992). Dilutions used for immunoblotting experiments were: 1:10,000 (DM1A), 1:5,000 (DM1B), 1:200 (6-11B-1), 1:100,000 (anti-NTT), 1:1,000 (SDL.3D10) and 1:10,000 (GT335).

Cell culture and labeling

Neurons were isolated from fetal mouse brain at 15 days of gestation and allowed to develop in primary culture for up to four week, as described previously (Berwald-Netter et al., 1981). These cultures are composed mostly of neuronal cells with a very slight contamination by astrocytes and fibroblast-like cells (≤5% for each). In some experiments, nocodazole (4.5 mM) was added to the culture medium at a final concentration of 4.5 μM; DMSO, the drug solvent, was added to control cultures (1/1000, v/v). Posttranslational labeling was performed by incubating cells in phosphate buffered saline (PBS) containing 3.7 MBq/mL [^{3}H]glutamate (Amersham, O.74-1.85 TBq/mmol) in the presence of (100 μg/ml) cycloheximide, a strong inhibitor of protein synthesis. These conditions resulted in the specific labeling of the polyglutamyl lateral chain (Eddé et al., 1992; Audebert et al., 1993). The radioactivity resulting from [^{3}H]glutamate incorporation into tubulin was determined after SDS-PAGE. We have previously described a convenient method that avoids solubilization of gel pieces (Audebert et al., 1993). Proteins were transferred onto nitrocellulose as described below and the membrane was stained with Ponceau Red. Tubulin bands were then cut out and processed directly for liquid scintillation counting.

Preparation of tissue and cell protein extracts

Swiss mice were sacrificed by decapitation and brains were immediately removed and homogenized in cold MEM buffer (0.1 M MES, pH 6.7, 2 mM EGTA, and 1 mM $MgCl_2$) containing a protease inhibitor mixture (10 μg/ml aprotinin, 10 μg/ml leupeptine and 1 mM phenylmethylsulfonyl fluoride). High-speed supernatants were recovered after centrifugation (100,000 ***g***, 4°C, 45 minutes) and immediatly treated for SDS-PAGE or two-dimensional (2-D) PAGE. Cell cultures were washed twice and harvested in cold PEM buffer (0.1 M PIPES, pH 6.9, 2 mM EGTA, 1 mM $MgCl_2$) containing the protease inhibitor mixture and were lyzed by adding 1% (w/v) NP40. Whole extracts were either treated directly for SDS-PAGE or 2-D PAGE, or centrifuged (15,000 ***g***, 4°C, 10 minutes) to obtain supernatant and pellet fractions. In some experiments, soluble and cytoskeletal fractions were prepared as previously described (Audebert et al., 1993) using the following microtubule stabilizing buffer: 0.1 M PIPES, pH 6.9, 5 mM EGTA, 5 mM $MgCl_2$, 20% (v/v) glycerol, 5% (v/v) DMSO and 0.25% (w/v) Triton X-100. Protein content was determined according to Bradford (1976) using bovine serum albumin as standard.

Electrophoresis

SDS-PAGE was performed according to Laemmli (1970) on an 8% acrylamide/0.11% bisacrylamide slab gel containing 0.1% (w/v) SDS (Merck, 90% pure). 2-D PAGE was performed as previously described (Wolff et al., 1982), except that pH 5.0-6.0 ampholytes (Serva) were used. Gels were stained with Coomassie Blue, soaked in amplify (Amersham) for 15-20 minutes, dried, and submitted to fluorography using Kodak XAR-5 films.

Immunoblotting

Proteins from SDS-PAGE or 2-D PAGE were transferred onto nitrocellulose as described previously (Towbin et al., 1979). Membranes were stained with Ponceau Red, saturated for 1 hour with TBS (Tris buffered saline: 20 mM Tris, pH 7.5, 136.8 mM NaCl) containing 5% low-fat milk, and incubated overnight with the primary antibody diluted in TBS containing 0.1% Tween-20. After washing in TBS containing 0.2% Tween-20, one-dimensional blots were incubated for 2 hours with 3.7 kBq/ml ^{125}I-labeled anti-mouse or anti-rabbit secondary antibodies (Amersham, 28-111 TBq/mmol) for quantification. Blots were then washed, dried and submitted to autoradiography for 16 to 72 hours. After autoradiography, the immunoreactive bands were cut off the nitrocellulose sheets and processed for liquid scintillation counting. 2-D blots were incubated with peroxidase-labeled anti-mouse IgG secondary antibodies and detection was carried out using the enhanced chemiluminescent method (ECL, Amersham). Successive incubations of the blots with different primary antibodies were performed by rapidly washing the blot in PBS between two incubations. This method enables the relative positions of the different immunoreactive spots to be located very precisely.

RESULTS

Glutamylated α- and β-tubulin accumulate during mouse brain development and neuronal differentiation in culture

The relative abundance of glutamylated tubulin was determined at different stages of post-natal brain development. Supernatant fractions of brain protein extracts were submitted to SDS-PAGE and immunoprobed with a general antibody directed against α-tubulin (DM1A) or with a specific antibody recognizing the glutamylated forms of both α- and β-tubulin (GT335). The abundance of α-tubulin, relative to the soluble protein content, increased during the early stages of develop-

ment, reaching a maximal value at day 7, and decreased thereafter to a level ~2-fold lower than the initial value (Fig. 1a). On the other hand, glutamylated α- and β-tubulin accumulated continuously throughout postnatal development, reaching at one month a level ~2.5-fold higher than the initial value (Fig. 1b).

Changes in glutamylated tubulin, as well as in acetylated α-tubulin and NTT, were also analyzed in mouse brain neurons developing in culture for 2, 8, 16, 21, and 28 days. After homogenization at 4°C, supernatant (Fig. 2) and pellet fractions (Fig. 3a) were analyzed by SDS-PAGE and immunoblotting using, in addition to the antibodies described above, a general antibody against β-tubulin and specific antibodies for acetylated α-tubulin and NTT. Fig. 2 shows that the amount of α- and β-tubulin in the supernatant fraction increased about 2-fold during the first week in culture, was maintained at a high level during the second week and decreased markedly afterwards to reach at day 28 a level similar to that observed at day 2. The amount of glutamylated α-tubulin also increased ~2-fold during the first week but was then maintained at a high level until day 28, whereas glutamylated β-tubulin accumulated progressively during the entire period of culture. The developmental expression of NTT was similar to that of glutamylated α-tubulin but that of acetylated α-tubulin was quite different; in particular, after a marked increase during the first week of culture, the abundance of acetylated α-tubulin decreased progressively to reach at day 28 a very low level, similar to that observed at day 2 (Fig. 2).

