Association of tubulin carboxypeptidase with microtubules in living cells

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Tubulin carboxypeptidase is the enzyme that releases the C-terminal tyrosine from α-tubulin, converting tyrosine-terminated (Tyr) to detyrosinated (Glu) tubulin. The present study demonstrates that this enzyme is associated with microtubules in living cells. We extracted cultured cells (COS-7) with Triton X-100 under microtubule-stabilizing conditions and found tubulin carboxypeptidase activity in the cytoskeleton fraction. We ruled out, by using several control experiments, the possibility that this result was due to contamination of the isolated cytoskeletons by non-associated proteins contained in the detergent fraction or to an artifact *in vitro* during the extraction procedure. The associated carboxypeptidase activity showed characteristics similar to those of brain tubulin carboxypeptidase and different from those of pancreatic carboxypeptidase A. In comparison with cultures at confluence, those at low cell density contained small (if any) amounts of carboxypeptidase activity associated with microtubules. In addition, the enzyme was shown to be associated only with cold-labile microtubules. The tubulin carboxypeptidase/microtubule association was also demonstrated in Chinese hamster ovary, NIH 3T3 and PC12 cells. Interestingly, this association was not observed in cultured embryonic brain cells. Our results demonstrate that tubulin carboxypeptidase is indeed associated with microtubules in living cells. Furthermore, the findings that this association occurs with a subset of microtubules and that its magnitude depends on the degree of confluence of the cell culture indicate that it could be part of the mechanism that regulates the tyrosination state of microtubules.

Key words: detyrosination, post-translational modification, tyrosination.

INTRODUCTION

Among other post-translational modifications, tubulin can be subjected to detyrosination at the C-terminus of the α-subunit by the action of tubulin carboxypeptidase [1–3]. Detyrosinated tubulin (Glu tubulin) can be retyrosinated by a distinct enzyme called tubulin tyrosine ligase. Tubulin carboxypeptidase acts slowly and mainly on microtubules, whereas the ligase acts rapidly and only on non-assembled tubulin [4,5]. Therefore stable microtubules are highly detyrosinated because the carboxypeptidase has enough time to exert its activity before microtubules disassemble. This is not so in highly dynamic microtubules and consequently these structures contain low levels of Glu tubulin and high levels of the tyrosinated (Tyr) form. This has been well documented [6–10] and at present a high content of Glu tubulin is considered to be a marker of stable microtubules in living cells.

We had found [11] that tubulin carboxypeptidase associates with microtubules *in vitro*. Recently we demonstrated [12] that in brain extracts this association is modulated by phosphorylation/dephosphorylation processes. From these results we conceived the idea that the association of the carboxypeptidase with microtubules could be part of the mechanism that regulates the tyrosination state of microtubules. Owing to the eventual implications that this could have in the functioning of microtubules we considered it important to investigate this hypothesis. As a first step, in the present study we investigated whether tubulin carboxypeptidase is associated with microtubules in living cells. Previous studies [7–9,13] reported the accumulation of Glu tubulin in preformed microtubules in cells under different conditions. However, this observation does not demonstrate whether the carboxypeptidase acts in a soluble form or in association with microtubules. The investigation of this question was complicated by the fact that neither an adequate biochemical enzyme assay nor an antibody specific for tubulin carboxypeptidase was available. Therefore in this study the association of the carboxypeptidase with microtubules was investigated by determining the activity of the enzyme in the cytoskeleton fraction obtained by extraction of cultured cells with detergent under conditions that eliminate lipids and soluble compounds, allowing the identification of microtubule-associated proteins [14]. In turn, the enzymic activity was assayed by quantifying the reaction product (Glu tubulin) contained in the isolated cytoskeletons as a function of the incubation time *in vitro*. Double immunofluorescence and densitometry after immunoblotting with antibodies specific for Glu and Tyr tubulin (Glu and Tyr antibodies respectively) were used in the analysis. We found that tubulin carboxypeptidase associates with microtubules in a variety of cultured cells. In COS cells the enzyme associates only with cold-labile microtubules and the magnitude of the association is higher as cell density increases. Interestingly, even in circumstances in which a significant amount of enzyme activity is associated with microtubules, these structures remain mainly tyrosinated, suggesting the necessity of one or more other factors or signals besides the association of carboxypeptidase with microtubules to start massive detyrosination.

MATERIALS AND METHODS

Chemicals

Phosphocellulose P11 was from Whatman. Pancreatic carboxypeptidase A (CPA), PMSF, EGTA, heparin, ATP, aprotinin,

Abbreviations used: CHO, Chinese hamster ovary; CPA, pancreatic carboxypeptidase A; Δ2 tubulin, tubulin dimer containing α-tubulin lacking the two last C-terminal (tyrosine and glutamic) residues; Glu antibody, rabbit polyclonal antibody against Glu tubulin; Glu microtubules, microtubules rich in Glu tubulin; Glu tubulin, tubulin dimer containing α-tubulin lacking the C-terminal tyrosine, exposing a glutamic residue at that end; Tyr antibody, rat monoclonal antibody against Tyr tubulin; Tyr microtubules, microtubules rich in Tyr tubulin; Tyr tubulin, tubulin dimer containing a tyrosine residue at the C-terminus of its α-tubulin subunit.

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benzamidine, 1,10-phenanthroline, Paclitaxel (taxol), 4-chloronaphth-1-ol, cytochalasin D, Triton X-100 and Mes were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Colchicine was from Merck (Darmstadt, Germany). Nitrocellulose (Hybond-C, 0.45 μm) was from Amersham. FluorSave was from Calbiochem (La Jolla, CA, U. S.A).

Antibodies

Glu antibody was prepared in our laboratory by the method of Gundersen et al. [15], obtaining a product with similar properties (specificity and titre). Rat monoclonal YL1/2 antibody (Tyr antibody) specific for Tyr tubulin was from Sera-Lab (U.K.). Rabbit polyclonal antibody against Δ2 tubulin (anti-Δ2) was a gift from Dr. Paturle-Lafanechère (INSERM, Grenoble, France). Mouse monoclonal DM1A antibody specific for total α-tubulin, rabbit antiserum specific for mouse IgG (whole molecule), rhodamine-conjugated goat anti-rabbit secondary antibody, fluorescein-conjugated goat anti-mouse secondary antibody and peroxidase-conjugated Protein A were purchased from Sigma Chemical Co.

