INTRODUCTION

Polyglutamylation is an original posttranslational modification of tubulin widely spread among evolutionarily distant species from protozoa to mammals, where the modification was first discovered (Eddé et al., 1990; Redeker et al., 1992; Alexander et al., 1991; Rüdiger et al., 1992; Schneider et al., 1998; Bré et al., 1994; Smertenko et al., 1997; Pucciarelli et al., 1997). Polyglutamyl side chains, composed of a various number of L-glutamate units, are linked to the γ-carboxyl group of a glutamate residue near the C terminus of the α- and β-tubulin subunits. Side-chains bearing up to 11 and 17 units have been reported for brain and axonemal tubulin, respectively (Redeker et al., 1998; Rüdiger et al., 1995; Geimer et al., 1997). Different experimental approaches indicate that polyglutamylation could play crucial roles in the regulation of interactions between structural or motor MAPs and microtubules (MTs) (Boucher et al., 1994; Larcher et al., 1996; Gagnon et al., 1996). Polyglutamylated tubulin is particularly abundant in brain and neuronal cells (Wolff et al., 1992; Audebert et al., 1994). Numerous studies have also reported its occurrence at high levels in axonemes of cilia and flagella of various organisms, from human sperm (Fouquet et al., 1994) to trichomonads, one of the earliest diverging eucaryotes (Delgado-Viscogliosi et al., 1996; Schneider et al., 1998). The occurrence of polyglutamylated tubulin is, however, not restricted to neurons or spermatozoa; low levels have also been found in other cell types or tissues (Wolff et al., 1992). More recently, polyglutamylated tubulin was detected in proliferating cells of different origins (HeLa, KE37, NIH3T3…) where it is associated with the centrioles, the spindle MTs and the midbody. This cell-cycle dependent distribution was correlated with a significant increase in glutamylated tubulin at mitosis (Bobinnec et al., 1998a), suggesting a role for polyglutamylation also in cell division.

SUMMARY

Polyglutamylation is a posttranslational modification of tubulin that is very common in neurons and ciliated or flagellated cells. It was proposed to regulate the binding of microtubule associated proteins (MAPs) and molecular motors as a function of the length of the polyglutamyl side-chain. Though much less common, this modification of tubulin also occurs in proliferating cells like HeLa cells where it is associated with centrioles and with the mitotic spindle. Recently, we partially purified tubulin polyglutamylase from mouse brain and described its enzymatic properties. In this work, we focused on tubulin polyglutamylase activity from HeLa cells. Our results support the existence of a tubulin polyglutamylase family composed of several isozymic variants specific for α- or β-tubulin subunits. In the latter case, the specificity probably also concerns the different β-tubulin isotypes. Interestingly, we found that tubulin polyglutamylase activity is regulated in a cell cycle dependent manner and peaks in G2-phase while the level of glutamylated tubulin peaks in mitosis. Consistent results were obtained by treating the cells with hydroxyurea, nocodazole or taxotere. In particular, in mitotic cells, tubulin polyglutamylase activity was always low while glutamylation level was high. Finally, tubulin polyglutamylase activity and the level of glutamylated tubulin appeared to be inversely related. This paradox suggests a complex regulation of both tubulin polyglutamylase and the reverse deglutamylase activity.

Key words: Tubulin, Microtubule, Glutamylation, Polyglutamylation, Posttranslational modification, Mitosis, Cell cycle
described its basic enzymatic properties. We showed that the enzyme catalyzes the sequential and MgATP-dependent addition of L-glutamate onto tubulin molecules. Moreover, MTs are much better substrates than unpolymerized tubulin; this supports the observation that the modification is associated preferentially with MTs. Interestingly, we found that mouse brain TPG is able to glutamylate both α- and β-tubulin from mouse brain but only α-tubulin from HeLa cells (Regnard et al., 1998). Since glutamylation occurs mostly on β-tubulin in HeLa cells, this observation suggests that there might be a different TPG involved in β-tubulin glutamylation in these cells.