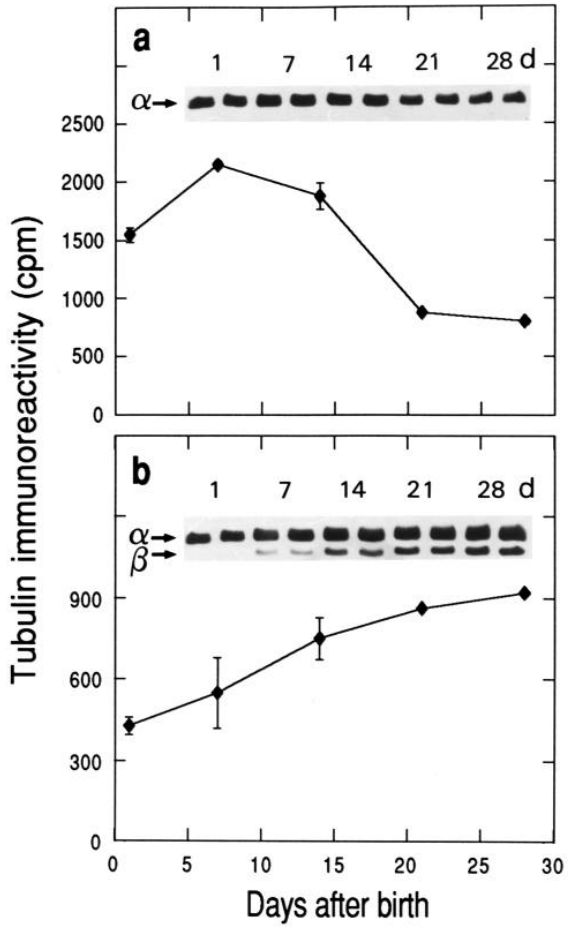

Fig. 1. Accumulation of glutamylated tubulin during mouse brain development. Supernatant fractions of brain protein extracts, prepared from 1-, 7-, 14-, 21-, and 28-day-old mice were submitted to SDS-PAGE, transferred to nitrocellulose and incubated with DM1A (a) or GT.335 (b). Insets show the tubulin region of the autoradiograph of nitrocellulose membrane after incubation with ^{125}I-labeled anti-mouse IgG antibodies. No other labeled protein was detected. Each sample was analyzed in duplicate (1.5 and 3 μg/lane of the soluble protein extracts were loaded on (a) and (b), respectively). The immunoreactive bands were excised from the membranes and processed for liquid scintillation counting. The results are expressed as cpm/μg of total proteins present in each sample and are means ± sd (n=2).

Neuronal differentiation in culture was accompanied by a progressive increase in the tubulin content in the pellet fraction (Fig. 3a). This was observed for all the tubulin species analyzed but was much more pronounced for acetylated α-tubulin and NTT. For instance, at day 28, the relative proportion of these forms in the pellet fraction reached ~60% for acetylated α-tubulin and ~40% for NTT, whereas that of whole and glutamylated tubulin did not exceed 20-30%. This experiment shows that acetylated α-tubulin is particularly enriched in cold-insoluble material, which agrees with previous observations of Sale et al. (1988). To take into account the contribution of the pellet fraction we analyzed whole cell homogenates (Fig. 3b). The data obtained were very similar to those described above for the supernatant fraction, except that the decrease of acetylated α-tubulin during the late phases of the culture was much less pronounced.

Stabilization of the α-tubulin glutamylation/deglutamylation reactions during neuronal differentiation

A question arising from these results is whether the increase in the relative abundance of glutamylated tubulin is related to an increase in the rate of glutamylation and/or to a decrease in the rate of deglutamylation. The former possibility was tested by measuring the posttranslational incorporation of [^{3}H]glutamate into tubulin at various times in culture. At each time point, equal amounts of whole protein extracts were analyzed by SDS-PAGE followed by fluorography (Fig. 4a). Three radioactive bands were detected, two bands comigrating with α- and β-tubulin, and a third one migrating at the front of migration (M_r <14 kDa), the nature of which is not yet known. Quantification of the radioactivity associated with the α-tubulin band revealed that glutamate incorporation changed with time in culture: it increased in the early stages in culture, reaching a maximal level at 4 days, and then progressively decreased until day 21 (Fig. 4b). Concerning β-tubulin, quantification was not possible because of its low level of radioactivity, but examination of the fluorograph (Fig. 4a) reveals a change similar to that of α-tubulin. Unexpectedly, these results suggest that the rate of glutamylation decreases during a period in which the abundance of glutamylated tubulin increases and reaches its maximal level (compare with Fig. 2).

The parameters of the deglutamylation reaction were determined by performing pulse-chase experiments in which neurons were first incubated with [^{3}H]glutamate and then allowed to chase for increasing times in a non-radioactive medium. Previous data, obtained with 6-day neurons, showed that the labeling of α-tubulin was progressively lost during the period of chase with biphasic kinetics, indicating the existence

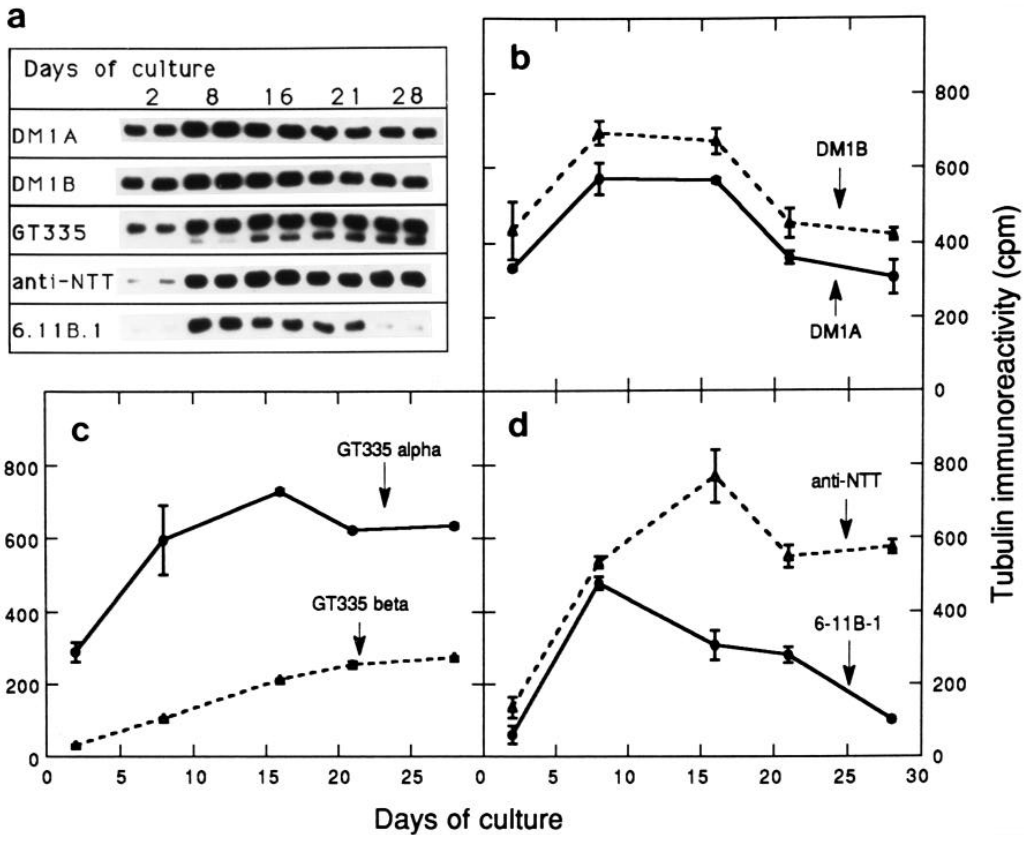

Fig. 2. Changes in glutamylated, acetylated and non-tyrosinatable tubulin during neuronal differentiation in culture. Embryonic mouse brain neurons were cultured for 2, 8, 12, 16, 21 and 28 days. At each time point, supernatant fractions (3 μg proteins/lane) were submitted to SDS-PAGE and immunoprobed with DM1A, DM1B, GT335, 6-11B-1, and anti-NTT. (a) Tubulin regions of the corresponding autoradiographs. No other protein was detected. (b-d) Quantitative changes were determined as described in the legend of Fig. 1. Data are expressed as cpm/μg of soluble proteins loaded on the gels and are means ± sd (*n*=2-4).