Cell cultures

COS-7, NIH 3T3, PC12 and Chinese hamster ovary (CHO) cells were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% (v/v) fetal bovine serum (Serono) at 37 °C in an air/CO_2 (19:1) incubator. Cells were plated at the desired density on plastic Petri dishes (60 or 100 mm in diameter) that contained one or two small coverslips, then grown for 2 days until they reached the desired final density. Routinely, culture medium was renewed at 24 h. Unless stated otherwise, all treatments involving cells were performed at 37 °C. Drugs added to culture medium were prepared in DMSO so that the final solvent concentration did not exceed 0.2% (v/v).

Isolation of the soluble and cytoskeleton fractions

For analysis of confluent and sparse cells, 60 and 100 mm Petri dishes respectively were used. After the cells had been washed with microtubule-stabilizing buffer [90 mM Mes (pH 6.7)/1 mM EGTA/1 mM $MgCl_2$/10% (v/v) glycerol], they were routinely extracted with 2.5 or 6 ml (for 60 or 100 mm dishes respectively) of MSB containing 10 μM taxol, 0.5% (v/v) Triton X-100 and protease inhibitors (10 μg/ml aprotinin, 0.5 mM benzamidine, 5 μg/ml *o*-phenanthroline and 0.2 mM PMSF) at 37 °C for 4 min with frequent gentle agitation. The detergent extract (hereafter called the soluble fraction) was then sucked off and stored under 0 °C; the structures that remained bound to the dishes (the cytoskeleton fraction) were rapidly washed twice with 5 or 12 ml (for 60 or 100 mm dishes respectively) of prewarmed MSB. The isolated cytoskeletons were processed immediately to determine carboxypeptidase activity.

Determination of tubulin carboxypeptidase activity associated with the cytoskeleton

The activity of tubulin carboxypeptidase was determined from the increase in the amount of Glu tubulin as a function of the incubation time of the isolated cytoskeleton preparations. Immediately after isolation, cytoskeletons (contained in several dishes) were incubated at 37 °C in microtubule-stabilizing buffer (2.5 or 6 ml for 60 and 100 mm dishes respectively) containing 5 μM taxol and protease inhibitors. After different periods, once the coverslips had been removed, the dishes were processed for immunoblots to determine the amount of Glu tubulin as described below. The coverslips were processed for immunofluorescence to detect the Glu microtubules (those rich in Glu tubulin) and Tyr microtubules (those rich in Tyr tubulin).

Immunoblots

After incubation, the incubation media were discarded and the cytoskeletons were immediately dissolved in 150 or 390 μl (for 60 and 100 mm Petri dishes respectively) of sample buffer [16]. After 1 min at 90 °C the extract was transferred to a plastic tube and stored at −70 °C until immunoblotting was performed. The soluble fraction was processed to eliminate the detergent from the protein [17]. The resulting protein pellet was dissolved in sample buffer (an equal volume to that of the cytoskeleton counterpart), heated at 90 °C for 1 min and stored at −70 °C until use. Soluble and/or cytoskeleton samples were subjected to SDS/PAGE [10% (w/v) gel] [16] and then transferred to nitrocellulose sheets [18]. When indicated, this was done in duplicate by running two identical gels in parallel. One of the nitrocellulose sheets was subjected to exhaustive treatment with CPA as described below. After blocking for 1 h with 5% (w/v) fat-free dried milk dissolved in TBS containing 0.1% (v/v) Triton X-100, blots were treated for 1 h at room temperature with one of the following antibodies: Glu, Tyr (YL 1/2), DM1A or Δ2 antibody (diluted 1:200, 1:1000, 1:1000 or 1:10000 respectively). After washing, the sheet that had been treated with DM1A was incubated with rabbit anti-mouse IgG (dilution 1:200) and the sheet that had been treated with YL 1/2 was incubated with rabbit anti-rat IgG (dilution 1:200). All sheets were then incubated for 1 h at room temperature in the presence of horseradish peroxidase conjugated to Protein A (dilution 1:1000). After washing, colour was developed by using 4-chloronaphth-1-ol as the chromogen. In some experiments, after the blocking step and before incubation with the primary antibody, one of the two nitrocellulose sheets was incubated in 10 ml of TBS containing 10 μg/ml CPA. After 30 min at 37 °C, the sheet was exhaustively washed with TBS containing 0.1% (v/v) Triton X-100 and treated as described above with primary antibodies. Because CPA converts all of the Tyr tubulin to the Glu form, this CPA-treated sheet was used to determine the amount of total tubulin.

Quantification of Glu tubulin

After colour development, immunoblots that had been treated with Glu antibody were dried and scanned with a Shimadzu densitometer (model CS-930) in reflectance mode at 600 nm. Frequently the colour intensity was heterogeneous across individual bands, so each band was scanned entirely in a single step by using a 6 mm slit.

The absorbance determined for each band in the nitrocellulose sheet that had not been treated with CPA represented the amount of Glu tubulin present in that particular sample of cytoskeleton. In contrast, the absorbance determined for the equivalent band in the CPA-treated sheet represented the maximal amount of Glu tubulin that could be obtained (total detyrosinable tubulin) in that sample. This was based on the observation that treatment with CPA resulted in the total removal of C-terminal tyrosine from tubulin (see Figure 3D). Throughout this work, the amount of Glu tubulin in a particular cytoskeleton sample is expressed as a percentage of the total detyrosinable tubulin, calculated as $100A_{noCPA}/A_{CPA}$, where A_{noCPA} is the absorbance of the sample not treated with CPA and A_{CPA} is the absorbance of the CPA-treated sample.

Provided that absorbance values of numerator and denominator correspond to identical samples, this expression is not

dependent on the amount of protein loaded. This is an important advantage because it thereby becomes unnecessary to load identical protein samples for comparison between different cytoskeleton preparations. When very different colour intensities were obtained for a particular sample in the CPA-treated and untreated nitrocellulose sheets, eventual conflicts derived from the non-linearity of the assay were overcome by repeating the immunoblots, modifying the volumes of the samples so as to obtain absorbances differing by less than 50%.