To further investigate the functional properties of glutamylation in non-neuronal cells, we measured TPG activity in total extracts or partially purified enzymatic fractions from HeLa cells and compared its properties to that of TPG from mouse brain. Our data support the existence of different isoform variants of TPG, specific for α- and β-tubulin, and even for each or subsets of β-tubulin isotypes. Moreover, we measured the variation in TPG activity and the accumulation of glutamylated tubulin during the cell cycle. We find that both TPG activity and the level of glutamylated tubulin appear to be cell cycle dependent but inversely correlated. To our knowledge, this is the first time that an enzyme activity catalyzing a posttranslational modification of tubulin is shown to be regulated during the cell cycle.

MATERIALS AND METHODS

Cell culture and synchronization

HeLa cells were grown in DMEM containing 10% fetal calf serum, 2 mM glutamine, 100 i.u. penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 5% CO₂ atmosphere. Thymidine-synchronized cells were obtained by 16-hour double thymidine block (2 mM thymidine) with a 10-hour release period in the presence of deoxycytidine (2 mM). Progression in the cell cycle was followed by FACs analysis using a FACScan Becton Dickinson flow cytometer. Cells blocked in mitosis were collected after an 18-hour incubation with 1 μM taxotere or nocodazole (Sigma, France). 18-hour treatment with 2.5 mM hydroxyurea (HU) (Sigma, France) was performed to block the cells in S-Phase.

Extraction procedure and partial purification

3-day-old mouse brains were homogenized in buffer B (50 mM BICINE, pH 9.0, 1 mM EGTA, 1 mM MgCl₂, 0.01% TX-100, 1 mM DTT) and partially purified TPG (Fraction IV) was prepared from these extracts as previously described (Regnard et al., 1998). The extraction procedure and partial purification of TPG from HeLa cells was adapted from that used for mouse brains. The cells were grown in 15 cm diameter culture dishes until they reached 15-20 × 10⁶ cells per dish, then washed with cold buffer B containing 0.1 M NaCl. The cells were scraped in a minimum volume and kept in melting ice. Triton X-100 was added to a final concentration of 0.1% as well as 10 mM benzenesulfonyl fluoride each at 10 μg/ml. After sonication, the homogenate was centrifuged 45 minutes at 50,000 g (4°C), giving the supernatant (S) and pellet (P) fractions. The pellet was re-extracted in buffer B containing 0.5 M NaCl and 0.4% Triton X-100 and centrifuged in the same conditions, giving fractions S' and P'. For partial purification, the supernatant fraction S was loaded on a phosphocellulose column equilibrated with buffer B (6-8 mg proteins per ml of stacked gel). TPG was eluted from the column by a step gradient of 0.35 M NaCl. Active fractions were quick frozen and stored as small aliquots at −80°C. They were desalted prior to the enzyme assays to avoid inhibition of the enzyme activity by salts. In synchronization experiments, TPG activity was measured in total extracts prepared as described above, quick frozen in small aliquots and stored at −80°C prior to the assay.

TPG assay

Enzyme activity was measured in the standard assay conditions previously described (Regnard et al., 1998). Briefly, reaction mixtures (20-100 μl) containing 50 mM Tris-HCl, pH 9.0, 2 mM ATP (equilibrated to pH 7 with NaOH), 8 mM MgCl₂, 2.5 mM DTT, 10 μM taxotere, L-[³H]glutamate (45-55 Ci/mmol, Amersham, UK) and 0.1 mg/ml taxotere-stabilized MTs, were incubated at 30°C for 40 minutes. In these conditions the assays are performed at near saturating MT concentration (Regnard et al., 1998). Brain or HeLa MTs were used as indicated in the text. To increase the radioactive signal, L-[³H]glutamate was concentrated 5- to 10-fold under speed vacuum and added at a final concentration of up to 66 μM. All protein samples of HeLa cells and mouse brains were added at a final concentration of 0.4 mg/ml in the assay (Fig. 1), except for the partially purified brain TPG (fraction IV) which was used at 40 μg/ml (Fig. 3). It has to be noted that the tubulin content of the protein samples to be assayed is minor compared to the amount of the added MT substrate. Salt concentration was always maintained at ≤15 mM to avoid inhibition of the enzyme activity. Reaction mixtures were submitted either to 1D-PAGE or to 2D-PAGE and fluorography. Exposures were performed at −80°C using Kodak (Rochester, NY) XAR-5 films after enhancement with amplify (Amersham, UK). Quantitations were done by scintillation counting of the α- and β-tubulin bands after 1D-PAGE and electrotransfer onto nitrocellulose sheets, as previously described (Regnard et al., 1998).