of two populations decaying at different rates: the major population (accounting for 65% of the total population) lost its radioactivity with a half-life of ~1 hour and the remaining 35% decayed with a half-life of ~19 hours. Evidence was obtained that this decay was related to the occurrence of a two-rate component of the deglutamylation reaction (Audebert et al., 1993). The results obtained here with 15-day neurons also show a biphasic kinetics for the deglutamylation of α-tubulin (Fig. 5). However, the major population (57%) decayed with a half-life of 2 hours (instead of 1 hour) and the minor one (43%) with a half-life of 20-22 hours (instead of 19 hours). Thus, a 2-fold decrease in the initial rate of deglutamylation of

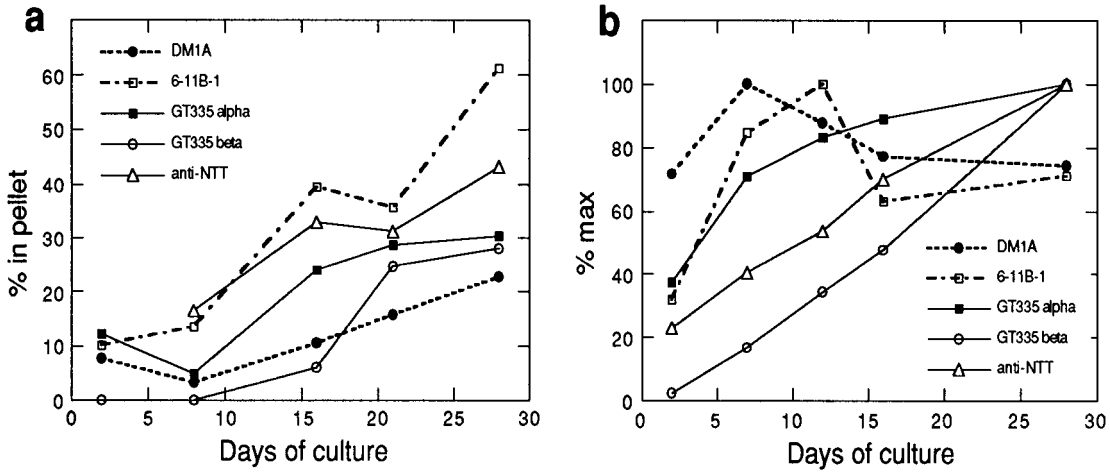

Fig. 3. (a) Accumulation of tubulin in the pellet fraction during neuronal differentiation in culture. The relative proportion of each tubulin species in the pellet fraction was measured as follows: at each time point, the pellet was dissolved in a volume equal to that of the supernatant fraction. Equal volumes of supernatant and pellet fraction were submitted to SDS-PAGE and immunoprobed with the diverse antibodies (not shown). The results are expressed as the percentage of cpm detected for each tubulin species in the pellet fraction relative to the sum of cpm detected in the pellet plus supernatant fractions. (b) Changes in tubulin levels in whole cell homogenates. Data are expressed as the percentage of the maximum value obtained with each antibody. Identical amounts of whole cells homogenates (3 μg/lane) were immunoprobed with the diverses antibodies (autoradiographs not shown).

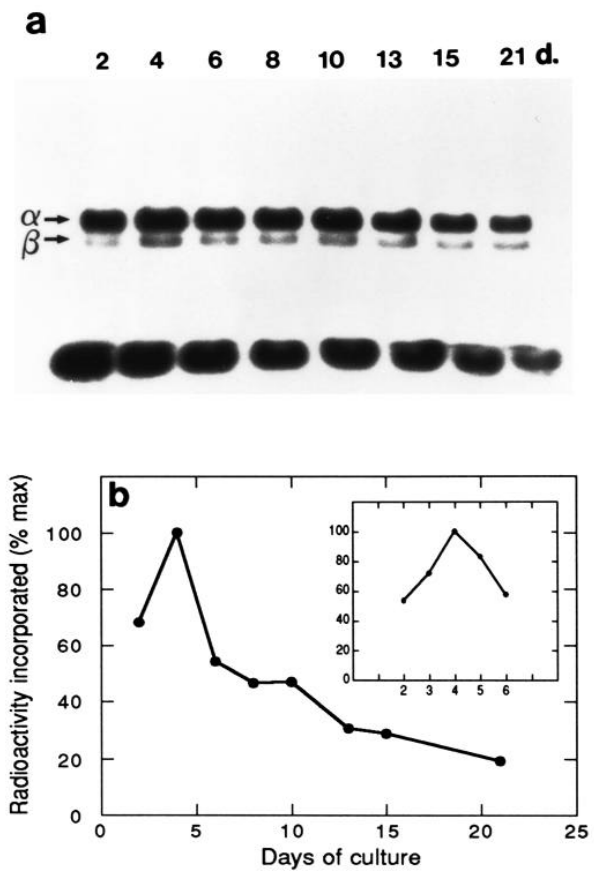

Fig. 4. Posttranslational incorporation of [^{3}H]glutamate into tubulin. Neurons were allowed to develop in culture for the times indicated and then incubated with [^{3}H]glutamate for 2 hours, in the presence of cycloheximide. Identical amounts (25 μg) of whole protein extracts were analyzed by SDS-PAGE. (a) Fluorograph of the Coomassie Blue-stained gel. (b) Quantification of the radioactivity incorporated into α-tubulin was determined as described in Materials and Methods and is expressed as the percentage of the maximal value (obtained at day 4: 3600 cpm). (Inset) to confirm the presence of a peak at day 4, the experiment was repeated in the day 2 to 6 range.

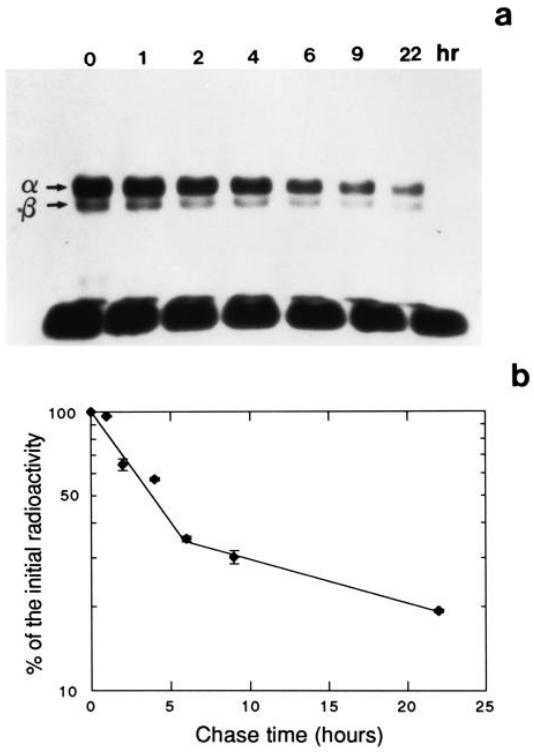

Fig. 5. Deglutamylation of tubulin in day 15 neurons. Day 15 neurons were incubated with [^{3}H]glutamate for 2 hours, in the presence of cycloheximide and then allowed to chase for the times indicated in a non-radioactive culture medium. Identical amounts (40 μg) of whole protein extracts were analyzed by SDS-PAGE. (a) Fluorograph of the Coomassie Blue-stained gel. (b) The radioactivity associated with α-tubulin was determined and plotted as a function of chase time. The results are expressed as the percentage of the initial radioactivity and are means ± sd of two determinations.

