Journal of Cellular Biochemistry 46:134–142 (1991)

A Combination of Posttranslational Modifications Is Responsible for the Production of Neuronal α-Tubulin Heterogeneity

Bernard Eddé, Jean Rossier, Jean-Pierre Le Caer, Yoheved Berwald-Netter, Annette Koulakoff, François Gros, and Philippe Denoulet

Laboratoire de Biochimie Cellulaire, Collège de France, 75231 Paris Cedex 05 (B.E., Y.B.-N., A.K., F.G., P.D.) and Laboratoire de Physiologie Nerveuse, C.N.R.S., 91198 Gif s/Yvette, France (J.R., J.-P. Le C.)

Abstract We describe the presence of α-tubulin and MAP2 acetyltransferase activities in mouse brain. The enzyme(s) copurified with microtubules through two cycles of assembly-disassembly. Incubation of microtubule proteins with [^{3}H]acetyl CoA resulted in a strong labeling of both α-tubulin and MAP2. To determine the site of the modification, tubulin was purified and digested with Glu-C endoproteinase. A unique radioactive peptide was detected and purified by HPLC. Edman degradation sequencing showed that this peptide contained ϵ^N-acetyllysine at position 40 of the α-tubulin molecule. This result demonstrates that mouse brain α-tubulin was acetylated at the same site as in *Chlamydomonas*. Isoelectric focusing analysis showed that acetylated α-tubulin was resolved into five isoelectric variants, denoted α3 and α5 to α8. This heterogeneity is not due to acetylation of other sites but results from a single acetylation of Lys^{40} of an heterogeneous population of α-tubulin isoforms. These isoforms are produced by posttranslational addition of one to five glutamyl units. Thus, neuronal α-tubulin is extensively modified by a combination of modifications including acetylation, glutamylation, tyrosylation, and other yet unknown modifications.

Key words: neuronal differentiation, tubulin heterogeneity, tubulin acetylation, tubulin glutamylation, MAP2 acetylation

Brain tubulin is characterized by a very high level of heterogeneity [4,5,10,11,32], essentially due to the contribution of neuronal cell types [8,12,21]. This heterogeneity relies, in part, on the expression of several tubulin isogenes [for review, see 29] and essentially on chemical modifications of the corresponding primary products. For instance, pulse-chase labeling of cultured neurons with [^{35}S]methionine showed that, among the eight isoelectric variants of α-tubulin expressed in these cells, as many as seven were produced by posttranslational modifications [6].

Posttranslational modifications of tubulin could play crucial roles in the formation and the specialization of particular microtubule structures. Directly or throughout interactions with microtubule associated proteins (MAPs), posttranslational modifications may affect assembly, stability, and functions of microtubules. We will focus, in this report, on the relation between two posttranslational modifications, acetylation and glutamylation, in the generation of brain tubulin heterogeneity.

Acetylation was first identified in *Chlamydomonas*, where it was involved in the synthesis of an electrophoretic variant of α-tubulin (called α3) which was associated predominantly with the flagellar microtubules [17,18]. The modified isoform was specifically labeled after incubation of the cells with [^{3}H]acetate, in the presence of a strong inhibitor of protein synthesis, cycloheximide, and biochemical analyses provided good evidence that this label corresponded, at least partially, to the acetylation of the ε-amino group of a lysine residue [18]. A monoclonal antibody (6-11B-1), raised against tubulin from the axonemes of sea urchin sperm, was shown to recognize selectively the acetylated α3 isoform of *Chlamydomonas* [24]. Searching for the binding

Abbreviations used: MAP, microtubule-associated protein; MTP, microtubule proteins; HPLC, high performance liquid chromatography; PTH, phenylthiohydantoine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; 2D-PAGE, two-dimensional PAGE.

Received November 2, 1990; accepted January 17, 1991.

site of 6-11B-1, Le Dizet and Piperno [16] found a modified residue near or within the epitope recognized by the antibody. This residue was characterized as ϵ^N-acetyllysine and localized at position 40 of the α-tubulin molecule.

Acetylation of α-tubulin is thought to occur in a wide variety of cell types and organisms, from microorganisms to mammals [25], including brain cells [2,3,7,8]. This conclusion was based on immunocytochemical and immunoblotting analyses performed with specific antibodies, and on a complementary approach which consisted to incubate cell cultures with radioactive acetate in the presence of cycloheximide and to search for acetate-labeled α-tubulin isoforms. However, it is not absolutely certain whether the binding of specific antibodies with mammalian α-tubulin was due to the same epitope described in *Chlamydomonas,* nor is there direct evidence that demonstrates acetylation of α-tubulin in any other organism than *Chlamydomonas.*

We recently characterized a new modification of neuronal α-tubulin consisting of the posttranslational addition of several glutamyl units on the gamma-carboxyl group of Glu^{445} and showed that acetate-labeling of α-tubulin in cultured mouse brain neurons was mostly due to this modification, through the metabolic conversion of acetate into glutamate via acetyl CoA and part of the tricarboxylic acid cycle [9]. It was particularly surprising that the different glutamylated α-tubulin isoforms comigrated, in resolutive isoelectric focusing gels, with isoforms reacting specifically with 6-11B-1 antibody. These results raise several questions: does acetylation of α-tubulin occur in neurons, and in this case, what is its quantitative importance and what is the modified amino acid residue(s)? What is the basis for the heterogeneity of acetylated α-tubulin? What is the relation between acetylation and glutamylation, that is, can these two posttranslational modifications occur concomitantly on the same α-tubulin molecules?

