Regulation of Cytoplasmic Tubulin Carboxypeptidase Activity during Neural and Muscle Differentiation: Characterization Using a Microtubule-Based Assay†

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ABSTRACT: A cycle of posttranslational modification of α-tubulin has previously been described in higher eukaryotes, in which a C-terminal tyrosine residue is removed and replaced by two complementary cytoplasmic enzymes. The activity of the detyrosinating enzyme, tubulin carboxypeptidase (TCP), and its potential for regulating the level of detyrosinated (Glu) subunits in microtubules (MTs) is of great interest, since TCP catalyzes the primary modification of tubulin and since the level of Glu α-tubulin in MTs increases during a variety of differentiative and morphogenetic events. As a first step in examining the role of TCP in cellular morphogenesis, it was necessary to develop an assay for TCP with sufficient sensitivity and specificity to detect TCP activity during these events. Unlike previously described assays for TCP, ours makes use of the affinity TCP exhibits for MTs. NGF-induced neurite outgrowth in PC-12 cells was accompanied by a moderate (~2-fold) increase in TCP activity, while myogenesis of L_6 cells resulted in an almost insignificant decrease in activity. Measurements of TCP activity during differentiation were correlated with the level of extract Tyr tubulin, which increased (~37%) during neurite outgrowth and was unchanged during myogenic differentiation. Our results suggest that TCP activity is regulated relative to its substrate, Tyr tubulin, and that changes in MT dynamics, rather than enzymatic activities, are the primary determinants of MT posttranslational modification state during differentiation. In addition, the assay we have devised for TCP and the characterization of TCP during differentiation may allow the future delineation of the mechanism(s) of regulation of TCP and the role this enzyme plays in modulating MT function during differentiation.

The cytoskeletal constituent tubulin undergoes a unique pair of posttranslational modifications. These modifications, which were first described in 1973 by Barra and co-workers (Barra et al., 1973), consist of the reversible addition of a tyrosine residue to the C-terminus of the α-tubulin polypeptide. In vitro and in vivo, tubulin protomers are tyrosinated by the enzyme tubulin tyrosine ligase (TTL;[1] Raybin & Flavin, 1977; Argarana et al., 1977), while microtubule (MT) polymers are detyrosinated by a tubulin-specific cytosolic carboxypeptidase (TCP; Kumar & Flavin, 1981, Arce & Barra, 1983).

The tyrosinating enzyme, TTL, has been purified and extensively characterized (Raybin & Flavin, 1977; Murofushi, 1980; Schroder et al., 1985), while the detyrosinating enzyme, TCP, has remained largely unexplored. Although its activity was identified in rat brain tissue in 1977 (Hallak et al., 1977), it has not yet been purified to homogeneity. Moreover, no specific assay for the measurement of TCP activity has been described, although some attempts have been made, using crude TCP-containing fractions, to assay TCP and to compare its properties to those of other carboxypeptidases (Argarana et al., 1980; Kumar & Flavin, 1981).

Molecular cloning of tubulin genes from a variety of tissues and from diverse organisms has revealed that, with only a few exceptions (testis tissue; Pratt et al., 1987; Hecht et al., 1988), the most abundantly expressed form of α-tubulin is synthesized in the tyrosinated (Tyr) form (Sullivan, 1988). Thus, the primary modification of α-tubulin is its detyrosination by TCP. In proliferating cells from vertebrates most MTs are highly dynamic and are enriched in Tyr tubulin, while a minor subset of less dynamic MTs is enriched in Glu tubulin, due to the polymer-dependent detyrosinating activity of TCP (Gundersen et al., 1987). Since the tyrosination state of cytoplasmic tubulin dimers is closely regulated in vivo (Gundersen et al., 1987; Webster et al., 1987) and since detyrosination of a stable MT subset occurs, but does not stabilize cytoplasmic MTs directly (Webster et al., 1990), it is likely that the function of TCP activity is to distinguish stable MTs biochemically from dynamic ones, allowing each MT subset to perform particular functions. In this way, tubulin detyrosination may help to specify MT function during differentiation and morphogenesis.