α-tubulin occurs between day 6 and day 15, which could balance the decrease in the rate of glutamylation observed above and could explain, at least in part, the accumulation of this tubulin species occurring during this period. Concerning β-tubulin, the fluorograph shown in Fig. 5a reveals a progressive decrease in the radioactivity associated with the corresponding band, indicating that deglutamylation of β-tubulin also occurred. However, because of the low level of radioactivity, it was not possible to determine precisely the parameters of this reaction.

The length of the β-tubulin polyglutamyl chain increases markedly during neuronal differentiation

We have shown previously that the diverse tubulin isoelectric variants separated by 2-D PAGE correspond mainly to glutamylated forms differing by their degree of glutamylation, i.e. the number of glutamyl units present in their lateral polyglutamyl chain (Eddé et al., 1990, 1992). To determine whether the accumulation of glutamylated tubulin occurring during neuronal differentiation is accompanied by a change in the degree of glutamylation, 2-D blots of whole protein extracts were immunodetected first with GT335 (Fig. 6, left column) and then with DM1A plus DM1B (Fig. 6, middle column). Several stretches of isoforms belonging to α-, β′- and β-tubulin were observed. Individual spots were resolved better at shorter exposures and were numbered accordingly.

The heterogeneity of glutamylated α-tubulin, as detected with GT335, was already very high at 2 days in culture (forms α2-α8) and remained the same at the different stages analyzed except that a more basic variant (α1) was detected from day 14 onwards. Since this form was also detected by anti-NTT (not shown), it likely corresponds to monoglutamylated NTT, in which the acidic shift provoked by the first added glutamyl unit is balanced by a basic shift due to the absence of the subterminal residue Glu # 450. When the blots were re-incubated with DM1A, an additional variant (denoted α1* and indicated by a large arrow) was detected. α1* also migrated at the α1 position but can be distinguished from the monoglutamylated NTT form by a slightly higher position in the 2-D gels. α1* was abundant at day 2, progressively decreased during differentiation, and became undetectable from day 21 onwards. Since this variant was detected only by DM1A it is very likely that it corresponds to the unmodified isotype(s) previously shown to migrate at the same position (Denoulet et al., 1988).

In contrast to α-tubulin, the heterogeneity of β-tubulin increased markedly during differentiation. In our 2-D gels, this subunit is resolved into two series of isoelectric variants differing slightly by their apparent M_r. The minor series, denoted β′, corresponds to the neuron-specific class III β-tubulin and the major one to the expression of other β-tubulin isogenes. The reactivity of the minor β′-tubulin series with GT335 was compared to that observed with anti-class III β-tubulin (SDL.3D10) (Fig. 6, left and right columns). Only two variants (β1′ and β2′) were detected at day 2 by SDL.3D10, neither of them reacting with GT335. We have shown previously that β1′ corresponds to the primary translational product of the corresponding isogene, and β2′ to its phosphorylated derivative (Denoulet et al., 1988; Eddé et al., 1989). During differentiation, more acidic β′-tubulin forms appeared and accumulated to the detriment of β1′ and β2′. These additional