We demonstrate here the presence of an acetyltransferase activity in mouse brain that acetylates α-tubulin on a single residue (Lys40), identical to that found in *Chlamydomonas.* Acetylation of α-tubulin occurs also in homogeneous cultures of either neurons or astroglia and at the same site. However, acetylation represents a minor modification whereas the different glutamylated forms account for almost 50% of neuronal α-tubulin. Our results show that neuronal α-tubulin is extensively modified by a combination of modifications including glutamylation, acetylation, tyrosylation, and other yet unknown modifications.

MATERIALS AND METHODS

Cell Cultures

Homogeneous cultures of mouse brain neurons and astroglia were performed as described previously [1,9].

Purification of Microtubule Proteins

Ten-day-old mice were used. Brains were dissected immediately after the sacrifice and rapidly homogeneized in cold buffer A (100 mM PIPES, pH 6.8, 2 mM EGTA, 1 mM $MgCl_2$, 1 mM GTP) using a teflon-glass potter (10 strokes at 1200 rpm). High speed supernatant was obtained by centrifugating the crude homogenate at 10,000*g* for 10 min (2°C) to eliminate the cellular debris and then at 100,000*g* for 60 min (2°C). Supernatant was then subjected to cycles of assembly-disassembly at 37°C in buffer A containing 4 M glycerol. In some experiments supernatant was submitted to PD-10 column (Pharmacia) chromatography.

Assay for Acetyltransferase Activities

The assays for acetyltransferase activities were carried out mainly as described by Greer et al. [13]. High speed supernatant, once-, and twice-cycled MTP (3 mg/ml) were incubated at 37°C for 90 min, in buffer A containing 15 μCi/ml [^{3}H]acetyl CoA (Amersham, 2–5 Ci/mmol) and 1 mM fresh GTP. Aliquots of the reaction media were processed for radioactive counting by precipitation with 10% (w/v) TCA onto Whatman 3MM filters. MTP preparations could be stored at −80°C for six months without detectable loss of acetyltransferase activity. They were then centrifuged at 10,000*g* (10 min, 4°C) before use.

Tubulin Purification and Proteolysis

After in vitro acetylation, tubulin and MAPs were purified using the taxol-induced microtubule assembly procedure [30]. Tubulin from cell cultures was purified using the mini-scale procedure described by Serrano et al. [28]. Digestion of tubulin with 1/20 (w/w) endoproteinase Glu-C (Boehringer-Mannheim) was performed in ammonium bicarbonate buffer, 50 mM, pH 7.8. Incubations were carried out for 18 hr at 37°C.

Under these conditions, the enzyme was described to cleave only Glu-X peptidic bonds [14].

HPLC Analysis

The samples of tubulin digests were diluted in 5% acetonitrile (Prolabo, UV grade) and 1% TFA (Applied Biosystems) and centrifuged in Eppendorf tubes at 10,000*g* for 2 min. The pellet, which contained unsolubilized material (usually 40–50% of the original preparation), was discarded. The supernatant was analyzed by HPLC (Waters-Millipore) using analytical (2.1 × 220 mm) or semi-preparative (4.6 × 220 mm) reversed phase RP 300 columns (Brownlee). Flow rates were 0.2 and 0.9 ml/min, respectively. Solution A was 0.1% TFA and solution B was 0.09% TFA, 70% acetonitrile. The elution gradient was linear: (95% A, 5% B) to (50% A, 50% B) in 60 min and (50% A, 50% B) to (0% A, 100% B) in 20 min, followed by a 10 min wash in (0% A, 100% B). The peptides were detected by their absorbance at 214 nm and collected manually. Aliquots of the collected fractions were processed for liquid scintillation counting in 10 ml of Picofluor 15 (Helvett Packard). Radioactive fractions were partially evaporated under vacuum and chromatographed again on a C18 reverse phase column (2.1 × 220 mm, Brownlee) using the same elution gradient as described above at a flow rate of 0.2 ml/min.

Amino Acid Sequencing

Peptides were sequenced using a 470A gas-liquid amino acid sequencer (ABI). 50% aliquots of the PTH-aa (phenyl-thiohydantoïne amino acids) were analyzed with a 120 PTH-aa analyzer (ABI) using the standard program developed by ABI on a C18-PTH column. The elution gradient was linear from (89% A, 11% B) to (62% A, 38% B) in 27 min. Solution A was 0.07 M ammonium acetate, 5% THF, in water and solution B was pure acetonitrile. Aliquots (40%) of the PTH-aa were stored and processed for liquid scintillation counting. PTH-ϵ^Nacetyllysine was synthesized using commercial ϵ^Nacetyllysine (Sigma) and purified by HPLC. This PTH derivative had, in our system, a retention time of 12.4 min comprised between that of PTH-Glu (9.9 min.) and PTH-Ala (12.9 min.).