Previous work has demonstrated that the cycle of tyrosination/detyrosination is altered in differentiating cells (in concert with MT dynamics), such that an oriented array of MTs enriched in Glu tubulin is elaborated during muscle differentiation (Gundersen et al., 1989), directed cell motility (Gundersen & Bulinski, 1988), and neurite outgrowth (Wehland & Weber, 1987a; Lim et al., 1989; Bulinski & Gundersen, 1991). Although information concerning the proportions of Glu and Tyr tubulin as well as the level of TTL activity has been reported for cells undergoing such events, the level of activity of TCP has not been accurately determined. Since the rapid modification of MTs, mediated by TCP activity, may be crucial during these MT-dependent differentiative events, and since the paucity of information

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[1] Abbreviations: CPA, -B, or -E, carboxypeptidase A, B, or E; CPI, carboxypeptidase inhibitor from potato; Glu, detyrosinated; MT, microtubule; TCP, tubulin carboxypeptidase; TTL, tubulin tyrosine ligase; Tyr, tyrosinated.

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concerning this potentially important cellular enzyme limits our understanding of the specialized role of modified MTs, our goal was to devise a specific and sensitive assay for TCP activity, in order to permit its characterization and quantification in cells that have been induced to differentiate. These results may allow us to assess the relative contribution of TCP activity, relative to the known parameters of TTL activity and MT dynamics, in generating posttranslationally modified MTs during cellular morphogenesis.

Materials and Methods

Materials. [^{14}C]Tyrosine (NEC-289E, ~500 mCi/mmol) was obtained from New England Nuclear Research Products, Boston, MA. Tissue culture chemicals, powdered media, and sera were obtained from GIBCO, Inc. (Grand Island, NY); all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Preparation of Polymeric TCP Substrate. A 10 mg/mL solution of porcine or bovine brain microtubule protein (including both tubulin and MAPs) was prepared by two cycles of temperature-dependent assembly/disassembly purification (Vallee, 1986) and was radiolabeled with 0.1 mM [^{14}C]tyrosine during a 45-min incubation in PEM (0.1 M PIPES, pH 6.7, 0.1 mM EGTA, 0.1 mM $MgCl_2$) supplemented with 0.1 M KCl, 12.5 mM $MgCl_2$, 3 mM ATP, 1 mM GTP, and 0.22 mg of partially purified tubulin tyrosine ligase [~100 pmol/(min·mg); Murofushi, 1980], in a final volume of 2 mL. Tubulin was then purified by either DEAE or phosphocellulose chromatography (Vallee, 1986), dialyzed for 1 h against PEM to remove KCl and any remaining free [^{14}C]tyrosine, and frozen as small aliquots in liquid nitrogen. Specific activity of substrate tubulin was adjusted to 7000–22 000 cpm/60 μg by addition of unlabeled tubulin. Radiolabeled tubulin was used to make polymeric tubulin during the assay, which was then used as the substrate for TCP. This tubulin polymerized fully in the presence of 20 μM taxol during routine experiments.

Preparation of Cytosolic Extracts. Rat pheochromocytoma cells (PC-12; Greene & Tischler, 1976) were obtained from Erik Schweitzer (UCLA). Proliferating cells were grown in DMEM supplemented with 5% (v/v) fetal bovine serum and 10% horse serum. At 70–90% confluence, the cells from 4–12 (100-mm) dishes were gently scraped with a rubber policeman, washed twice in 5 mL of warm (37 °C) Earle's balanced salt solution (EBSS) and once in 5 mL of warm PEM, and lysed by sonication (3–5 pulses of 5-s duration using 20-W power, Branson Ultrasonics Model 205, Danbury, CT) in 1–2 mL of ice-cold PEM, such that the buffer volume was approximately twice the volume of the pelleted cells. The crude extract was centrifuged at 541000*g* for 10 min in a Beckman table-top ultracentrifuge (TL-100, Beckman Instruments, Palo Alto, CA), and the supernatant was diluted with cold PEM to a protein concentration of 1 mg/mL (100 μg of total protein/tube). Proliferating cells (35–50% confluent) were induced to differentiate by adding NGF (final concentration = 50 μg/mL) to the culture medium and refreshing it, every other day, for 5–7 days. These highly differentiated cultures were washed in warm EBSS in the dish, scraped, pelleted, and prepared as described above.