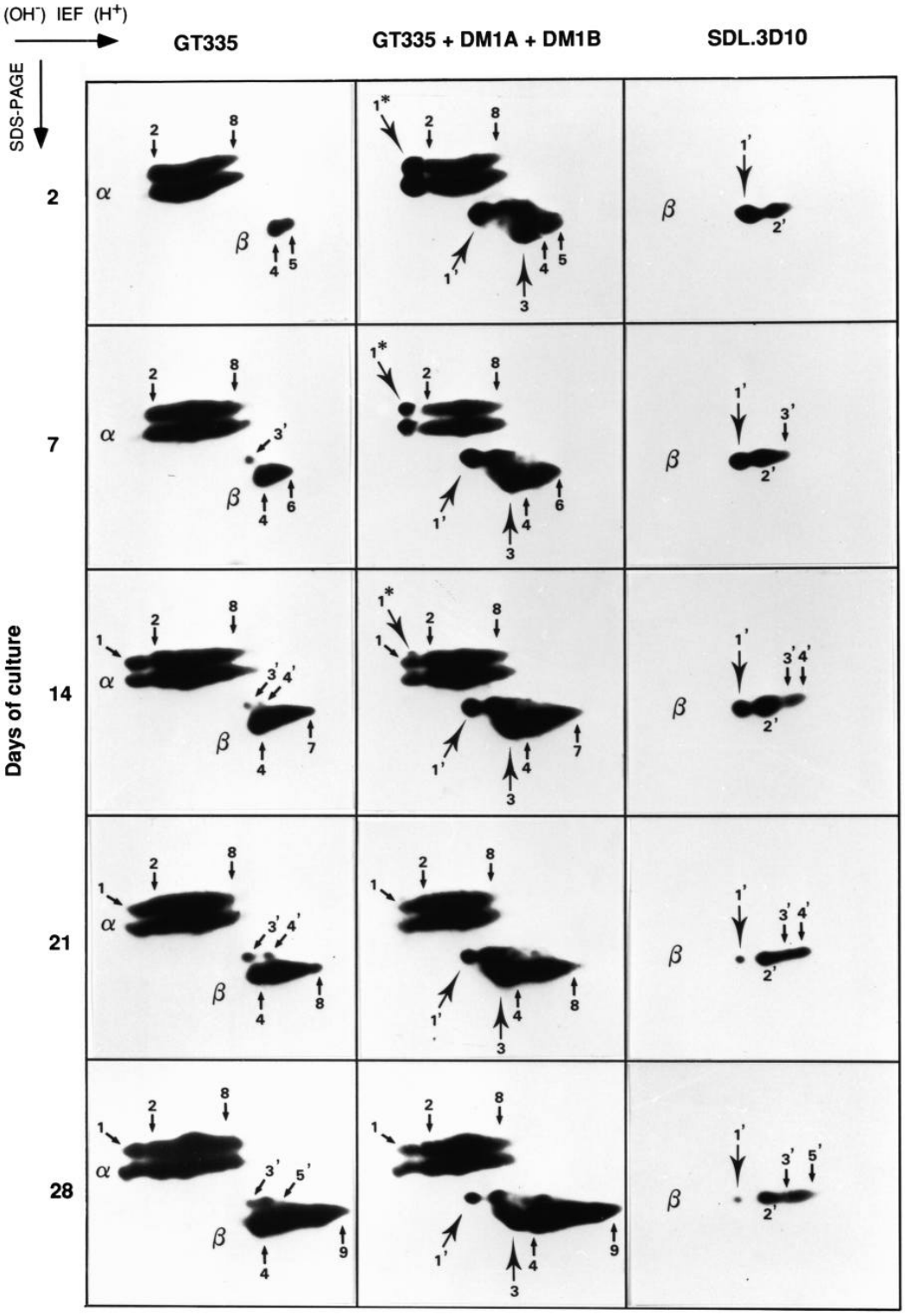

Fig. 6. 2-D PAGE analysis of polyglutamylated α- and β-tubulin. Whole protein extracts, from neurons cultured for 2, 7, 14, 21 or 28 days, were separated by 2-D PAGE and immunodetected with GT335 (left panels). Glutamylated α-, β-, and β′-tubulin isoforms are indicated by small arrows. After washing, the same blots were re-incubated successively with DM1A and DM1B to reveal additional non-glutamylated forms of α- and β-tubulin (middle panels). These forms, namely the unmodified isotypes α1*, β3 and β1′, are indicated by large arrows. No attempt was made to distinguish other β′-tubulin isoforms because they were poorly resolved from the major β-tubulin ones. Instead, duplicate blots were incubated in parallel with SDL.3D10, a monoclonal antibody directed against the neuron-specific class III β′-tubulin (right panels). In addition to the unmodified β1′ isotype and its phosphorylated derivative β2′, this antibody stained more acidic isoforms also recognized by GT335. It should be noted that α-tubulin migrated as two stretches differing slightly by their apparent M_r. To our knowledge, the molecular basis of this phenomenon has not yet been explained. We refer here to the isoelectric variants independently of their migration in the second dimension. Only tubulin regions are shown.

spots were labeled by both GT335 and SDL.3D10, which indicates that they correspond to glutamylated forms of class III β′-tubulin. For the major β-tubulin series, a marked increase in the number of isoforms recognized by GT335 was observed throughout differentiation. Only two glutamylated isoforms (β4, β5) were detected at day 2 but additional more acidic forms (β6 to β9) appeared and accumulated successively until day 28 of culture. Incubation with DM1B revealed an additional more basic unglutamylated form (β3), previously shown to correspond to the major class II β-tubulin primary translational product (Denoulet et al., 1988), which seems to slightly decrease during time in culture. These results indicate that an increase in the degree of glutamylation of β- and β′-tubulin occurs progressively during neuronal differentiation.

Effect of microtubule depolymerizing drugs

The above results revealed qualitative and quantitative differences in the glutamylation of α- and β-tubulin subunits. In the following experiments, we compared the behavior of the two subunits with regard to the effect of microtubule depolymerizing drugs.

Neurons (21-day) were incubated with nocodazole. Under these conditions, the glutamylation of both α- and β-tubulin was almost completely inhibited, as judged by posttranslational incorporation of radioactive glutamate (Fig. 7a). This inhibition was effective despite the fact that the vast majority (~80%) of tubulin remained associated with the cytoskeletal fraction, even after a 4 hour exposure to the drug (Fig. 7b). Indeed, incubation with nocodazole led to only a slight transfer of whole as well as of glutamylated α- and β-tubulin from the cytoskeletal to the soluble fraction. This contrasts with the dramatic effect of nocodazole on the degree of glutamylation of α-tubulin. Fig. 8 shows 2-D blots of cytoskeletal and soluble fractions of 21-day neurons exposed or not to nocodazole. In control cells, glutamylated α- and β-tubulin present in the cytoskeletal fraction exhibits a complex 2-D pattern similar to that observed in total cell extracts (compare with Fig. 6). In the soluble fraction, the heterogeneity of these tubulin species was much lower, consisting of only 3 α- and 3 β isoforms. These results reveal an asymmetrical partition of glutamylated tubulin isoforms depending on their degree of glutamylation. Upon nocodazole treatment, the 2-D heterogeneity of glutamylated α-tubulin in the cytoskeletal fraction was strongly reduced, the more glutamylated forms α5-α8 becoming undetectable. This disappearance is unlikely to be due to a selective degradation of these forms since the total level of tubulin was unchanged during nocodazole treatment (Fig. 7b). Instead, it must reflect the occurrence of a deglutamylation reaction, which became preponderant because of the inhibition of the glutamylation reaction. In contrast to α-tubulin, β-tubulin heterogeneity remained unchanged during nocodazole treatment suggesting that deglutamylation of this subunit, if any, occurs very slowly.

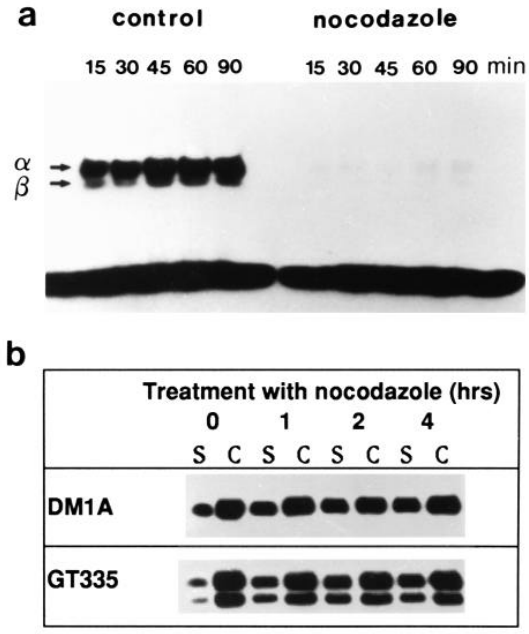

Fig. 7. Effect of nocodazole on the glutamylation and the partitioning of glutamylated tubulin. (a) Day 21 neurons were incubated with [^{3}H]glutamate, in the presence of cycloheximide, for the time indicated. Nocodazole or DMSO (control) was added 1 hour prior to the beginning of labeling. Identical amounts (40 μg) of whole protein extracts were submitted to SDS-PAGE and labeled proteins were detected by fluorography. (b) Day 21 neurons were incubated with nocodazole for the time indicated. Soluble (S) and cytoskeletal (C) fractions were prepared as described in Materials and Methods and adjusted to the same volume. Identical volumes of each fraction were submitted to SDS-PAGE and immunoprobed with DM1A or GT335. Only tubulin regions of the blots are presented because no other proteins were detected.