Gel Electrophoresis and Fluorography

Denaturating 9.5 M urea (Schwartz Mann) cylindrical IEF gels [22] were performed as described by Wolff et al. [32], using pH 4–6 LKB ampholines (2%, w/v). For 2D-PAGE, IEF gels were performed with a broader pH range ampholines (LKB) consisting of a mixture of pH 3.5–10 (20%) and 5–7 (80%). The gels were soaked with the equilibration buffer of O'Farrell [22] but for 15 min instead of 2 hr, and the second dimension was performed on a 8% acrylamide (LKB) slab gel (24 × 13 cm) containing 0.1% (w/v) sodium dodecyl sulfate (90% pure, Merck). One dimensional SDS-PAGE [15] (8 cm long) was performed as described for the second dimension of 2D-PAGE. After staining with Coomassie blue, the gels were soaked 20 to 30 min with Amplify (Amersham), dried, and submitted to fluorography using XAR5 films (Kodak Inc.).

RESULTS

α-Tubulin and MAP2 Acetyltransferase Activities Copurified with MTP

The presence of α-tubulin acetyltransferase activity in mouse brain was assayed by incubating brain extracts with [^{3}H]acetyl CoA. The labeled proteins were analyzed by SDS-PAGE and detected by fluorography (Fig. 1, lanes 1 and 2). When using a cytosolic fraction (high speed supernatant, denoted S1), a single labeled protein species was detected with an apparent MW slightly higher than MAP1 (denoted X in Figure 1, lanes 1). When S1 was desalted before being assayed, two additional bands comigrating with α-tubulin and MAP2 were labeled (Fig. 1, lanes 2). Labeling of these two latter proteins was markedly increased when the assay was performed with once-cycled or twice-cycled microtubule proteins (MTP) whereas labeling of protein X became undetectable (Fig. 1, lanes 3 and 4). In order to confirm the identity of the radioactive polypeptides, tubulin and MAPs were purified using taxol and then analyzed by 2D-PAGE. Figure 2 shows that the major labeled species was recovered with the tubulin fraction and comigrated with the acidic part of the α-tubulin spot (Fig. 2a,b) whereas the minor labeled species was recovered with the MAPs fraction and comigrated with MAP2 (Fig. 2c,d). These results show the presence, in mouse brain, of α-tubulin and MAP2 acetyltransferase activities which copurify with microtubules.

In Vitro Acetylated α-Tubulin Is Heterogeneous

The 2D-PAGE analysis presented in Figure 2 suggested that the label corresponding to α-tubulin, although restricted to the acidic part of the

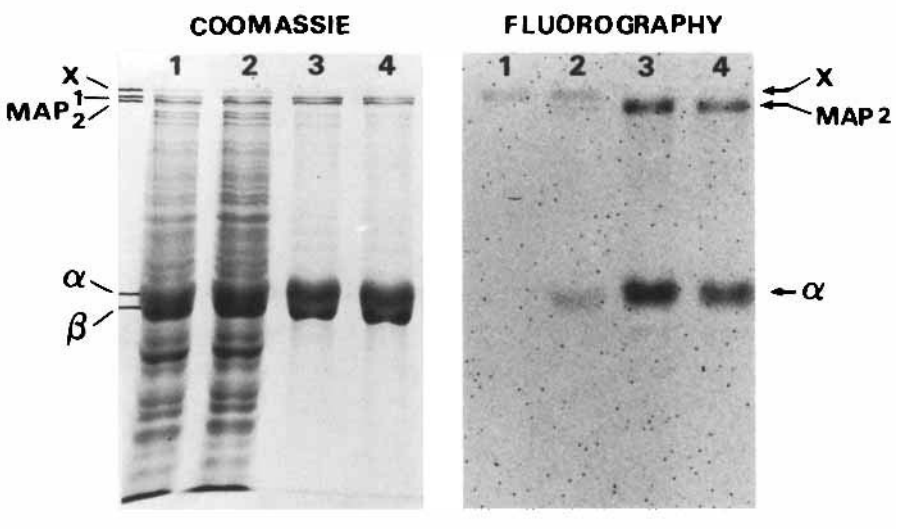

Fig. 1. In vitro acetylation of mouse brain proteins. High speed supernatant (S1), before (lanes 1) or after (lanes 2) desalting on a G25 column, once-cycled (lanes 3) and twice-cycled (lanes 4) MTP were incubated with 16 μCi/ml [^{3}H]acetyl CoA for 1 hour at 37°C. 30 μg of S1 and 10 μg of MTP were loaded on the gel (SDS-PAGE, 8 cm in length). **Left:** Coomassie blue staining. **Right:** fluorograph of the same gel exposed for 7 days. α and β refer to as tubulin subunits. Note that protein X is present in S1 sample but did not copurify with MTP.

α-tubulin spot, is heterogeneous. This could be much better investigated by resolutive isoelectric focusing (IEF) analysis. In this experiment, once-cycled MTP was incubated with [^{3}H]acetyl CoA for 10 to 120 min. After each time, tubulin was immediately purified using taxol and analyzed by IEF. Coomassie blue staining of the focusing gel shows that, at this stage of development (10 days after birth), mouse brain tubulin is resolved into six α-tubulin (denoted α1–α6) and eight β-tubulin (denoted β3–β10) isoforms. In addition, as previously reported [8], very acidic α (α7, α8) and very basic β (β′1, β′2) isoforms comigrated in the intermediate region of the gel, marked by an asterisk (Fig. 3, lane CBS). The corresponding fluorographs show that, among the eight α-tubulin isoforms, as many as five (α3, α5–α8) were labeled (Fig. 3, fluorography). This labeling increased with time, reaching a plateau value at 60 min, but no variation of the relative intensity of the different labeled bands was observed.