MT preparation

Taxotere-stabilized MTs from 3-day-old and adult mouse brain and from HeLa cells were purified in the presence of 1 mM GTP and 20 μM taxotere as described (Vallee, 1982). They were carefully resuspended at a concentration of ~10 mg/ml in warm 50 mM MES, pH 6.6, 1 mM EGTA, 1 mM MgCl₂, 0.1 mM GTP, 20 μM taxotere and stored as small aliquots at −80°C. We showed previously that MTs prepared and stored in this way were stable under the assay condition used (Regnard et al., 1998).

1D- and 2D-PAGE

1D-PAGE (Laemmli, 1970) was performed using 8% acrylamide, 0.11% bisacrylamide, slab gels containing 0.1% (w/v) SDS (90% pure, Merck, Darmstadt, Germany). Isoelectric focusing in 9.5 M urea was performed as described (Wolff et al., 1982) except that pH 5.0-6.0 amphotolys (Serva, Heidelberg, Germany) were used for the experiments presented in Figs 2 and 3 while pH 3.5-10 amphotolys (Serva, Heidelberg, Germany) were used for the experiment shown in Fig. 5b. The second dimension was performed on 24 cm slab gels in the same conditions as for 1D-PAGE.

Western blot analysis and quantitation of glutamylated tubulin level

Anti-cyclin A2 mAb (Desdouets et al., 1996) was diluted 1:5000. α- and β-tubulin were immunodetected by the monoclonal antibodies DM1A (1:5000, Amersham, UK) and TUB 2.1 (1:2000, Sigma, St Louis, MO), respectively. Horseradish peroxidase-conjugated secondary antibody (Bioys, Compiègne, France) was diluted 1:7500. mAb GT335 (Wolff et al., 1992) recognizes glutamylated α- and β-tubulin in various tissues and cells. However, in total extracts from HeLa cells, the level of glutamylated tubulin is too low to be detected directly by western blot after 1D-PAGE. To increase the sensitivity of the detection, GT335 was biotinylated with NHS-LC-biotin (Pierce, IL), according to the manufacturer’s instructions. Simultaneous incubation of GT335 (0.4 μg/ml) and streptavidin conjugated to
Determination of protein concentration
Protein concentrations were measured in triplicates by the Bradford method (Bio-Rad, Hercules, CA), with bovine serum albumin as standard.

RESULTS
Distinct TPGs are responsible for the glutamylation of α- and β-tubulin subunits
TPG activity was measured in supernatant and pellet fractions prepared either from 3-day-old mouse brain or from HeLa cells as described in Materials and Methods. Analysis of the radioactive products after 1D-PAGE and fluorography revealed that HeLa cell supernatant exhibits a preferential activity towards β-tubulin, while brain supernatant exhibits a preferential activity towards α-tubulin (Fig. 1). Quantitation of the radioactivity incorporated into each subunit gave an α/β ratio of 1:3 in the former case and 2.5:1 in the latter. Since the same MT substrates were used in the two cases, these data suggest the existence of two distinct TPG activities, α-TPG and β-TPG, responsible for the glutamylation of α- and β-tubulin subunits, respectively. Both seem to be present in brain and in HeLa cells but with a different relative abundance or efficiency.

Earlier attempts to separate α- and β-TPG activities from brain were unsuccessful (Regnard et al., 1998). However, these activities could be partially separated during the initial solubilization step of HeLa cells. Indeed, although some variability occurred from one experiment to another, β-TPG is always solubilized more efficiently than α-TPG. Moreover, α-TPG activity could be further salt-extracted from the pellet fraction (Fig. 1). This differential solubility is the first biochemical evidence that α- and β-TPG are distinct enzymes.