It is known that total brain tubulin is composed of three species: tyrosinated, detyrosinated and non-tyrosinable tubulin [19,20]. This last species was characterized as lacking the two C-terminal residues (Tyr and Glu); it was therefore called Δ2 tubulin [21,22]. Analysis by immunoblot of COS cytoskeleton preparations showed practically no Δ2 tubulin in freshly prepared cytoskeletons and less than 5% of the total tubulin in cytoskeletons that had been incubated for 2 h under the conditions described above for the determination of tubulin carboxypeptidase associated with cytoskeletons. We therefore consider that the percentage of Glu tubulin with respect to total detyrosinable tubulin as calculated above is a good approximation to the percentage of Glu tubulin with respect to total tubulin.

Immunofluorescence

At the end of the incubation time of the isolated cytoskeletons, the preparations on the coverslips were fixed with 0.25% (v/v) glutaraldehyde and 2% (w/v) paraformaldehyde in PBS (30 min at room temperature), then stored at 2–4 °C in PBS containing 0.02% sodium azide until use. Fixed cytoskeletons were incubated with 2% (w/v) BSA in PBS (PBS/BSA) for 30 min and then stained by double indirect immunofluorescence with the use of Glu and Tyr antibodies (1:200 and 1:500 dilutions respectively). Secondary antibodies were fluorescein-conjugated goat anti-rat IgG and rhodamine-conjugated goat anti-rabbit IgG and were used simultaneously at 1:200 dilution in PBS/BSA. Coverslips were mounted in FluorSave and observed for epifluorescence on an Axioplan microscope (Zeiss, Cologne, Germany).

Protein determination

Proteins in the soluble and cytoskeleton fractions were precipitated quantitatively by the method of Wessel and Flügge [17]. This procedure eliminates the need for Triton X-100 and SDS, allowing protein determination by the method of Lowry et al. [23] without interference.

RESULTS

Isolation of the soluble and cytoskeleton fractions

Because the quality of the isolated cytoskeleton fraction with regard to its integrity and the complete elimination of soluble (non-associated) components was crucial for this work, we investigated the distribution of total protein and tubulin between the soluble and cytoskeleton fractions. Figure 1(A) shows the electrophoretic pattern of proteins of each fraction. The cytoskeleton fraction (lane 3) contained a smaller variety of polypeptides than the soluble fraction (lane 2). The corresponding immunoblot (Figure 1B) shows that most tubulin was recovered in the cytoskeleton fraction (compare lanes 5 and 6). To estimate the efficiency of the extraction method to eliminate non-associated proteins from the cytoskeleton fraction, cells were incubated for 1 h in the presence of 100 μM colchicine to disassemble the microtubule network; they were then extracted

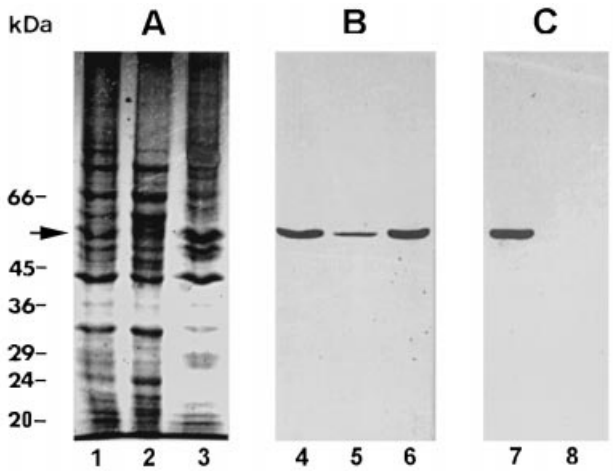

Figure 1 Electrophoretic analysis of total protein and tubulin recovered in the soluble and cytoskeleton fractions

COS cells were grown to approx. 70% confluence and the soluble and cytoskeleton fractions were isolated. Aliquots from both fractions and from whole cells, equivalent to the same number of cells, were subjected to electrophoresis in duplicate. One gel was stained with Coomassie Blue (**A**); the other was used for blotting and immunostaining with DM1A (**B**). Lanes 1 and 4, whole cells; lanes 2 and 5, soluble fraction; lanes 3 and 6, cytoskeleton fraction. In a separate experiment (**C**), before extraction with detergent, the cells were incubated for 1 h at 37 °C in the presence of 0.1 mM colchicine (added to the culture medium) and then the soluble (lane 7) and cytoskeleton (lane 8) fractions were isolated and analysed for tubulin by immunoblotting with DM1A. The positions of molecular mass markers are indicated at the left. The arrow indicates the position of α-tubulin.

in the routine manner. Under these conditions, practically all of the tubulin was recovered in the soluble fraction (Figure 1C, lanes 7 and 8). This estimate was confirmed by a quantitative analysis of protein and tubulin (Table 1). As can be seen, the ratio of tubulin to total protein in the cytoskeleton fraction was severalfold higher than that in the soluble fraction. A second detergent extraction of cytoskeletons did not alter significantly either the pattern of proteins or the amount of tubulin (results not shown), confirming the effectiveness of the method in preserving the integrity of microtubules. The recovery of practically all of the tubulin (more than 95%) in the soluble fraction when cells were preincubated in the presence of colchicine is an important result because it indicates that proteins that are not associated with the cytoskeleton can be removed efficiently. It therefore seems reasonable to assume that any compound that is present in the cytoskeleton fraction should be considered as being associated in some way with this structure rather than as a contaminant.