Localization of the Acetylated Residue

Purified [^{3}H]-acetylated tubulin was digested by Glu-C endoproteinase (which cleaves polypeptides at the C-side of Glu residues) and the proteolytic peptides were separated by reversed phase HPLC with a C8-RP300 column (Fig. 4a). The labeled material eluted with a retention time of 55–56 min. This fraction was further purified using a C18 column (Fig. 4b). A unique radioactive peptide, denoted b.GC1, was then eluted at 66 min. The amino acid sequence of this peptide was determined by automatic Edman degradation and gave 13 pmoles of His-Gly-Ile-Gln-Pro-Asp-Gly-Gln-Met-Pro-Ser-Asp-Aclys-Xaa-Ile-Gly-Gly-Xaa-Asp. No other amino acid sequence was found. Although two amino acids, indicated as Xaa, could not be clearly identified, the rest of the sequence unambiguously matches that of mouse α-tubulin genes [31] from amino acids 28–46. The C-terminal part of b.GC1 could not be determined, due to the low amount of material, but it probably extended to Glu^{55} as expected for a Glu-C cleavage. At the thirteenth cycle of the Edman degradation, no PTH-Lys was obtained but rather a radioactive PTH-derivative with a retention time corresponding to that of a synthetic PTH-ϵ^Nacetyllysine. These results demonstrated that mouse brain α-tubulin was acetylated at the same site as in *Chlamydomonas*.

The relative abundance of acetylated α-tubulin was determined by comparing the molar amount of b.GC1, as estimated from the amino acid sequencing data, and the molar amount of tubulin digests used for HPLC purification. Starting from 3 nmoles of tubulin digests loaded onto the first C8-RP300 column (Fig. 4a), we obtained about 13 pmoles of b.GC1 as estimated from the release of PTH-Gly^{29} during the second cycle of the Edamn degradation (since the release of PTH-His is generally poor, we did not

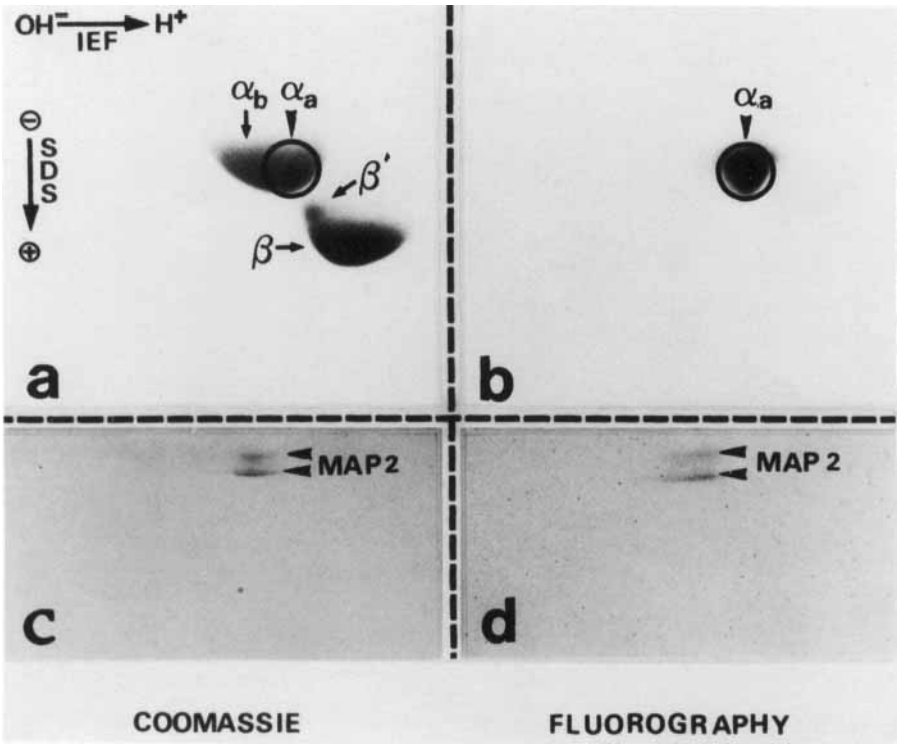

Fig. 2. 2D-PAGE analysis of acetylated α-tubulin and MAP2. MTP was incubated with [^{3}H]acetyl CoA for 1 h; tubulin and MAPs were then purified using taxol [30] and analyzed by 2D-PAGE. 20 μg of tubulin (a,b) and 5 μg of MAPs (c,d) were loaded on the gels. **a,c:** Coomassie blue staining. **b,d:** Fluorographs exposed for 5 d (b) and 10 d (d), respectively. The radioactive α-tubulin spot was localized at the right acidic part of the Coomassie blue stained α-tubulin spot. Note that MAP2 splits, in our 2D-gels, into two components of slightly different apparent *MWs*. α_a and α_b indicate the acidic and basic parts of the α-tubulin spot, respectively. β and β' indicate the major and the minor β-tubulin components present in brain. Only tubulin and MAPs regions are presented. No other proteins were detected in the whole 2D-gels.