DISCUSSION

In this report, we analyze the developmental expression of posttranslationally modified forms of tubulin, and particularly

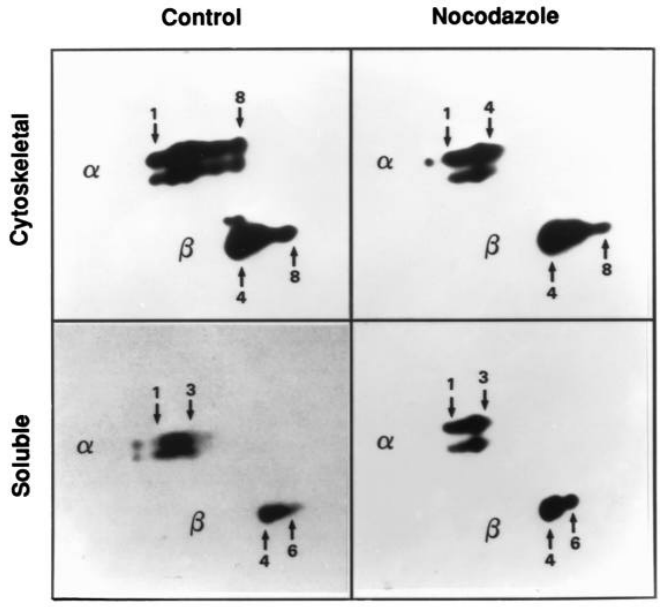

Fig. 8. Effect of nocodazole on the degree of glutamylation of tubulin. Day 21 neurons were incubated for 4 hours with nocodazole or DMSO (control). Soluble and cytoskeletal fractions were submitted to 2-D PAGE and immunodetected with GT335. Only tubulin regions are shown.

that of glutamylated α- and β-tubulin, during neuronal differentiation. Going from the observation that glutamylated tubulin markedly increased during brain development in vivo, we have focused our study on a cellular model of neuronal differentiation. Cultures of embryonic mouse brain cells were previously shown to be composed of >90% neuronal cells, which differentiate rather synchronously and acquire many properties characteristic of mature neurons, such as the ability to generate and propagate action potentials and to transmit information through synapses (Berwald-Netter et al., 1981; Chelly et al., 1990). Because little, if any, cell death occurs within up to one month in culture, this model is particularly useful to study the late phases of neuronal differentiation. The validity of this model is further supported by observations that the increase in tubulin heterogeneity occurs with a time course remarkably similar to that observed during brain development in vivo (Denoulet et al., 1988).

Changes in tubulin levels during neuronal differentiation in culture were measured by analyzing whole cell extracts, as well as supernatant and pellet fractions, by quantitative immunoblotting. The ~2-fold increase in whole tubulin levels observed during the first week in culture is obviously related to the continuous need of microtubules underlying the formation of the dense axodendritic network. The subsequent decrease in the overall tubulin level could be due to a decrease in the density of microtubules accompanying the appearance of neurofilaments in the stabilized processes (Hoffman and Cleveland, 1988) and/or to the disappearance of some processes during neuronal maturation. Initial phases of neuronal differentiation are also accompanied by a marked accumulation of all the posttranslationally modified forms tested. Glutamylated α- and β-tubulin and NTT continue to accumulate as differentiation proceeds but acetylated α-tubulin reaches its maximal level at about day 12 and decreases thereafter. In addition, acetylated α-tubulin becomes largely associated with cold-insoluble microtubules in the pellet fraction as opposed to glutamylated α- and β-tubulin, which remain largely in a cold-soluble form. Thus, the developmental patterns of the different PTMs are not identical.

Enrichment of glutamylated tubulin was confirmed by 2-D PAGE, which distinguishes unmodified isotypes from their glutamylated derivatives, the latter migrating generally at more acidic positions. Unmodified forms α1*, β1′ and β3 decrease markedly during differentiation: α1* and β1′ become almost undetectable from day 21 onwards whereas β3 remains abundant up to day 28. This result indicates that almost all α- and β′-tubulin, but not all β-tubulin, become posttranslationally modified during neuronal differentiation. The disappearance of α1* together with the fact that the vast majority of acetylated α-tubulin and NTT is under a glutamylated form (Eddé et al., 1991; Paturle-Lafanechère et al., 1991) indicate that, in mature neurons, almost all α-tubulin is polyglutamylated. These results also indicate that β-tubulin is much less glutamylated than α-tubulin, which is also supported by posttranslational labeling with [^{3}H]glutamate and immunodetection with GT335.

Even more striking is the difference in the degree of glutamylation of α- and β-tubulin. The heterogeneity of α-tubulin is already very high in young neurons and does not change during differentiation, except the slight basic shift due to the accumulation of NTT. On the contrary, the degree of glutamylation of β-tubulin is very low in young neurons and increases progressively throughout differentiation to reach the length of 6 units found in adult brain (Redeker et al., 1992; Rüdiger et al., 1992). In addition, the more acidic variants, i.e. those bearing the highest number of glutamyl units, are always the least abundant. A similar evolution is observed for class III β′-tubulin, although the highest number of glutamyl units found at day 28 (3: β3′-β5′) is lower than that found in adult brain (Alexander et al., 1991). Except for class III β′-tubulin, the changes observed during neuronal differentiation in culture were very similar to that found in brain during development (not shown). These quantitative and qualitative differences cannot be explained by a differential availability of glutamylatable α- and β-tubulin isotypes. For instance, staining of class III β′-tubulin with SDL.3D10 is already high at day 2 but limited to the unmodified isotype β′1 and its phosphorylated derivative β′2. Class II β-tubulin, the major brain isotype migrating at the β3 position (Denoulet et al., 1988), is very abundant but weakly glutamylated in 2-day neurons. The alternative hypothesis that glutamylation is catalyzed by several isotype-specific isoenzymes is consistent with the sequence divergence around the sites of glutamylation: GEGEEEGEE(Y) for mα1 and mα2, EQGEFEEEE… for class II mβ2, and GEMYEDDDEE… for class III mβ6 (β′-tubulin) (Eddé et al., 1990; Alexander et al., 1991; Redeker et al., 1992; Rüdiger et al., 1992). It is striking that at least in the first two sequences a relatively high number of E residues are present in the close vicinity of the glutamylated residue (underlined). As none of these neighboring E residues were found to be glutamylated, a strong selectivity towards the single site of glutamylation is expected. It is very unlikely that a single enzyme could have such a degree of selectivity for different isotypes. It thus appears that the marked differences between polyglutamylation of α- and β-tubulin are likely due to differential regulation of the isoenzymes involved.

Surprisingly, enrichment of glutamylated tubulin during differentiation in culture was accompanied by a decrease in the posttranslational incorporation of radioactive glutamate into both subunits, suggesting that their rate of glutamylation was reduced. In this case, accumulation of glutamylated tubulin could be explained by a greater decrease in the rate of deglutamylation. We previously demonstrated the occurrence of a complex reverse deglutamylation of α-tubulin in 6-day neurons. The rate of this reaction was shown to be dependent on the length of the polyglutamyl chain, higher rates being observed for the removal of the distal units from longer chains (4-6 units) compared to shorter ones (1-3 units) (Audebert et al., 1993). The biphasic kinetics of decay, observed here with 15-day neurons, illustrates this phenomenon but the higher rate of deglutamylation of α-tubulin is ~2-fold lower in 15 day- than in 6 day-neurons. These findings indicate that the turnover of glutamyl units onto α-tubulin slows down as neuronal differentiation proceeds. Although it was difficult to get quantitative information about the deglutamylation kinetics of β-tubulin, incubation of cultured neurons with nocodazole almost completely inhibits the glutamylation reaction and, thus, provides an indirect way for analyzing the process of deglutamylation. Under these conditions, the heterogeneity of α-tubulin is strongly reduced, indicating that the longer polyglutamyl chains (4-6 units) were rapidly shortened to 3 units whereas the shorter chains (1-3 units) remained relatively

resistant. By opposition, the heterogeneity of β-tubulin remains unchanged during nocodazole treatment indicating that, in this case, short and long chains exhibit a similar resistance to deglutamylation. Since deglutamylation of β-tubulin is clearly observed by pulse chase experiments, it is likely that this reaction proceeds at a low and uniform rate.