Tubulin carboxypeptidase activity is associated with native microtubules

Cytoskeletal preparations obtained from cultured COS cells were incubated at 37 °C for different periods to determine whether Tyr microtubules were converted to Glu microtubules. At the end of each incubation period, the preparations were analysed by immunoblot and double immunofluorescence with Glu and Tyr antibodies as described in the Materials and methods section. Figure 2 shows the results of the immunofluorescence study. At zero time of incubation, microtubules were brightly stained by the Tyr antibody (Figure 2B), whereas only few microtubules, especially near the centrosome, were faintly stained with the Glu antibody (Figure 2A), indicating that in freshly isolated cyto-

Table 1 Distribution of tubulin and total protein between the soluble and the cytoskeleton fractions

Cells were grown to near-confluence in 60 mm Petri dishes; the soluble and cytoskeleton fractions were isolated as described in the Materials and methods section. Proteins from both fractions were precipitated quantitatively by the method of Wessel and Flügge [17]. Total protein was determined by the method of Lowry et al. [23] and tubulin by densitometry on immunoblots treated with DM1A. Values corresponding to the soluble and cytoskeleton fractions were calculated as percentages of the sum of both fractions. Recovery values were calculated as the sum of the values of the soluble and cytoskeleton fractions with respect to those measured in the whole cell. For colchicine treatment, the drug was added (100 μM final concentration) to the culture medium 1 h before extraction with detergent. Values are means $\pm$ S.D. for three independent experiments.

Drug added before extraction	Preparation	Tubulin (%)	Total protein (%)	Ratio of tubulin to total protein
None	Whole cell	100	100	1
	Soluble fraction	18 $\pm$ 4	60 $\pm$ 3	0.30
	Cytoskeleton fraction	82 $\pm$ 4	40 $\pm$ 3	2.05
	Recovery (soluble + cytoskeleton)	95 $\pm$ 5	93 $\pm$ 5	
Colchicine	Soluble fraction	96 $\pm$ 4	68 $\pm$ 6	1.41
	Cytoskeleton fraction	4 $\pm$ 2	32 $\pm$ 5	0.12
	Recovery (soluble + cytoskeleton)	92 $\pm$ 5	93 $\pm$ 4	

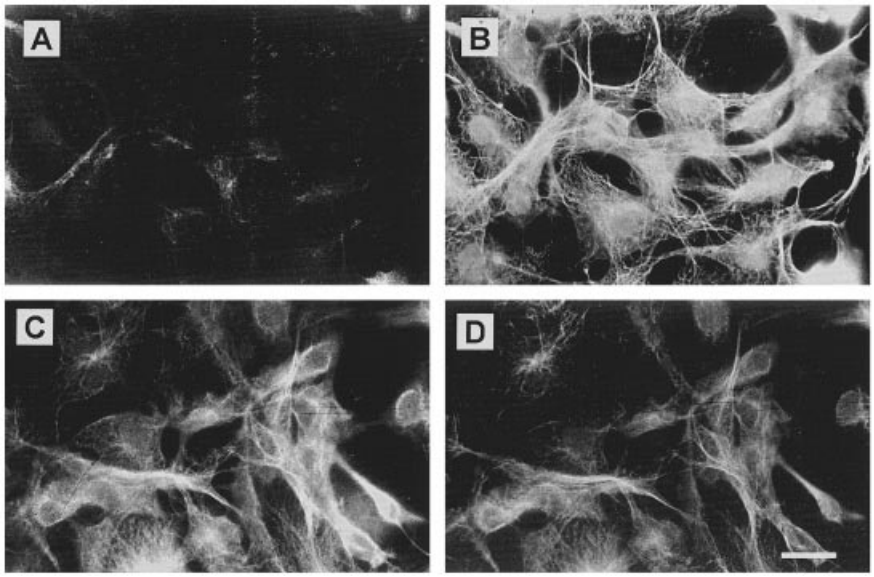

Figure 2 Visualization of Glu microtubules and Tyr microtubules by double immunofluorescence, showing that detyrosination occurs during the incubation of isolated cytoskeletons

COS cells were grown to near-confluence on glass coverslips; cytoskeletons were isolated. The samples were fixed immediately (**A**, **B**) or after a 2 h incubation period at 37 °C (**C**, **D**) and double-stained with Glu (**A**, **C**) or Tyr (**B**, **D**) antibodies. Scale bar, 20 μm.

skeletons, microtubules consist mainly of Tyr tubulin. In isolated cytoskeletons that had been incubated for 2 h, the immunostaining of the Tyr microtubules (Figure 2D) was significantly lower than that at zero time, whereas microtubules rich in Glu tubulin (Figure 2C) were now clearly seen throughout the whole cell, indicating that tubulin carboxypeptidase was active in these preparations. Inspection of many fields in preparations similar to those shown in Figure 2 revealed that cytoskeletons of all of the cells were detyrosinated homogeneously, that is, there were no particular cells that behaved differently from others. Furthermore, immunofluorescence analysis of the microtubule network of single cells did not allow us to distinguish between different degrees of detyrosination in individual cytoplasmic microtubules.

The results from the qualitative analysis presented above were confirmed by immunoblot analysis with antibodies against both tubulin isoforms. Cytoskeleton preparations isolated from COS cells were incubated for different periods of time. Figure 3(A) shows that at zero time of incubation (lane 1) Glu tubulin stained as a faint band, increasing in intensity at longer durations of incubation, indicating the presence of carboxypeptidase activity in these cytoskeleton preparations. Correspondingly, Tyr tubulin staining (Figure 3B) decreased as a function of the incubation period. When the nitrocellulose sheet with the transferred proteins was treated with CPA before being immunostained (Figure 3C), the intensity of the Glu tubulin band at any time of incubation was higher than that of the corresponding band in the equivalent blot without treatment with CPA (Figure 3A), indicating that Tyr tubulin was not limiting for the activity of tubulin carboxypeptidase. Treatment with CPA was effective in releasing all of the C-terminal tyrosine from α-tubulin (Figure 3D). Therefore the bands in Figure 3(C) stained with Glu tubulin after treatment with CPA represented total tubulin for each