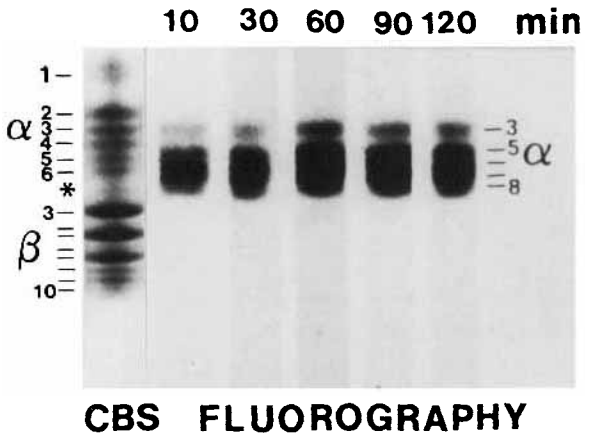

Fig. 3. Acetylated α-tubulin is heterogeneous. MTPs were incubated with [^{3}H]acetyl CoA for the time indicated. Tubulin was then purified and analyzed by resolutive IEF. A Coomassie blue staining of one of these gels is presented (lane CBS); the other lanes correspond to fluorographs exposed for 3 days.

take it into account). Assuming a sequencing efficiency of 30–50%, and taking into account the aliquots of b.GC1 used for liquid scintillation counting, we estimated the amount of b.GC1 present in the initial preparation to be 50–80 pmoles. By this method, acetylated α-tubulin represents about 2–3% of the total α-tubulin present in brain.

α-Tubulin Acetylation in Cultured Neurons and Astroglia

The presence of acetylated α-tubulin in mouse brain neurons and astroglia was sugested by immunoblotting and immunofluorescence experiments using specific antibodies [2,3,7,9]. In order to confirm these results, we performed cell labeling experiments with [^{3}H]acetate and analyzed the radioactive proteolytic peptides of tubulin. Fifteen d-embryonic mouse brain neurons were cultured for one week and then incubated with [^{3}H]acetate, in the presence of a strong

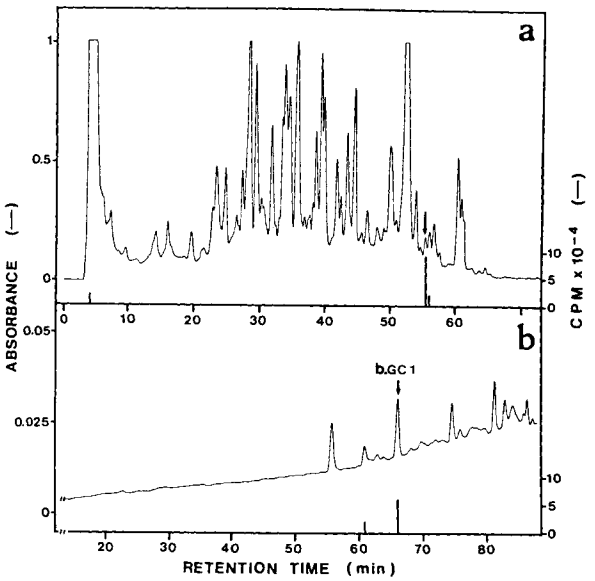

Fig. 4. HPLC purification of the in vitro acetylated α-tubulin peptide. In vitro [^{3}H]-acetylated tubulin (300 μg, 100,000 cpm) was purified and digested by Glu-C endoproteinase. **a:** The whole peptides were loaded first on a C8-RP300 column (2.1 × 210 mm). **b:** The radioactive fraction was then further purified on a C18 column. A single radioactive fraction was then detected and denoted b.GC1. Absorbance was monitored at 215 nm.

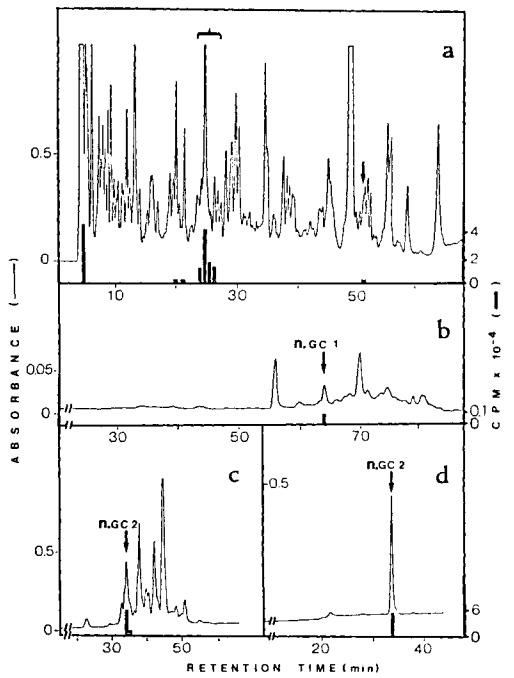

Fig. 5. HPLC purification of the acetate-labeled tubulin peptides of cultured neuronal cells. Mouse brain neurons were cultured for 1 week and then incubated with [^{3}H]acetate, in the presence of cycloheximide. Tubulin was purified (20 μg, 100,000 cpm), mixed with unlabeled mouse brain tubulin (200 μg), and digested with Glu-C endoproteinase. **a:** The whole peptides were loaded on a C8-RP300 column (4.6 × 210 mm). **b:** A minor radioactive fraction with RT of 51 min. was further purified on C18 column and denoted n.GC1. **c:** The major radioactive fractions eluting with RT of 24–27 min were pooled and further purified on a C18 column. **d:** The radioactive fraction detected in c and denoted n. GC2 was re-chromatographed under the same conditions.