TPG activities specific for tubulin isotypes
The following experiments were conducted to characterize further the properties of the α- and β-TPG from mouse brain and from HeLa cells. The structure of the polyglutamyl side chain of brain tubulin is not yet known in detail, however, the first unit of the side chain is added obligatorily via a γ-carboxyl linkage. In the case of α-tubulin, the second and third units were shown to be bound mostly via α-carboxyl linkages (Redeker et al., 1991, 1996; Wolff et al., 1994). Thus, the question arises of whether there are distinct activities responsible for the initiation and elongation steps. For this purpose, we prepared different MTs for use as substrates in the glutamylation reactions. These MTs were purified from young mouse brain (3-day-old), adult mouse brain and HeLa cells and differ in their amount of polyglutamylated tubulin and in the length of the polyglutamyl side-chains. In 2D-PAGE analysis and Coomassie blue staining, MTs from HeLa cells (Fig. 2A) are resolved into mainly two species corresponding to unglutamylated α- and β-tubulin. Young mouse brain MTs exhibit numerous isoelectric variants of α-tubulin, essentially due to the various numbers of glutamyl units in the side chains, but a limited heterogeneity of the β subunit (Fig. 2B). This latter increases during post-natal development (Audebert et al., 1994), and reaches a high level in the adult (Fig. 2C). It has to be noted that the satellite spots denoted β’ correspond to the neurospecific class III β-tubulin isotype and are present only in brain MTs (Fig. 2B,C).

Glutamylation of these different substrates was performed using TPGs isolated from HeLa cells or brain. To increase the sensitivity of the assay and to eliminate any contamination by the endogenous tubulin, we used a phosphocellulose-enriched fraction of HeLa TPG and fraction IV from brain. 2D-PAGE analysis of the radiolabeled products showed that the glutamylation patterns are dependent on the heterogeneity of the MT substrates (Fig. 3). With the mostly non glutamylated HeLa MT substrate, the major labeled products correspond to the monoglutamylated isoforms (Fig. 3A,B), with the exception that HeLa β-tubulin seems not to be recognized by

![Image](329x362 to 554x721)
the brain enzyme (see below). Using the already polyglutamylated brain MTs, the labeling was heterogeneous and corresponds to the isoforms initially present (Fig. 3C-F). These data led us to conclude that both HeLa and brain TPGs are able to use unglutamylated isoforms of α- and β-tubulin to initiate, and already glutamylated isoforms to elongate polyglutamyl chains. The data presented above showed also interesting differences between the behavior of HeLa and brain TPGs. In particular, HeLa β-tubulin is an efficient substrate for HeLa enzyme (Fig. 3A) but not for brain enzyme (Fig. 3B). This lack of activity is probably not due to a deficient initiation by brain TPG, since monoglutamylated β-tubulin products were clearly observed with young brain MTs (Fig. 3D, long arrowheads). We suggest that this lack could be related to the expression of different tubulin isotypes in the two systems, given that the tubulin multigene family is very well conserved between mouse and human (Sullivan and Cleveland, 1986).

Indeed, most β-tubulin isotypes are glutamylatable at glutamylation sites that do not share any obvious consensus sequence (except that they are particularly rich in acidic residues). Whereas class II and III β-tubulin are the major isotypes expressed in adult brain, class I and IVa are less represented (Banerjee et al., 1988). On the contrary, class I and IVb are the most abundant isotypes in HeLa cells (Lewis et al., 1985, 1987). The absence of glutamylation of HeLa β-tubulin by the brain enzyme could be explained by the presence in brain of activities able to glutamylate class II, but not class I and IV isotypes. Another observation strongly supports the existence of an isotype specificity of β-TPG. Indeed, when brain MTs were used as substrate, class III β-tubulin (β’) which migrates in our 2D gels slightly above the

**Fig. 2.** MTs purified from HeLa cells (A), young (3-day-old) (B) and adult (C) mouse brain were submitted to 2D-PAGE and stained with Coomassie blue. Different protein amounts were loaded on the gels (10 μg (A), 15 μg (B) and 20 μg (C)) to visualize and resolve the tubulin isoforms present in each sample. The shift of tubulin isoforms towards the acidic side of the gels (right) is proportional to the number of glutamyl units in the side chain. Note the particular migration of class III β-tubulin isoforms (denoted here β’) which are separated from the main β-tubulin stretch (B and C). Monoglutamylated tubulin in HeLa MTs is too minor to be detected by Coomassie staining. Only the tubulin regions are presented.