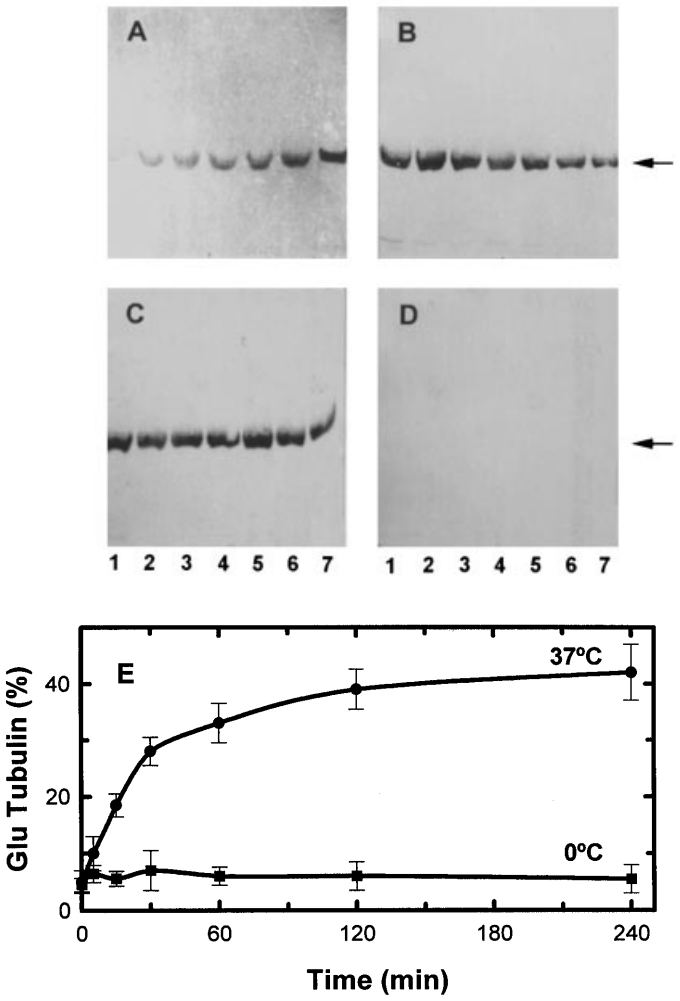

Figure 3 Amount of Glu and Tyr tubulin in isolated cytoskeletons as a function of the incubation duration

COS cells were grown to approx. 70% confluence and the cytoskeleton fraction was isolated and incubated at 37 °C as described in the Materials and methods section. After incubation for the stated durations, samples were subjected to SDS/PAGE and immunoblotting with Glu (**A**, **C**) and Tyr (**B**, **D**) antibodies. Before immunostaining, the nitrocellulose membranes (**C**, **D**) were incubated with pancreatic CPA, which produced the full detyrosination of tubulin. In these panels the amount of protein loaded in each lane was half of that loaded in (**A**) and (**B**), which were not treated with CPA. Arrows at the right indicate the position of α-tubulin. Lanes 1–7 correspond to 0, 5, 10, 30, 60, 120 and 240 min of incubation respectively. (**E**) the amount of Glu tubulin is shown as a function of the incubation time, as determined from densitometric scans of blots obtained such as those shown in (**A**) and (**C**) (for details see the Materials and methods section). To show the temperature dependence of detyrosination, the cytoskeletons were incubated at 37 °C (●) or 0 °C (■). Values are means ± S.D. for four independent experiments.

corresponding sample. Finally, it is important to note that no band other than those corresponding to α-tubulin can be seen in any lane of the blots, indicating a good specificity of the antibodies as well as the absence of endoprotease activity from the cytoskeleton preparations.

The amount of Glu tubulin as a percentage of total detyrosinable tubulin was calculated as described in the Materials and methods section from a densitometric analysis of several blots similar to those shown in Figures 3(A) and 3(C) and averaged from independent experiments. Results show that the amount of Glu tubulin increased gradually during the incubation of the cytoskeleton preparations. (Figure 3E). At zero time of incubation (freshly isolated cytoskeletons), the amount of Glu tubulin was very low (approx. 4%) but increased up to nearly 40% after 2–3 h of incubation of the cytoskeletons. Practically no detyrosination occurred if the incubation was at 0 °C. Incubation periods of longer than 3–4 h produced excessive detachment of cytoskeletons from the dish. We were therefore unable to determine whether this percentage of Glu tubulin was the maximal value that could be reached by incubation of the cytoskeletons *in vitro*.

These results demonstrate the presence of a carboxypeptidase activity associated with the cytoskeletons. It should be noted that similar results were obtained when more drastic extraction conditions were used, such as increasing 10-fold the volume of the extraction buffer, twice repeating the extraction step, increasing the concentration of Triton X-100 to 2% (v/v), or combining two or more of these conditions (results not shown). Furthermore, similar results were obtained when the incubation of cytoskeletons was continued in the extraction buffer instead of washing. These results indicate that the presence of compounds contained in the soluble fraction neither inhibited nor stimulated the detyrosination of microtubules, at least under the experimental conditions described.

Finally, when the cells were treated, before extraction, with cytochalasin D to disrupt actin microfilaments, the magnitude of the carboxypeptidase activity associated with the cytoskeleton was similar to that of control cells (without cytochalasin) (results not shown). This indicates that microfilaments do not act as a support for the enzyme.

Association of tubulin carboxypeptidase activity with cold-labile and cold-stable microtubules

It is known that many types of tissue and cultured cell possess subsets of microtubules with properties different from the average. One example is that of cold-stable microtubules, which do not depolymerize when maintained at 0 °C. When confluent COS cells were kept in the cold for 30 min before extraction with detergent, resistant microtubules accounted for approx. 20% of the total. This figure was calculated by densitometry of Western blots (results not shown). Figure 4(B) shows (1) that cold-stable microtubules were depleted of Glu tubulin and (2) that the content of Glu tubulin did not increase as a function of incubation time, indicating that these microtubules had no associated carboxypeptidase activity. Consequently, the activity of tubulin carboxypeptidase was associated with cold-labile microtubules. In addition, we found that stabilizing microtubules by incubating the cells in the presence of 20 μM taxol for 10 min before exposure to cold prevented the disassembly of cold-labile microtubules; coincidentally, carboxypeptidase activity was found in the cytoskeleton fraction in a magnitude similar to that found in the control. These results indicate that the enzyme became a soluble entity when its support, that is cold-labile microtubules, was disassembled. Besides the obvious importance of the existence of subsets of microtubules containing (or not) associated carboxypeptidase, it should be noted that the fact that the enzyme became detergent-soluble when microtubules disassembled (by treatment with cold) reinforces the idea that the enzyme was actually bound to microtubules and not adventitiously retained in the detergent-extracted cells.