inhibitor of protein synthesis (cycloheximide). Astroglial cells were isolated from newborn mouse brain, cultured for three weeks, and labeled under the same conditions. After cell incubation with [^{3}H]acetate, tubulin was purified, mixed with unlabeled mouse brain tubulin, and digested with Glu-C endoproteinase. Radioactive peptides were purified by HPLC. In the case of neurons, several radioactive fractions were recovered (Fig. 5a). The minor fraction eluting at 51 min was further purified on a C18 column and was then eluted at 64 min, a retention time similar to that obtained for b.GC1 on the same column (Fig. 5b). Automatic Edman degradation showed that the sequence of n.GC1 matches that of mouse α-tubulin genes from amino acids 28–43. Again PTH-ϵ^Nacetyllysine was found at position 40.

The same analysis was performed with cultured astroglial and showed the presence of a radioactive peptide (denoted g.GC1) eluting with the same retention time as b.GC1 (Fig. 6). Although the amino acid sequence of g.GC1 was not determined, it is very likely that the radioactive moiety associated with this peptide is also due to acetylation of Lys-40.

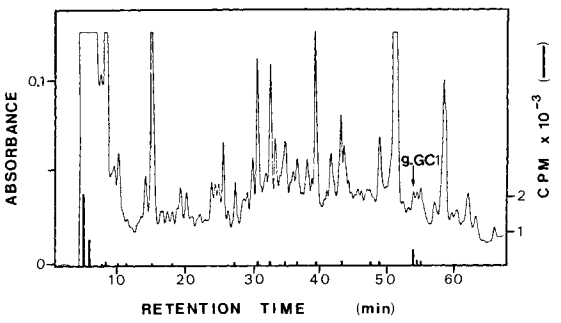

Fig. 6. HPLC analysis of acetate-labeled tubulin peptides of cultured astroglial cells. Mouse brain astroglia were cultured for 3 weeks and incubated with [^{3}H]acetate, as described for neurons. Purified tubulin (10 μg, 1,500 cpm) was digested with Glu-C endoproteinase and the whole peptides were loaded on a C8-RP300 column (2.1 × 210 mm). Radioactive counting showed a weak radioactive peak with RT of 54 min, similar to that obtained for b.GC1 on the same column.

Glutamylation Is a Major Modification of Neuronal α-Tubulin

The major radioactive fraction eluting at 23–26 min (Fig. 5a) was further purified on the C18 column and gave an apparently homogeneous peak, denoted n.GC2, with a retention time of 34 min (Fig. 5c,d). The amino acid sequence of n.GC2 was determined by Edman degradation and contained 1 nmol of Val-Gly-Val-Asp-Ser-Val-Glu-Gly-Glu-Gly-X-Glu-Glu-Gly, which matches the sequence of two mouse α-tubulin isogenes, M α1 and M α2, from amino acids 435–448. No other amino acid sequence was observed. This sequence differed from that deduced from the nucleotide sequence at position 445 (eleventh cycle of the Edman degradation): instead of obtaining PTH-Glu no PTH-amino acid could be detected at the corresponding cycle. This observation is consistent with a modified Glu^{445} residue. We recently showed that the corresponding modification is not an acetylation but corresponds to the posttranslational addition of one to five glutamyl units on the γ-carboxyl group of Glu^{445} [9]. Thus, in cultured neurons, [^{3}H]acetate is used to label at least two different modifications of α-tubulin. One corresponds to the direct addition of acetate, probably via its activated form acetyl CoA, and is identical to the modification observed in vitro. The other one represents the addition of several glutamyl units onto Glu^{445}. In this latter case, radioactive acetate is used to label cellular glutamate via the entry of acetyl CoA into the tricarboxylic acid cycle and α-ketoglutarate.

The relative abundance of acetylated and glutamylated tubulin was determined by comparing the molar amounts of recovered n.GC1 (50 pmoles) and n.GC2 (1 nmole), as estimated from amino acid sequencing data, and the molar amount of tubulin digests (2 nmoles) loaded onto the first C8-RP300 column (Fig. 5a). The values obtained were corrected by the sequencing efficiency factor and took into account the aliquots used for scintillation counting. These results showed that glutamylated α-tubulin is much more abundant than acetylated α-tubulin (around 50% vs. 2.5% of total α-tubulin, respectively).

In the case of glial cells we could not detect any radioactive peptide in the region corresponding to GC2 (Fig. 6). Thus, contrary to acetylation, posttranslational glutamylation is absent in this cell type, or present at a level too low to be detected with our technique.