**Fig. 3.** Partially purified TPG fractions from HeLa cells (A,C,E) and from brain (B,D,F) were assayed with the three types of MTs shown in Fig. 2 and the labeled products were analyzed by 2D-PAGE and fluorography. HeLa MT (A,B), young brain MT (C,D) and adult brain MT (E,F) were all added at the same concentration in the assay (0.1 mg/ml). The short arrowheads indicate the position of unglutamylated isoforms reported from the corresponding Coomassie blue stained gel (not shown), and the long arrowheads indicate the position of the monoglutamylated isoforms. β’ denotes the neuronal class III β-tubulin isoforms present in brain (D and F).
main β-tubulin stretch was clearly labeled with the brain enzyme (Fig. 3D,F) but not with the HeLa enzyme (Fig. 3C,E). These data show that enzymatic forms able to glutamylate class II β-tubulin are present in brain but absent in HeLa cells. Thus, we can conclude that there are not only different TPGs specific for α- and β-tubulin subunits, but also isozymes specific for each or at least subsets of β-tubulin isotypes. Isozymic variants of α-TPG could not be evidenced here because all MT substrates contain almost identical α-tubulin isotypes (α1 and/or α2) glutamylated similarly with HeLa and brain TPG.

**TPG activity varies with the cell cycle**

In a previous report, we showed that glutamylated tubulin is associated with the mitotic apparatus in dividing HeLa cells. An increase in the abundance of glutamylated β-tubulin at mitosis was also suggested (Bobinnec et al., 1998b). To investigate a possible regulation of this process, we followed TPG activity in HeLa cells synchronized by a double thymidine block. Enzyme assays were carried out with total extracts to take into account α- and β-TPG activities without any bias due to their differential solubility. Moreover, we verified that activity measured in total extracts was equal to that measured in supernatant plus pellet fractions. Fig. 4 illustrates the variation of TPG activity during the cell cycle. The maximum activity (set to 100%) was observed in G2-phase and then dropped progressively to ~40% in G1-phase. Interestingly, this decrease seemed to begin before the onset of mitosis and was slower than the rate of progression through mitosis, as revealed by the disappearance of G2/M cells. It has to be noted that, for an unknown reason, a high variability of the results obtained in four independent experiments was observed for S-phase data points.

The data presented above correspond to the sum of α- and β-tubulin incorporation. Similar results were obtained when the two subunits were considered independently (not shown). This means that α- and β-TPG activities are regulated coordinately during the cell cycle. Interestingly, similar results were obtained when brain instead of HeLa MTs were used as substrates (not shown).

**The peak of TPG activity and the peak of glutamylated tubulin are not synchronous in the cell cycle**

As shown above, TPG activity was maximal in G2-phase and decreased during M-phase, although previous immunofluorescence data strongly suggest an accumulation of glutamylated tubulin in mitosis (Bobinnec et al., 1998b). This discrepancy led us to quantify in parallel the levels of both glutamylated tubulin and TPG activities. The sensitivity of the anti-glutamylated tubulin mAb GT335 was improved by biotinylation, allowing immunodetection of the antigen in total HeLa cell extracts after 1D-PAGE. This is illustrated in Fig. 5a where the reactivity of GT335 was probed against total brain (2 μg, lane 1) or HeLa (20 μg, lane 2) extracts, as well as taxotere-purified HeLa tubulin (5 μg, lane 3). A quantitative analysis indicated that the amount of glutamylated β-tubulin in HeLa cells did not exceed 4-5% of the total β-tubulin content (data not shown). The specificity of the response was assessed by 2D-PAGE analysis of HeLa extract (Fig. 5B). A major labeling was associated with β-tubulin and a weak one with α-tubulin. Two additional faint spots, more acidic than tubulin,
were also detected but this apparent cross-reactivity was very minor compared to that associated with \( \beta \)-tubulin.

Fig. 6 shows in parallel, the changes of TPG activity, glutamylated \( \beta \)-tubulin and cyclin A2 levels for one of the synchronization experiments presented in Fig. 4. Immunodetection with GT335 revealed that a low level of glutamylated \( \beta \)-tubulin was maintained during S- and G2-phases, similar to that found in asynchronous cells. When the cells progressed through mitosis, as shown by the degradation of cyclin A2, a 5-fold increase in glutamylated \( \beta \)-tubulin occurred (Fig. 6). Strikingly, this sharp peak appeared ~2-3 hours after the peak of TPG activity, leading to the apparent paradox that the glutamylation level increased when the enzyme activity decreased.