Effect of carboxypeptidase inhibitors on the activity associated with the cytoskeleton

To characterize the carboxypeptidase activity associated with the cytoskeletons, we investigated the effect of different compounds

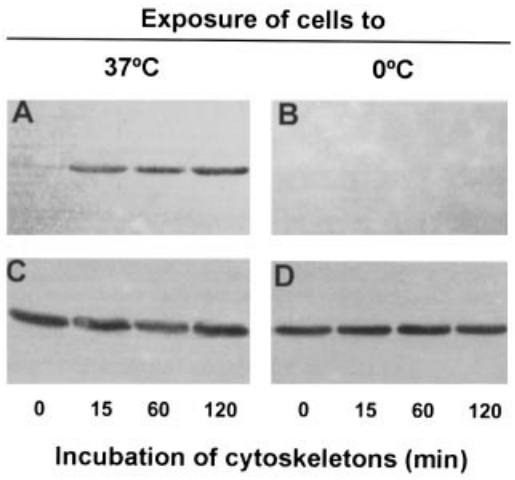

Figure 4 Effect of low temperature on carboxypeptidase/microtubule association

COS cells were grown to approx. 70% confluence. The dishes were exposed to 0 °C for 30 min before isolation of the cytoskeletons. The isolation procedure was then done as routinely except that it was performed at 0 °C and taxol was omitted from the extraction buffer. As a control, cells that had been maintained at 37 °C were also processed in parallel. The cytoskeletons isolated from both sets of cells were incubated at 37 °C for the stated periods and then processed for Western blotting and treated with Glu antibody as described in the legend to Figure 3. (**A**, **C**) Control cells maintained at 37 °C; (**B**, **D**) cells exposed to 0 °C. Before immunostaining, nitrocellulose membranes were treated (**C**, **D**) or not (**A**, **B**) with CPA. Owing to the small amount of tubulin in the cytoskeletons from cold-exposed cells, it was necessary to concentrate these samples to achieve loading amounts of total tubulin comparable to those from controls.

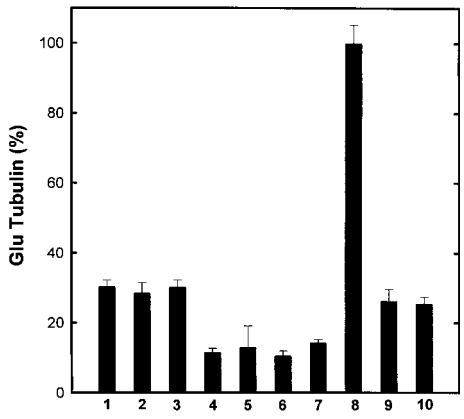

Figure 5 Effect of carboxypeptidase inhibitors on detyrosination of cytoskeletal microtubules

COS cells were grown to 70% confluence and the cytoskeletons were isolated and incubated as routinely to measure detyrosination activity in the presence of the following compounds: 1, none; 2, carboxypeptidase inhibitor from potato (CPI; 100 μg/ml); 3, 8-hydroxyquinoline-5-sulphonic acid (10 mM); 4, $ZnCl_2$ (1 mM); 5, NaCl (0.5 M); 6, heparin (50 μg/ml); 7, ATP (10 mM); 8, CPA (2 μg/ml); 9, CPA (2 μg/ml) plus CPI (100 μg/ml); 10, CPA (2 μg/ml) plus 8-hydroxyquinoline-5-sulphonic acid (10 mM). After incubation at 37 °C for 30 min, the amount of Glu tubulin was measured as described in the Materials and methods section. Values are means ± S.D. for four independent experiments.

previously recognized for their inhibitory effects on partly purified rat-brain tubulin carboxypeptidase. As can be seen in Figure 5, $ZnCl_2$ (final concentration 1 mM), NaCl (0.5 M), heparin (50 μg/ml) and ATP (10 mM) inhibited the carboxypeptidase activity associated with the cytoskeleton in magnitudes similar to those reported for the previously characterized rat-brain tubulin carboxypeptidase [1,24–27]. In contrast, the pancreatic carboxypeptidase inhibitors potato CPA inhibitor and 8-hydroxyquinoline-5-sulphonic acid did not inhibit the carboxypeptidase activity associated with cytoskeletons (Figure 5, bars 1–3) when tested at concentrations that effectively inhibited CPA (Figure 5, bars 8–10).

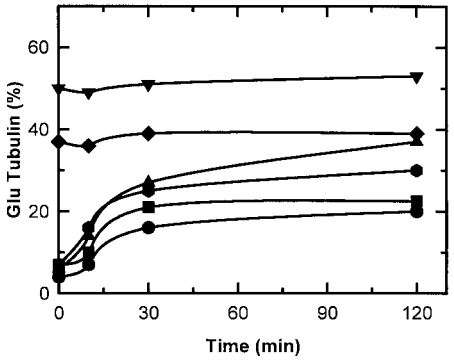

Figure 6 Association of tubulin carboxypeptidase with microtubules in different cell types

The course of detyrosination in cytoskeletons isolated from different cell types was determined as described for COS cells in the legend to Figure 3(E). Symbols: ▲, COS cells; ■, CHO cells; ●, NIH 3T3 cells; ⬢, PC12 cells; ◆,▼, respectively freshly obtained and 3-day-cultured cells isolated from brains of 7-day-old chick embryos.

Tubulin carboxypeptidase/microtubule association occurs in different types of cultured cell

We investigated whether the association of tubulin carboxypeptidase with microtubules was a phenomenon particular to COS cells or was common to other types of cell. We found that CHO, NIH 3T3 and PC12 cells also contained this enzyme in association with microtubules (Figure 6). Although it seems that the amount of enzyme associated depended on the type of cell, this should be taken with caution because, as demonstrated below for COS cells, the magnitude of the association depended on the density of the cultured cells and this parameter was not taken into account when the present experiments were performed.