Proliferating rat L_6 myoblasts (ATCC No. CRL 1458, Rockville, MD) were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum. Cells from a total of 15–30 (100-mm) dishes were washed twice in 3 mL of warm EBSS, scraped from the dish, washed once with 5 mL of warm PEM buffer, resuspended in 0.5–1.0 mL of cold PEM, and gently sonicated (5–10 pulses of 10-s duration using 10-W power). Cell lysis was monitored using a microscope to obtain gently lysed cells and to prevent excessive disruption of lysosomes. As for the PC-12 cell homogenates, L_6 cell homogenates were pelleted at high speed; however, 500 μg of L_6 cell extract protein was used to permit detection of TCP activity in extracts of these cells. Myoblasts were induced to differentiate by changing the serum content of the culture medium to 2% horse serum. These cultures (8–12 dishes), which contained almost exclusively myotubes, were prepared identically to the differentiated PC-12 cultures, and sonication was again closely monitored.

The brains of one or two euthanized mice (Swiss Webster, retired adult breeders) were minced and homogenized in 1.5–2 volumes of cold PEM, centrifuged at 541000*g* for 10 min, and diluted to 1.25 mg/mL (250 μg of total protein/tube). Protein determination in all cases was made using the BCA reagent, with BSA as a standard.

Assay of TCP Activity in Cytosolic Extracts. (*A*) *Polymeric TCP Assay.* Cell or brain tissue extracts (100–500 μg) were mixed with 60–120 μg of [^{14}C]tubulin substrate, and taxol was added to a final concentration of 20 μM. The mixture was incubated for 10 min at 37 °C to allow maximal tubulin polymerization and TCP binding to the MT substrate, and then the mixture was centrifuged (436000*g* at 25 °C) for 4 min to pellet the MTs. The supernatant was removed, the MT pellet was resuspended in 100 μL of PEM (or, in some experiments, as described in the Results, in other buffers or PEM containing inhibitors or activators of enzymes related to TCP), and the resuspended MT pellet was incubated for 15–30 min at 37 °C. Altering the concentration of taxol over the range from 5 to 20 μM had no effect on the measured activity. Following the incubation, 0.5–1.0 mg of BSA was added as carrier protein and the sample protein was precipitated with 10% (w/v) TCA at 4 °C. Other fractions (e.g., samples of the original cytosolic extract, and the supernatant from the centrifugation of TCP and MTs) were TCA-precipitated by the same procedure. TCA-precipitated samples were centrifuged (245000*g*); both TCA-soluble and -insoluble radioactivity was measured in a liquid scintillation counter (Model LS-1800, Beckman Instruments, Palo Alto, CA). All data values are the result of duplicate determinations.

(*B*) *Monomeric TCP Assay.* Extracts were obtained as described and mixed directly with 60–120 μg of substrate tubulin or with tubulin plus inhibitors. The mixtures were then incubated for 30 min at 37 °C and TCA-precipitated, and the TCA-soluble radioactivity was counted as before.

Determination of Tyr Tubulin Content. Tyr tubulin quantification was performed on samples whose duplicates were assayed for TCP. Extract and MT substrate were incubated as described, but after centrifugation, the pellet was resuspended in cold PEM containing 10–100 mM calcium chloride and incubated on ice for 1–2 h to fully depolymerize the taxol-stabilized MTs. 5 M urea was included in the buffer where necessary, to effect complete solubilization. These samples were then either electrophoresed and western-blotted (Laemmli, 1970; Towbin et al., 1979) or adsorbed to nitrocellulose using a slot-blotting apparatus (Schleicher and Schuell, Keene, NH). For both procedures, the nitrocellulose was stained with an antibody specifically reactive with Tyr tubulin (Gundersen et al., 1984). Immunoreactivity was quantified by scanning samples on a video densitometer and comparing values to those of bovine brain tubulin standards, in which the proportion of Tyr tubulin subunits has been previously determined (unpublished results). For this study, we have assumed that all α-tubulin is of either the Tyr or the

Glu form. Recently, Paturle et al., (1989) isolated a brain tubulin species that could not be tyrosinated by purified tubulin tyrosine ligase preparations (Schroder et al., 1985); however, neither its presence in extracts from other cell and tissue types nor its biological significance has been determined. If non-tyrosinatable tubulin were present in our tubulin standard preparations, then the proportion of Tyr tubulin subunits would be somewhat less than presently calculated, necessitating a corresponding adjustment in calculating the TCP's specific activity.

Partial Purification of TCP from Murine Brain Tissue. Partially purified TCP was prepared from 5 g of murine brain tissue, using the protocol of Argarana et al. (1980).