DISCUSSION

We showed previously [8] that incubation of neurons with [^{3}H]acetate, in the presence of cycloheximide, resulted in the posttranslational labeling of the six most acidic variants of α-tubulin (α3–α8). Very recent data [9] and this report showed that this labeling, instead of being due to acetylation, was essentially due to the posttranslational addition of a various number of glutamyl units on the gamma-carboxyl group of Glu^{445}. However, immunoblotting experiments revealed [8] that isoforms with similar isoelectric points also reacted with a monoclonal antibody (6-11B-1) described to recognize selectively the acetylated form(s) of α-tubulin in *Chlamydomonas* [24]. These results indicated that acetylated α-tubulin can comigrate in IEF gels with glutamylated α-tubulin or alternatively that 6-11B-1 may recognize a distinct epitope in mouse brain cells.

In order to investigate this question we developed an in vitro assay specific for the acetylation reaction. Incubation of brain MTP with [^{3}H]acetyl CoA resulted in a strong labeling of α-tubulin and in a weak labeling of a protein species, which has been identified as MAP2 on two criteria: copurification with MAPs fraction and comigration, in 1D- and 2D-PAGE, with MAP2. MAP 1 was not labeled. Since metabolic conversion of acetyl CoA is unlikely to occur using MTP preparations, these two proteins were probably acetylated during the assay. In the case of α-tubulin, this conclusion was confirmed by the characterization of the modified radioactive residue (see below). Whether acetylation of these two proteins is due to the same enzyme is not known but the corresponding activities copurified with MTP throughout two cycles of assembly-disassembly (see Fig. 1). Both activities were also inhibited by a small MW compound(s) which is retained onto a G25 column (exclusion limit: 10 Kdaltons). Such an inhibitor has been found in *Chlamydomonas* where, along with a deacetylase activity, it could provide a key for understanding the preferential localization of α3-tubulin in the flagella [20]. In nerve cells, a preferential localization of acetylated microtubules in axons compared with dendrites or growth cones has been proposed [26]. The inhib-

itory activity evidenced in our experiments could be involved in this differential distribution.

The presence of an α-tubulin acetyltransferase activity in 3×-cycled MTP preparations of calf brain was reported but this activity was shown to be extremely weak [13]. We obtained a much higher activity using MTP preparations from 10-day-old mouse brain. This difference is likely related to the age of the animals used in the two studies. We found actually that in vitro acetylation of α-tubulin and MAP2 was markedly lower in MTP prepared from 30-day-old than from 10-day-old mice. Since adult α-tubulin is a good substrate for *Chlamydomonas* acetyltransferase [13,20] this result suggests that mouse brain α-tubulin acetyltransferase activity is developmentally regulated.

In order to characterize the nature and the site of the modification concerned by the in vitro assay, we digested [^{3}H]-acetylated tubulin with Glu-C endoproteinase and purified the radioactive peptide (denoted b-GC1) by reversed phase HPLC. By amino acid sequencing, b-GC1 was shown to contain a radioactive AcLys at position 40 of the α-tubulin molecule. A similar analysis of tubulin from neural cells in culture incubated with [3H]acetate indicated that, under in vivo conditions, α-tubulin was also acetylated at Lys40. Thus, acetylation of mouse brain tubulin occurred at the same site as in *Chlamydomonas* [16]. This result is important because it provides a molecular basis for the binding of anti-acetylated α-tubulin antibodies to mammalian α-tubulin.

The relative abundance of acetylated versus total α-tubulin in purified brain tubulin preparations was estimated to about 3%. It has to be noted that this value represents an estimation rather than an accurate measurement. In particular, the sequencing efficiency cannot be determined with precision and depends on the peptide being analyzed. Nevertheless, this value represents probably a slight underestimation because it did not take into account a possible loss of acetylated isoforms during tubulin purification. Indeed, Sale et al. [27] observed a selective enrichement of acetylated tubulin in cold pellet fractions which are discarded during microtubule purification.

Contrary to acetylation, glutamylation is a major modification of neuronal α-tubulin, concerning about 50% of total α-tubulin. In addition, under cell incubation with [^{3}H]acetate, almost all of the radioactivity (95%) associated with α-tubulin was recovered with the n-GC2 glutamylated peptides whereas only a very minor fraction of the radioactivity (1%) was recovered with the n-GC1 acetylated peptide. Thus, care must be taken when considering acetate labeling of α-tubulin in cultured cells as being due to acetylation of Lys40. This is probably true for certain cell types such as astroglia where most of the radioactivity associated with α-tubulin was recovered in a peptide (g-GC1) having a similar retention time as b-GC1, but it is obviously not the case for neurons.

Both acetylated and glutamylated α-tubulin are heterogeneous. IEF analysis showed that acetylated α-tubulin consists of five isoelectric variants, denoted α3 and α5 to α8, and that glutamylated α-tubulin is resolved into six variants, namely α3 to α8 [8]. The heterogeneity of glutamylated α-tubulin can be explained by the successive addition of one to at least five glutamyl units, each causing an acidic shift of pI [9]. Since we detected a single site of acetylation, the heterogeneity of acetylated α-tubulin can be explained by the presence of multiple substrates differing by their pI's and all undergoing a single acetylation. Glutamylated isotubulins are good candidates for such heterogeneous substrates, but our investigation suggests that other yet unknown modifications may produce a series of isoelectric variants which could also serve as substrates for acetylation (unpublished observations). Nevertheless, it is clear that the real heterogeneity of neuronal α-tubulin is much greater than what can be separated by IEF, each isoelectric band corresponding to several molecular entities, differing both in their amino acid sequence and in their posttranslational modification content. Tyrosylation and detyrosylation processes still increase this complexity; each of the isoelectric variants may or may not bear the C-terminal tyrosine. Consequently, a minimal value of α-tubulin heterogeneity in neurons can be estimated to over 20–30 molecular entities.