Figure 6 also shows that embryonic brain cells, despite containing a high Glu tubulin content compared with that of non-neuronal cells, seem not to have tubulin carboxypeptidase associated with microtubules because no increase in the amount of Glu tubulin could be observed with increasing incubation period. This was true of isolated cells both before culturing (round cells) and after several days in culture (cells with highly developed processes).

Association of tubulin carboxypeptidase with microtubules in sparse and confluent cells

During the course of these studies we observed much variation between the results from independent experiments. This was due to variations in the degree of confluence between cultures. We found that cells cultured at low density (10–20% confluence) contain practically no tubulin carboxypeptidase activity associated with microtubules (Figure 7). In contrast, microtubules of cells at 80–100% confluence showed a relatively high carboxy-

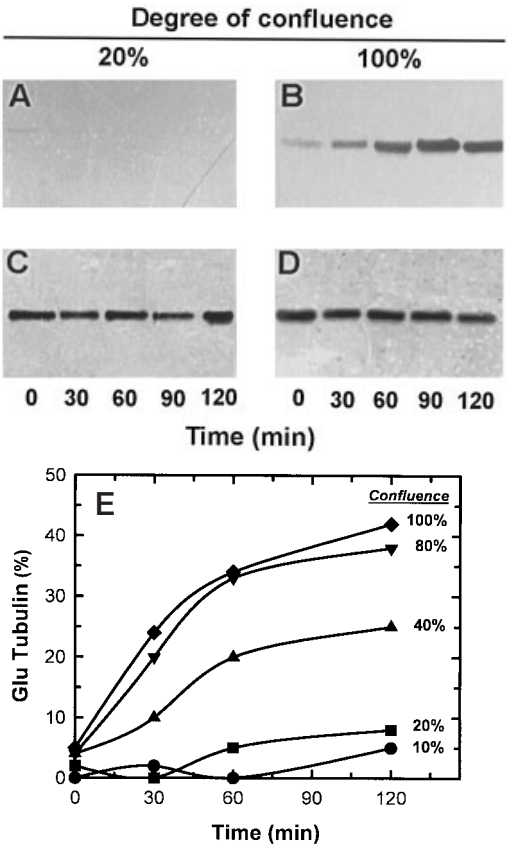

Figure 7 Association of tubulin carboxypeptidase with microtubules in cell cultures at different degrees of confluence

COS cell cultures were started in such a way that after 2 days they reached different cell densities. These densities were expressed in terms of the protein content of each culture as a percentage of that in a monolayer confluent culture. After the isolation of cytoskeletons, the course of detyrosination was determined as described in the legend to Figure 3. (**A**–**D**) Blots immunostained with Glu antibody, treated (**C**, **D**) and not treated (**A**, **B**) with CPA, corresponding to sparse (20% confluence) and confluent (100% confluence) cultures. To ensure the loading of similar amounts of tubulin, the volume of the solution used for the resuspension of cytoskeletons was adjusted in accordance with cell densities. The amount of tubulin loaded per well in (**A**) and (**B**) was double those in (**C**) and (**D**). (**E**) Detyrosination course of microtubules from cells cultured at the different cell densities indicated at the right.

peptidase activity. After 2 h of incubation, approx. 50% of the tubulin was detyrosinated. Tubulin that remained as Tyr tubulin after this incubation could be detyrosinated completely by a subsequent 1 h incubation with a preparation of tubulin carboxypeptidase partly purified from bovine brain (results not shown). Another interesting observation in Figure 7 is that microtubules from 80–100% confluent cells, despite containing a substantial amount of associated tubulin carboxypeptidase, are mainly in the tyrosinated state (note the low proportion of Glu tubulin at zero time incubation).

DISCUSSION

In 1983 we demonstrated [11] that tubulin carboxypeptidase can associate with microtubules during the reconstitution of these filaments *in vitro*. Recently we found that this association can be modulated by phosphorylation/dephosphorylation processes [12]. Here we have shown that this enzyme can also associate with microtubules in living cells. By extracting cells with detergent we isolated microtubule-containing cytoskeletons and detected the presence of the enzyme associated with them. Because an antibody against the enzyme was not yet available, we were compelled to monitor the presence of the enzyme by determining its catalytic activity in the cytoskeleton preparations. For this, native microtubules included in the cytoskeleton preparations were used as a substrate for the associated enzyme; the reaction product, Glu tubulin, was quantified as a function of the incubation time. Because a significant increment in the content of Glu tubulin was observed, we inferred that the enzyme was associated with microtubules.

Certainly, we have no direct evidence that the carboxypeptidase is associated with microtubules rather than with some other cytoskeletal element. However, because (1) treatment of the cells with cytochalasin D had no effect on the magnitude of the associated enzyme (result not shown), (2) cytoskeletons became free of carboxypeptidase activity when cold-labile microtubules were disrupted (Figure 4), (3) microtubules are the natural substrate of the enzyme [4,5,9], and (4) the enzyme associates with microtubules *in vitro* [11,12,26], we consider that the carboxypeptidase is associated with microtubules rather than with another cytoskeletal structure.

Another aspect to be considered is the identity of the carboxypeptidase associated with native microtubules in non-nerve cells. The response of the associated enzyme to several well-known carboxypeptidase inhibitors indicated (Figure 5) that it is obviously different from pancreatic CPA and carboxypeptidase B and quite similar to bovine brain tubulin carboxypeptidase. The low level of enzyme activity and the limited availability of the starting material makes it difficult to characterize COS cell carboxypeptidase biochemically in cell extracts. In fact, by using the most sensitive methods available [12,26] we attempted to measure carboxypeptidase activity in supernatants from the high-speed centrifugation of cell homogenates (cells sonicated after exposure to cold for 1 h). The carboxypeptidase could not be measured with accuracy owing to low levels of activity and high dispersion of values (results not shown).