A paradoxical relationship between TPG activity and tubulin glutamylation levels

To study in more detail the relationship between TPG activity and \( \beta \)-tubulin glutamylation in the cell cycle, we used different agents blocking the cells at different time-points in the cell cycle. TPG activity and glutamylated \( \beta \)-tubulin levels were measured in parallel and compared to those obtained for untreated asynchronous growing cells. In HeLa cells blocked at mitosis by either taxotere or nocodazole, glutamylated tubulin accumulated 2- to 4-fold, whereas TPG activity decreased to 25-30% as compared to the control cells (Fig. 7). These results are in agreement with those obtained by the double thymidine block synchronization experiments and confirmed that TPG activity is low in mitotic cells. Interestingly, both drugs had similar effects, suggesting that the polymerization state of tubulin has only a limited influence on this process. However, the accumulation of glutamylated tubulin, although observed in both cases, was 2-fold higher in taxotere than in nocodazole treated cells. This difference could be related to the preferential activity of TPG towards MTs (Regnard et al., 1998).

Cells blocked in S-phase were obtained by treatment with HU, a drug which inhibits the ribonucleotide reductase and provokes the arrest of DNA synthesis. FACS analysis showed that 90% of taxotere or nocodazole treated cells were blocked at mitosis, and ~75% of HU treated cells were blocked at the G1/S boundary and ~25% within the S-phase.

Fig. 7. HeLa cells were blocked either in mitosis with 1 \( \mu \)M taxotere (T) or 1 \( \mu \)M nocodazole (N), or in S-phase with 2.5 mM hydroxyurea (HU). Incubation times were 18 hours in all cases. TPG activity was measured in total extracts with HeLa MTs as substrates and the level of glutamylated \( \beta \)-tubulin was quantified by western blotting with the biotinylated GT335 mAb. Data are expressed as percentage of the value measured for untreated asynchronous growing cells (control cells, CTL) and are means ± s.d. of 2 independent experiments. FACS analysis showed that ~90% of taxotere or nocodazole treated cells were blocked at mitosis, and ~75% of HU treated cells were blocked at the G1/S boundary and ~25% within the S-phase.