Taken altogether, these results indicate that polyglutamylation and deglutamylation of α- and β-tubulin are catalyzed by distinct isoenzymes that exhibit some differences in their catalytic properties and are differentially regulated during neuronal differentiation.

The functions of tubulin PTMs remain largely unknown. Like acetylated α-tubulin (L'Hernault and Rosenbaum, 1985; Piperno and Fuller, 1985; Cambray-Deakin and Burgoyne, 1987; Black and Keyser, 1987; Sale et al., 1988; Black et al., 1989; Eddé et al., 1991) and NTT (Paturle-Lafanechère et al., 1994), glutamylated tubulin is preponderant in nerve cells but also in other stable structures such as axonemes and centrioles (Fouquet et al., 1994). Accumulation of tubulin PTMs during neuronal differentiation occurs concomitantly with the increased microtubule stability accompanying this process (see for review, Solomon, 1992). Functionally, this could be related to the transition from a marked flexibility of the growing neurites, which reorganize frequently in young neurons, to the progressive formation of long and stabilized neurites. The cytoskeleton of mature neurons is characterized by the presence of a dense network of microtubules cross linked with each other or with neurofilaments. This network supports the transport of organelles and various cellular components along very long distances. It is obvious that various microtubule associated proteins, including motor and structural proteins, are involved in these functions. Neuronal differentiation is also accompanied by the accumulation of microtubule-associated proteins, including MAP1, MAP2 and Tau (Drubin et al., 1985; Black et al., 1986; Larcher et al., 1992). Provoked expression of Tau in non-neuronal cells has been found to increase microtubule assembly and stability (Drubin and Kirschner, 1986), to bundle microtubules (Kanai et al., 1989) and, more strikingly, to provoke axon-like extensions (Knops et al., 1991; Baas et al., 1991). These observations indicate that Tau, and probably other structural MAPs, have a direct influence on microtubule stability and on the organization of the cytoskeletal network. How this function is regulated by PTMs of tubulin, and in particular polyglutamylation, is under investigation.

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REFERENCES

Alexander, J. E., Hunt, D. F., Lee, M. K., Shabanowitz, J., Michel, H., Berlin, S. C., Macdonald, T. L., Sundberg, R. J., Rebhun, L. I. and Frankfurter, A. (1991). Characterization of posttranslational modifications in neuron-specific class III β-tubulin by mass spectrometry. *Proc. Nat. Acad. Sci. USA* **88**, 4685-4689.

Audebert, S., Desbruyères, E., Gruszczynski, C., Koulakoff, A., Gros, F., Denoulet, P. and Eddé, B. (1993). Reversible polyglutamylation of α and β-tubulin and microtubule dynamics in mouse brain neurons. *Mol. Biol. Cell* **4**, 615-626.

Baas, P. W., Pienkowski, T. P. and Kosik, K. S. (1991). Processes induced by tau expression in SF9 cells have an axon-like microtubule organization. *J. Cell Biol.* **115**, 1333-1344.

Banerjee, A., Roach, M. C., Trcka, P. and Luduena, R. (1990). Increased microtubule assembly in bovine brain tubulin lacking the type III isotype of β-tubulin. *J. Biol. Chem.* **265**, 1794-1799.

Berwald-Netter, Y., Martin-Moutot, N., Koulakoff, A. and Couraud, F. (1981). Na^+-channel-associated scorpion toxin receptor sites as probes for neuronal evolution in vivo and in vitro. *Proc. Nat. Acad. Sci. USA* **78**, 1245-1249.

Black, M. M., Aletta, J. M. and Greene, L. A. (1986). Regulation of microtubule composition and stability during nerve growth factor-promoted neurite outgrowth. *J. Cell Biol.* **103**, 545-557.

Black, M. M. and Keyser, P. (1987). Acetylation of α-tubulin in cultured neurons and the induction of α-tubulin acetylation in PC-12 cells by treatment with nerve growth factor. *J. Neurosci.* **7**, 1833-1842.

Black, M. M., Baas, P. W. and Humphries, S. (1989). Dynamics of α-tubulin acetylation in intact neurons. *J. Neurosci.* **9**, 358-368.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgramm quantities of protein utilising the principle of protein dye binding. *Anal. Biochem.* **72**, 248-254.

Cambray-Deakin, M. A. and Burgoyne, R. D. (1987). Posttranslational modifications of α-tubulin: acetylated and detyrosinated forms in axons of rat cerebellum. *J. Cell Biol.* **104**, 1569-1574.

Chelly, J., Hamard, G., Koulakoff, A., Kaplan, J. C., Kahn, A. and Berwald-Netter, Y. (1990). Distrophin gene transcribed from different promoters in neuronal and glial cells. *Nature* **344**, 64-65.

Denoulet, P., Eddé, B., Jeantet, C. and Gros, F. (1982). Evolution of tubulin heterogeneity during mouse brain development. *Biochimie* **64**, 165-172.

Denoulet, P., Eddé, B., Henrique, D. P., Koulakoff, A., Berwald-Netter, Y. and Gros, F. (1988). The increase of tubulin heterogeneity during differentiation of neurons in primary culture is controlled mainly at the posttranslational level. In *Structure and Function of the Cytoskeleton* (ed. B.A. Rousset), pp. 231-238. John Libbey, London-Paris.

Drubin, D. G., Feinstein, S. C., Shooter, E. M. and Kirschner, M. W. (1985). Nerve growth factor-induced neurite outgrowth in PC12 cells involves the coordinate induction of microtubule assembly and assembly promoting factors. *J. Cell Biol.* **101**, 1799-1807.

Drubin, D. G. and Kirschner, M. W. (1986). Tau protein function in living cells. *J. Cell Biol.* **103**, 2739-2746.

Eddé, B., Jakob, H. and Darmon, M. (1983). Two specific markers for neural differentiation of embryonal carcinoma cells. *EMBO J.* **2**, 1473-1478.

Eddé, B., de Néchaud, B., Denoulet, P. and Gros, F. (1987). Control of isotubulin expression during neuronal differentiation of mouse neuroblastoma and teratocarcinoma cell lines. *Dev. Biol.* **123**, 549-558.

Eddé, B., Denoulet, P., de Néchaud, B., Koulakoff, A., Berwald-Netter, Y. and Gros, F. (1989). Posttranslational modifications of tubulin in cultured mouse brain neurons. *Biol. Cell* **65**, 109-117.

Eddé, B., Rossier, J., Le Caer, J. P, Desbruyères, E., Gros, F. and Denoulet, P. (1990). Posttranslational glutamylation of α-tubulin. *Science* **247**, 83-85.

Eddé, B., Rossier, J., Le Caer, J. P., Berwald-Netter, Y., Koulakoff, A., Gros, F. and Denoulet, P. (1991). A combination of posttranslational modifications is responsible for the production of neuronal α-tubulin heterogeneity. *J. Cell. Biochem.* **46**, 134-142.

Eddé, B., Rossier, J., Le Caer, J. P., Promé, J. C., Desbruyères, E., Gros, F. and Denoulet, P. (1992). Polyglutamylated α-tubulin can enter the tyrosination/detyrosination cycle. *Biochemistry* **31**, 403-410.