Results

Development of a Specific and Sensitive Assay for TCP. Our main goal in the present study was to examine TCP activity during cellular differentiation. Previous attempts to measure TCP activity (Hallak et al., 1977), even in adult brain tissue (the most abundant source), gave ambiguous results, due in large part to the lack of sensitivity of the assay and the variable contribution of the endogenous Tyr tubulin present in the extract to the [^{14}C]-labeled substrate tubulin. Therefore, in order to devise an assay for TCP that would meet our criteria of sensitivity and specificity, we made use of the enzyme's reported ability to associate with MTs (Arce et al., 1983; Kumar & Flavin, 1981; Gundersen et al., 1987). To prepare a "polymeric TCP substrate", we first incubated microtubule protein with [^{14}C]tyrosine and partially purified preparations of the tyrosinating enzyme, TTL. We then purified the tubulin, which was labeled with [^{14}C]tyrosine at its C-terminus, and polymerized it with the MT-stabilizing drug, taxol. We incubated cytosolic extracts from brain or from differentiating cells with the polymeric TCP substrate and allowed the extract TCP to bind to the polymer, which we subsequently isolated by centrifugation. The pellets contained polymeric substrate and cytosolic TCP; we quantified activity of the latter as [^{14}C]tyrosine released during an incubation of the pelleted MTs under conditions permissive for TCP activity.

One obvious complication in measuring the activity of TCP in cytosolic extracts is that, without exception, these extracts contain endogenous tyrosinated (Tyr) tubulin which, when sedimented with our [^{14}C]-labeled Tyr tubulin substrate, competes with the labeled tubulin and reduces its specific activity. Thus, in order to measure with accuracy the TCP activity sedimented from a cytosolic extract, it was incumbent upon us to determine the total level of Tyr tubulin present during the assay. Figure 1 shows one such measurement. As described in Materials and Methods, we used antisera specific for Tyr tubulin along with standard samples of predetermined Tyr tubulin content to assess the level of Tyr tubulin and, consequently, the specific activity of [^{14}C]tyrosine at the C-terminus of tubulin in the pelleted enzyme/substrate complexes. With the exception of the differentiated L_6 cell extracts, in every type of extract that we assayed (using 60–120 μg of substrate per 100–500 μg of extract), the endogenous Tyr tubulin contributed significantly to the total Tyr tubulin in the pellet. For example, we observed that ~26% (n = 3) of the pelleted Tyr tubulin, on average, was contributed by the cytosolic extract in assays of mouse brain extract, while 12–16% (n = 13) of the pelleted Tyr tubulin was derived from PC-12 cell extracts, regardless of their differentiation state. Thus, TCP activity was measured in cytosolic extracts by quantifying the TCP's product and determining the total specific activity derived from both exogenous ([^{14}C]-labeled) and endogenous (unlabeled) substrate present in the assay buffer.