DISCUSSION

Isozymic variability of TPG

In mammals, polyglutamylation concerns both tubulin subunits and also most isotypes which have diverging sequences essentially in the 15 last amino-acids containing the
glutamylation site(s) (Eddé et al., 1990; Redeker et al., 1992, 1998; Mary et al., 1994; Rüdiger et al., 1992; Alexander et al., 1991). The data obtained in this study support the existence of isozymic variants of TPG. First, α-TPG and β-TPG, specific for α- and β-tubulin, were biochemically separated (Fig. 1). Second, the cross reactions between TPGs and MTs from brain or HeLa cells indicate that TPGs must also be specific for one or several β-tubulin isotypes (Fig. 3). In particular, unambiguous data were obtained for the glutamylation of the neuronal class III β-tubulin, one of the most divergent isotypes. The results also support the existence of other isozymes since HeLa TPG but not brain TPG was able to glutamylate HeLa β-tubulin. The significant sequence divergence between class I (major in HeLa) and class II (major in brain) isotypes around the glutamylation site could account for this specificity. In contrast, it is difficult to draw a conclusion for the other isotypes because their relative abundance in the substrate MTs is too low. However, one can hypothesize that class Ia and IVb, which are strongly related and share an identical 14 amino acid C-terminal sequence, could be glutamylated by a single TPG isozyme. Whether the same isozyme would also glutamylate the class II isotype, which shares the sequence GFEEEEEE with class IV isotypes (the glutamylation site identified for class II and Ia isotypes is indicated in bold), remains an open question. In conclusion, at least three TPG isozymes could be responsible for the overall glutamylation of β-tubulin isotypes. In the case of α-tubulin, no difference was observed between brain and HeLa TPGs, probably because the α1/2 isotype is the major isotype in both systems. The existence of other α-TPGs specific for isotypes divergent from α1/2 could be suspected, in particular for α4 which has been shown to be glutamylated at two distinct sites (Redeker et al., 1998). Whether the molecular basis of the heterogeneity of TPGs concerns the whole enzyme, a catalytic subunit or an associated factor is a challenging issue. To which extent distinct TPGs are responsible for the initiation and elongation steps in the glutamylation reaction remains an unresolved question. If distinct enzymes are required, HeLa cells in which glutamylated tubulin is essentially monoglutamylated, would be expected to contain mostly initiation enzymes. However, HeLa TPG was shown to elongate efficiently polyglutamyl side chains of α- and β-tubulin from brain (Fig. 3F). On another hand, it seems unlikely that a single TPG could modify indifferently α- and γ-carboxyl groups of the substrate. Interestingly, this question has been addressed in the case of folic acid cofactors which exist mainly as polyglutamate derivatives. In mammals, folylpolyglutamates containing only γ-linkages are apparently synthesized by a single enzyme (Cichowicz and Shane, 1987). In Escherichia coli, α- and γ-linkages are present and two corresponding enzyme activities were separated (Ferone et al., 1986a,b). These examples highlight the importance of the type of the linkages in polyglutamyl chains and suggest that, in the case of tubulin, enzymes catalyzing γ-linkages (denoted γE) could differ from those catalyzing α-linkages (αE). Initiation of polyglutamyl side chains would involve only γE, while elongation could be driven by γE and/or αE. According to that model, the results obtained with HeLa TPG would imply that the same γE is able to both initiate and elongate side chains. Very recently, TPG was isolated from the trypanosomatid Crithidia fasciculata (Westermann et al., 1999). In this case, the enzyme activity was shown to glutamylate preferentially tubulin which is already glutamylated. While it is not known whether the reaction occurs via α- or γ-linkages, these results clearly indicate that this elongation enzyme is unable to initiate polyglutamyl side chains.

 Till now, there is no information about the quaternary structure of TPG. However, the enzyme partially purified from mouse brain seems to be organized in a multimeric structure of ≥300 kDa (Regnard et al., 1998, and unpublished results). The genes encoding the(s) polypeptide(s) are still unidentified. A purified enzyme fraction from C. fasciculata contains a single polypeptide of 40 kDa, perhaps corresponding to a catalytic subunit (Westermann et al., 1999). No sequence information has yet been published for this protein. The specificity towards tubulin subunits and isotypes could be due to isozymic variability of either catalytic polypeptides or associated subunits.

Subcellular localization of glutamylated tubulins and TPG enzymes

Compartmentalization of glutamylated tubulin was already described in highly asymmetrical cells, such as spermatozoa where axonemes but not MTs of the manchette are glutamylated (Fouquet et al., 1994). In HeLa cells, the very stable centriolar structure is highly enriched in glutamylated tubulin, but the dynamic MTs of the mitotic spindle are also glutamylated although with the exception of astral MTs (Bobinnec et al., 1998b). In addition, α- and β-glutamylated tubulins are differentially distributed. Glutamylated β-tubulin is major in the cytoplasmic tubulin pool, whereas glutamylated α-tubulin is found almost only in the centrioles (Fig. 5, lanes 2 to 4, and Bobinnec et al., 1998b). Thus, the compartmentalization of glutamylated tubulin is not solely related to MT stability but suggests also a differential distribution of TPG isozymes. Actually, the low extractability of α-TPG from HeLa cells (Fig. 1B,C) could be an indication of its association with centrosomes which are particularly insoluble cell components. Other mechanisms could also be involved in the compartmentalization, such as differential routing of glutamylated tubulin species within the cell and/or local protection from deglutamylation.