Field, J. F. and Lee, J. C. (1988). Analysis of tubulin proteins and peptides in neuronal and non-neuronal tissues using immobilized pH gradients. *Electrophoresis* **9**, 555-562.

Fouquet, J. P., Eddé, B., Kann, M. L., Wolff, A., Desbruyères, E. and Denoulet, P. (1994). Differential distribution of glutamylated tubulin during spermatogenesis in mammalian testis. *Cell Motil. Cytoskel.* **27**, 49-58.

Gard, D. L. and Kirschner, M. W. (1985). Polymer-dependent increase in phosphorylation of β-tubulin accompanies differentiation of a mouse neuroblastoma cell Line. *J. Cell Biol.* **100**, 764-774.

George, H. J., Misra, L., Fields, D. J. and Lee, J. C. (1981). Polymorphism of brain tubulin. *Biochemistry* **20**, 2402-2409.

Gozes, I. and Sweadner, K. J. (1981). Multiple tubulin forms are expressed by a single neurone. *Nature* **294**, 477-480.

Hoffman, P. N. and Cleveland, D. W. (1988). Neurofilament and tubulin expression recapitulates the developmental program during axonal

regeneration: induction of a specific β-tubulin isotype. *Proc. Nat. Acad. Sci. USA* **85**, 4530-4533.

Joshi, H. C. and Cleveland, D. W. (1989). Differential utilization of β-tubulin isotypes in differentiating neurites. *J. Cell Biol.* **109**, 663-673.

Joshi, H. C. and Cleveland, D. W. (1990). Diversity among tubulin subunits: toward what functional end? *Cell Motil. Cytoskel.* **16**, 159-163.

Kanai, Y., Takemura, R., Oshima, T., Ihara, Y., Yanagisawa, M., Masaki, T. and Hirokawa, N. (1989). Expression of multiple tau isoforms and microtubule bundle formation in fibroblasts transfected with a single tau cDNA. *J. Cell Biol.* **109**, 1173-1184.

Knops, J., Kosik, K. S., Lee, G., Pardee, J. D., Cohen-Gould, L. and McConlogue, L. (1991). Overexpression of tau in non-neuronal cells induces long cellular processes. *J. Cell Biol.* **114**, 725-734.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Larcher, J. C., Boucher, D., Ginzburg, I., Gros, F. and Denoulet, P. (1992). Heterogeneity of tau proteins during mouse brain development and differentiation of cultured neurons. *Dev. Biol.* **154,** 195-204.

Lee, M. K., Rebhun, L. I. and Frankfurter, A. (1990). Posttranslational modification of class III β-tubulin. *Proc. Nat. Acad. Sci. USA* **87**, 7195-7199.

Lewis, S. A., Lee, M. G. S. and Cowan, N. J. (1985). Five mouse tubulin isotypes and their regulated expression during development. *J. Cell Biol.* **101**, 852-861.

L'Hernault, S. W. and Rosenbaum, J. L. (1985). Chlamydomonas α-tubulin Is post-translationally modified by acetylation on the ∑-amino group of a lysine. *Biochemistry* **24**, 473-478.

Lim, S. S., Sammak, P. J. and Borisy, G. G. (1989). Progressive and spatially differentiated stability of microtubules in developing neuronal cells. *J. Cell Biol.* **109**, 253-263.

Luduena, R. F. (1993). Are tubulin isotypes functionally significant. *Mol. Biol. Cell* **4**, 445-457.

Miller, F. D., Naus, C. C. G., Durand, M., Bloom, F. E. and Milner, R. J. (1987) Isotypes of α-tubulin are differentially regulated during neuronal maturation. *J. Cell Biol.* **105**, 3065-3073.

Moura-Neto, V., Mallat, M., Jeantet, C. and Prochiantz, A. (1983). Microheterogeneity of tubulin proteins in neuronal and glial cells from the mouse brain in culture. *EMBO J.* **2**, 1243-1248.

Murphy, D. B. (1991). Functions of tubulin isoforms. *Curr. Opin. Cell Biol.* **3**, 43-51.

Paturle, L., Wehland, J., Margolis, R. L., and Job, D. (1989) Complete separation of tyrosinated, detyrosinated, and nontyrosynatable brain tubulin subpopulations using affinity chromatography. *Biochemistry* **28**, 2698-2704.

Paturle-Lafenechère, L., Eddé, B., Denoulet, P., Dorsselaer, A. V., Mazarguil, H., Caer, J. P. L., Wehland, J. and Job, D. (1991). Characterization of a major brain tubulin variant which cannot be tyrosinated. *Biochemistry* **30**, 10523-10528.

Paturle-Lafanechère, L., Manier, M., Trigault, N., Pirollet, F., Mazarguil, H. and Job, D. (1994). Accumulation of Δ2-tubulin, a major tubulin variant which cannot be tyrosinated, in neuronal tissues and in stable microtubule assemblies. *J. Cell Sci.* **107**, 1529-1543.

Piperno, G. and Fuller, M. T. (1985). Monoclonal antibodies specific for an acetylated form of α-tubulin recognize the antigen in cilia and flagella from a variety of organisms. *J. Cell Biol.* **101**, 2085-2094.

Redeker, V., Le Caer, J. P., Rossier, J. and Promé J. C. (1991). Structure of the polyglutamyl side chain posttranslationally added to α-tubulin. *J. Biol. Chem.* **266**, 23461-23466.

Redeker, V., Melki, R., Promé, D., Le Caer, J. P. and Rossier, J. (1992). Structure of tubulin C-terminal domain obtained by subtilisin treatment. The major α and β tubulin isotypes from pig brain are glutamylated. *FEBS Lett.* **313**, 185-192.

Rüdiger, M., Plessman, U., Klöppel, K. D., Wehland, J. and Weber, K. (1992). Class II tubulin, the major brain β-tubulin isotype is polyglutamylated on glutamic acid residue 435. *FEBS Lett.* **308**, 101-105.

Sale, W. S., Besharse, J. C. and Piperno, G. (1988). Distribution of acetylated α-tubulin in retina and in in vitro-assembled microtubules. *Cell Motil. Cytoskel.* **9**, 243-253.

Solomon, F. (1992). Neuronal cytoskeleton and growth. *Curr. Opin. Neurobiol.* **2**, 613-617.

Towbin, H., Stahelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Nat. Acad. Sci. USA* **76**, 4350-4354.

Wang, D., Villasante, A., Lewis, S. A. and Cowan, N. J. (1986). The mammalian beta-tubulin repertoire: hematopoietic expression of a novel beta-tubulin isotype. *J. Cell Biol.* **103**, 1903-1910.

Wolff, A., Denoulet, P. and Jeantet, C. (1982). High level of tubulin microheterogeneity in the mouse. Brain. *Neurosci. Lett.* **31**, 323-328.

Wolff, A., Néchaud, B. d., Chillet, D., Mazarguil, H., Desbruyères, E., Audebert, S., Eddé, B., Gros, F. and Denoulet, P. (1992). Distribution of glutamylated α- and β-tubulin in mouse tissues using a specific monoclonal antibody, GT335. *Eur. J. Cell Biol.* **59**, 425-432.

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