Acetylation and glutamylation occur at particular sites of the α-tubulin molecule. Acetylation is localized in the amino-terminal domain, previously shown to interact with Tau *protein [19]* while glutamylation is localized in the carboxy-terminal domain. This latter region is exposed on the surface of the microtubule lattice and interacts with some MAPs and with calcium. It is of particular interest to note that MAP2 and

cytoplasmic dynein were suggested to interact with EGEE or EGEEE [23], which is the precise site of glutamylation. Whether this modification affects the binding of these two proteins is under investigation.

ACKNOWLEDGMENTS

We thank C. Gruszczynski, and E. Desbruyères for excellent technical assistance and R. Guenard for providing taxol. This work was supported by grants from Collège de France, Centre National de la Recherche Scientifique (URA 115 and UPR 2212), Institut National de la Santé et de la Recherche Médicale (CRE 89.60005), Commission of the European Communities (CI.1-0508-FR-H), and Fondation pour la Recherche Médicale Française.

REFERENCES

1. Berwald-Netter Y, Martin-Moutot N, Koulakoff A, Couraud F: Proc Natl Acad Sci USA 78:1245–1249, 1981.
2. Black MM, Baas PW, and Humphries S: J Neurosc 9:358–368, 1989.
3. Cambray-Deakin MA, Burgoyne RD: J Cell Biol 104: 1569–1574, 1987.
4. Dahl JL, Weibel VJ: Biochem Biophys Res Commun 86:822–828, 1979.
5. Denoulet P, Eddé B, Jeantet C, Gros F: Biochimie 64:165–172, 1982.
6. Denoulet P, Eddé B, Pinto Henrique D, Koulakoff A, Berwald-Netter Y, Gros F: In Rousset BAF (ed): "The European Symposium on the Structure and Function of the Cytoskeleton." London, Paris: John Libbey Eurotext, 1988, pp 231–237.
7. Eddé B, De Néchaud B, Denoulet P, Gros F: Dev Biol 123:549–558, 1987.
8. Eddé B, Denoulet P, De Néchaud B, Koulakoff A, Berwald-Netter Y, Gros F: Biol Cell 65:109–117, 1989.
9. Eddé B, Rossier J, Le Caer JP, Desbruyères E, Gros F, Denoulet P: Science 247:83–85, 1990.
10. George HJ, Misra L, Fields DJ, Lee JC: Biochemistry 20:2402–2409, 1981.
11. Gozes I, Littauer UZ: Nature (Lond) 276:411–413, 1978.
12. Gozes I, Sweadner KJ: Nature (Lond) 294:477–480, 1981.
13. Greer K, Maruta H, L'Hernault SW, Rosenbaum JL: J Cell Biol 101:2081–2084, 1985.
14. Houmard J, Drapeau GR: Proc Natl Acad Sci USA 69:3506–3509, 1972.
15. Laemmli UK: Nature (Lond) 227:680–685, 1970.
16. Le Dizet M, Piperno G: Proc Natl Acad Sci USA 84:5720–5724, 1987.
17. L'Hernault SW, Rosenbaum JL: J Cell Biol 97:258–263, 1983.
18. L'Hernault SW, Rosenbaum JL: Biochemistry 24:473–478, 1985.
19. Littauer UZ, Giveon D, Thierauf M, Ginzburg I, Ponstingl H: Proc Natl Acad Sci USA 83:7162–7166, 1986.
20. Maruta H, Greer K, Rosenbaum JL: J Cell Biol 103:571–579, 1986.
21. Moura-Neto V, Mallat M, Jeantet C, Prochiantz A: EMBO J 2:1243–1248, 1983.
22. O'Farrell PH: J Biol Chem 250:4007–4021, 1975.
23. Paschal BM, Obar RA, Vallee RB: Nature 342:569–572, 1989.
24. Piperno G, Fuller MT: J Cell Biol 101:2085–2094, 1985.
25. Piperno G, Le Dizet M, Chang X: J Cell Biol 104:289–302, 1987.
26. Robson SJ, Burgoyne RD: Cell Motility Cytoskel 12:273–282, 1989.
27. Sale WS, Besharse JC, Piperno G: Cell Motility Cytoskel 9:243–253, 1988.
28. Serrano L, Diaz-Nido J, Wandosell F, Avila J: J Cell Biol 105:1731–1739, 1987.
29. Sullivan KF: Ann Rev Cell Biol 4:687–716, 1988.
30. Vallee RB: J Cell Biol 92:435–442, 1982.
31. Villasante A, Wang D, Dobner PR, Dolph P, Lewis SA, Cowan NJ: Mol Cell Biol 6:2409–2419, 1986.
32. Wolff A, Denoulet P, Jeantet C: Neurosci Lett 31:323–328, 1982.