Comparison of TPG activity and the level of glutamylation of tubulin in cells

Tubulin polyglutamylation is a very common modification in neurons and spermatocytes, but is also found in minor amounts in other tissues or cells in culture (Wolff et al., 1992; Fouquet et al., 1994; Bobinnec et al., 1998b, Ranganathan et al., 1998). Glutamylated β-tubulin accounts for ≤5% of the total tubulin in HeLa cells (Fig. 5), compared to >50% in brain (Audebert et al., 1994). Given in addition the huge difference in the tubulin contents (≤1% of total proteins in HeLa cells, ~20% in neurons), the relative amount of glutamylated tubulin to total proteins can be estimated to be 0.04% and 10%, respectively. However, the TPG activity when measured in asynchronous HeLa cell extracts is only ~3-fold lower than in brain extracts. Activities measured in other proliferating cells are in the same range (not shown). However, the level of glutamylated tubulin in cells is also determined by the rate of the reversal deglutamylation reaction, already evidenced in cultured neurons (Audebert et al., 1993). These results thus indicate that
the turnover of the modification is much faster in HeLa cells compared to brain. This difference could reflect the need of a rapid regulation in proliferating cells, compared to non-dividing differentiated neurons.

To our knowledge, we give here the first example of an enzyme activity involved in a posttranslational modification of tubulin which is regulated during the cell cycle. Indeed, when measured in synchronized cells, TPG activity was found to vary in a 3-fold range with a maximum in G2-phase, together with a 5-fold increase in glutamylated tubulin. However, the variation curves of these two related parameters are not linked, the peak of glutamylated tubulin being observed with 2-3 hours delay, at a moment when TPG activity is significantly lower than its maximal value (Fig. 6). Such a paradox was also observed in cells blocked with drugs at different stages of the cell cycle, glutamylated tubulin accumulating when TPG activity is low and vice versa. These results indicate that a complex regulation, probably implicating the deglutamylation reaction, takes place during the cell cycle. It is possible that this regulation concerns not only the level of glutamylated tubulin but also the turnover of the modification. For instance, the constant level of glutamylated tubulin maintained before mitosis could result from the parallel increase in glutamylating and deglutamylating activities, thus leading to a higher turnover of the modification. Changes in the turnover of glutamylation were also observed during neuronal development. Namely, an increase in glutamylated tubulin levels was observed together with a decrease in both glutamylating and deglutamylating activities (Audebert et al., 1993, 1994; Regnard et al., 1998). Because of the preferential activity of TPG towards MTs (Regnard et al., 1998), the increase in MT dynamics at mitosis (for a recent review see Cassimeris, 1999) should hinder the accumulation of glutamylated tubulin. This seems to be avoided by the increase in TPG activity which could first counterbalance this influence and then enable the accumulation of glutamylated tubulin in MTs of the spindle. This could also explain at least part of the delay between the two phenomena. Other levels of regulation, such as the cell cycle dependent expression of tubulin isotypes and/or TPG isozymes or variations of the half-life of tubulin, should not be excluded.

TPG, as well as the reverse deglutamylase activity, might be regulated at the transcriptional or posttranslational level, for instance by phosphorylation events. Whatever the level of regulation, it appears that α- and β-TPG activities are regulated coordinately. Actually, the α/β glutamylation ratio remains constant during a short term process such as cell cycle progression (this report) or a long term process such as neuronal differentiation (Regnard et al., 1998). Interestingly, an opposite variation of α- and β-TPG was observed when HeLa cells were treated with ALLN, an inhibitor of the proteasome and of calpain. A 2-fold decrease of α-TPG activity was observed together with a slight (~20%) increase in β-TPG activity, leading to a significant change in the α/β glutamylation ratio from 1:2 for untreated cells to 1:5 for ALLN treated cells (unpublished results). This is the first example of a non-concerted regulation of α- and β-TPG activities. Thus, it appears that the regulation processes could be common to the two activities or specific for each.

In non-neuronal cells, glutamylation of centrioles and cytoplasmic MTs occurs mainly on the α subunit for the former and on the β subunit for the latter. It is tempting to speculate that increased glutamylation of centrioles on α-tubulin might be necessary to overcome MT destabilization promoted in mitosis. This role could be achieved either by stabilizing the links between tubulin molecules, along or between protofilaments, or by regulating the binding of centrosomal proteins (Bobinnec et al., 1998a). In mitotic MTs, increased glutamylation of β-tubulin might regulate the binding of other partners, for instance molecular motors essential for mitotic progression. The differences in polyglutamyl chain lengths of centriolar and cytoplasmic MTs could represent an additional mechanism to recruit different MAPs. The length of the side chain has been indeed proposed to regulate the binding of MAPs and motors as a molecular potentiometer (Boucher et al., 1994; Larcher et al., 1996).

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