The occurrence of the association of carboxypeptidase with microtubules in NIH 3T3, PC12 and CHO cells as well as in COS cells (Figure 6) suggests that this is a property widely distributed in non-nerve cells, although it is premature to generalize the concept. Nevertheless, it was surprising that cells of nerve origin (isolated from chick brain embryos) whose enzyme activity content was relatively high and in which almost half of all tubulin was detyrosinated, contained carboxypeptidase that did not seem to be associated with microtubules because detyrosination after the isolation of cytoskeletons could not be observed (Figure 6). In this respect we can present three possible interpretations. First, the enzyme is actually not associated with microtubules; secondly, the enzyme is associated with microtubules in small, selected regions of the cells so that detyrosination seems negligible when averaged with total tubulin in the immunoblots; and thirdly, the enzyme remains associated with microtubules that have already been detyrosinated in the living cell and therefore this pool of carboxypeptidase cannot be detected by the system (enzymic assay) used in the present work. A combination of the second and third possibilities supports the idea that the enzyme would in fact be associated with microtubules but because most of them have already been detyrosinated (perhaps owing to stabilization of these structures), only those contained in selected regions of the cell where microtubules are mainly dynamic (perhaps the distal portions of the neural processes) could be a potential substrate for the associated carboxypeptidase. We have not further investigated these hypotheses, but in any case it should provide an important piece of evidence in describing the tyrosination/detyrosination machinery of the nervous cell.

Methodological considerations

The extraction procedure used in this work is based on that described by Solomon [14], which has been proved to be useful in identifying microtubule components in several types of cell, both established lines and primary cells [28–31]. Cells cultured on plastic Petri dishes were extracted with 0.5 % (v/v) Triton X-100 under microtubule-preserving conditions; the remaining cytoskeletons were used to detect the presence of tubulin carboxypeptidase activity. The extraction procedure typically removes lipids, small molecules and soluble proteins (approx. 70 % of the total cell proteins) [14]. The cytoskeleton fraction remains bound to the plastic surface and contains microtubules, microfilaments, intermediate filaments, nuclei and some membrane components [14,30]. Provided that the soluble elements have been completely eliminated, any compound remaining in the cytoskeleton fraction should be considered as either a constituent or 'associated' with this structure. To address these critical points we demonstrated that our extraction procedure was efficient in preserving native microtubules (Figures 1 and 2, and Table 1) and in eliminating the soluble elements from the cytoskeleton fraction (Figure 1 and Table 1). The recovery of minor (if any) amounts of tubulin in the cytoskeleton fraction when extraction was performed after microtubule disintegration by cell treatment with colchicine represents strong evidence that our procedure efficiently eliminates soluble components. This result greatly decreases the possibility that the carboxypeptidase activity found in the cytoskeleton fraction was due to contamination or was simply retained in the cytoskeleton network.

The possibility that the association reported here was due to an experimental artifact during the isolation of cytoskeletons can reasonably be ruled out from the following observations: first, tubulin carboxypeptidase associates with microtubules *in vitro* [11,12,26], and secondly, the association does not occur in sparse COS cells (Figure 7), nerve cells (Figure 6) or cold-treated COS cells (Figure 4). These results strongly indicate that no physical condition or chemical component of the extraction buffer induced the enzyme to associate with microtubules. We therefore conclude that tubulin carboxypeptidase is found in the cytoskeleton fraction because the enzyme was already associated with microtubules in the living cell.

Tubulin carboxypeptidase/microtubule association is regulated in the living cell

The association of tubulin carboxypeptidase with microtubules depends on the physiological status of the cell. In effect, as cell density varies, some kind of signal seems to be transmitted between cultured cells, inducing changes in the association between carboxypeptidase activity and microtubules (Figure 7). We do not know whether this is due to cell density itself or to transitions between dividing and quiescent states. Experiments with cells in the quiescent or activated state (by manipulation of serum concentration) will serve to address this question in the future. From the knowledge that the tubulin carboxypeptidase/microtubule association is modulated by phosphorylation/dephosphorylation processes [12] it is reasonable to consider the possibility that cell-cycle-regulated kinases such as cdcs (cell division cycle) and cdks (cyclin-dependent kinase) are involved in the signalling that regulates the carboxypeptidase/microtubule association as cell density varies. Perhaps a similar regulation system is responsible for the discrimination in the association of the enzyme with cold-labile and cold-stable microtubules (Figure 4). Although the division of cytoplasmic microtubules according to their response to cold is an artifice, it is useful in detecting the existence of differential microtubule subpopulations in the cell. This raises the interesting possibility that the enzyme is not homogeneously associated with all of the microtubules but to only a specific subset of them or to segments of microtubules as a predeterminant of their future detyrosination. This possibility could not be tested because the immunofluorescence technique that we used in this work had insufficient resolution.

Finally, it is an intriguing observation that even when a high activity of carboxypeptidase is associated with microtubules in confluent cultures, these polymers in the living cells are mainly in the tyrosinated state (note the low proportion of Glu tubulin in cytoskeletons at zero time of incubation in Figure 7). This can be easily explained on the basis of the highly dynamic behaviour of microtubules in dividing cells, as demonstrated previously [32,33]. Anyway, the question remains about the purpose of the cell in associating much of the carboxypeptidase with microtubules just reaching confluence. Given that elevated levels of Glu tubulin are found in cells undergoing morphogenesis [10] and already differentiated cells [8], we are tempted to assume that this greater association is one of the sequential events in preparing the cytoskeletal machinery for cell differentiation. As differentiation cannot proceed under our culture conditions, detyrosination of microtubules does not continue; we therefore found a majority of Tyr microtubules. It seems that the carboxypeptidase/microtubule association is not in itself enough to produce detyrosination of microtubules, but it could be a necessary condition. After association, variations in the microtubule dynamics could control the rate of detyrosination. Two extreme examples could be the situations in nervous cells where the enzyme could remain associated with microtubules that had been already detyrosinated because these were stable microtubules and, in contrast, in COS cells at confluence (Figure 7) in which the carboxypeptidase is associated with microtubules that remain with a high Tyr tubulin content because they are dynamic microtubules. At this point we should look again at the possibility that the enzyme does not associate homogeneously with all of the microtubules but to a selected population of microtubules or to selected segments of them. Although only speculative, it is an interesting hypothesis that the association of the enzyme with microtubules has the physiological role of selectively 'labelling' the microtubules or segments of them that are needed to be detyrosinated and that stabilization is the trigger for detyrosination. Further studies on this point are in progress in this laboratory.

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