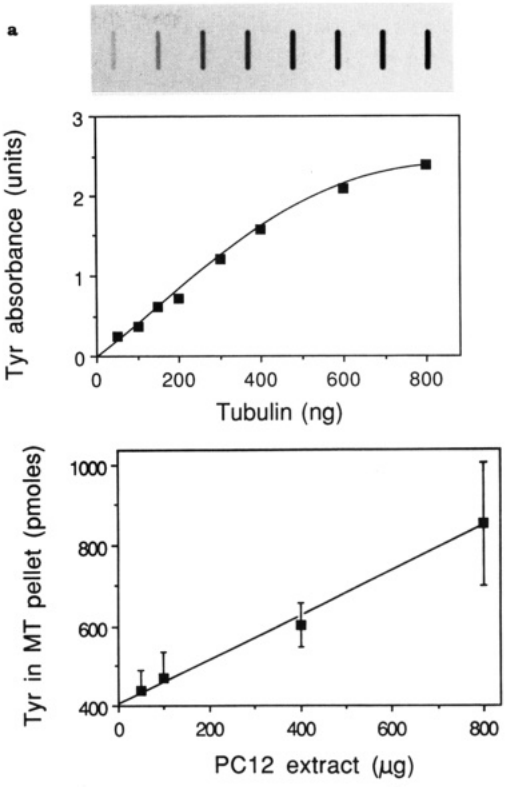

FIGURE 1: Measurement of Tyr tubulin in enzyme·MT complexes in a typical experiment. DEAE-purified tubulin standards (a), containing a known proportion of Tyr tubulin subunits, were blotted onto nitrocellulose, and Tyr reactivity was measured by densitometry. Absorbance was linear over the total tubulin concentration range from ~50 to 500 ng and then reached a plateau level. Blotted standards corresponding to each plotted value are shown above the graph. (b) The contribution of PC-12 tubulin to the total in the pelleted enzyme·MT complex was measured by mixing 60 μg of [^{14}C]-labeled substrate, 50–800 μg of extract, and taxol to a final concentration of 20 μM, incubating the mixture for 10 min as in a routine TCP assay, and then pelleting the complex at high speed. The pellet was resuspended in PEM buffer containing 100 mM Ca^{2+} (and 5 M urea if necessary to solubilize MTs), incubated on ice for 1–2 h, and blotted and scanned for Tyr tubulin intensity as in (a). Direct measurement of these values allowed the accurate determination of TCP activity in routine assays. Plotted values are the average of 4–6 determinations.

We determined the efficacy of our TCP assay in several ways. For example, as in the case of any enzyme assay, it was necessary to demonstrate that the amount of product ([^{14}C]tyrosine) measured was due to TCP activity in the added extract, rather than due to an activity endogenous to the substrate, and that this activity increased in proportion to the amount of extract added to the reaction mixture. Figure 2 shows that this was, in fact, the case; no activity was observed without addition of extract, and the measured TCP activity increased as increasing amounts of extract were assayed. In addition, we measured the time course of [^{14}C]tyrosine release (Figure 3), and we determined that the quantity of substrate used in routine assays was sufficient to saturate the level of enzyme in the cytosolic extracts (Figure 4). These results demonstrated that the measured release of [^{14}C]tyrosine was due to the activity of an enzyme present in the cytosolic extract and that our conditions might be expected to allow sensitive measurement of the TCP activity.

Although the experiments described above demonstrated our ability to measure TCP activity sedimenting with MTs, we wished to know whether the assay was an accurate representation of *total* cytosolic TCP activity. To examine total

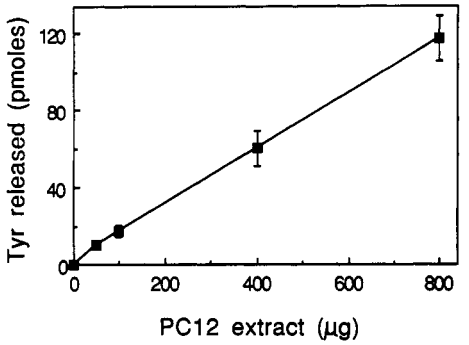

FIGURE 2: TCP activity increases linearly with added PC-12 extract. From 50 to 800 μg of PC-12 extract was mixed with 60 μg of labeled substrate and taxol to 20 μM, incubated for 10 min at 37 °C, centrifuged to obtain an enzyme·MT pellet, resuspended in 0.1 mL of PEM buffer, and assayed for 15 min at 37 °C. TCP activity was quenched by adding cold TCA to 10% (w/v) and was measured by counting TCA-soluble radioactivity. TCP activity (in picomoles released) was calculated as described in Materials and Methods. The activity in as little as 25 μg of extract was unambiguously measured under these conditions.

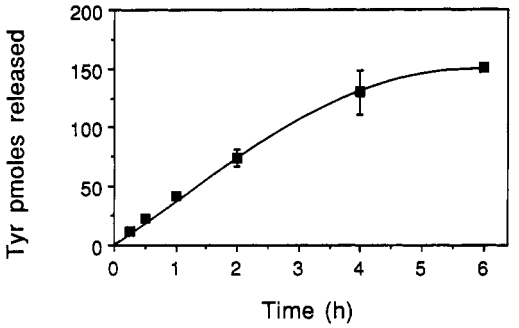

FIGURE 3: Time-course of TCP activity in PC-12 extracts. TCP activity in 100 μg of extract was assayed using 120 μg of labeled substrate, while identically prepared enzyme·MT pellets were measured for their content of Tyr tubulin. TCP activity increased linearly from 5 min to ~4 h and then reached a plateau by 6 h of incubation. Routinely, the assay time was from 15 to 30 min. Standard deviations not visible here were well within the heights of the symbols.

cytosolic TCP activity, it was necessary to quantify the proportion of TCP that was sedimented with the polymeric substrate and, consequently, was available for assay in the pellet fraction. To this end, we subjected murine brain extracts and PC-12 cell extracts to the assay protocol, sedimenting polymeric substrate with the TCP, as usual. We then determined the level of TCP activity remaining in the supernatant rather than sedimenting with the MTs by reassaying the supernatant material, using the polymeric TCP substrate. For both types of extract, TCP activity was not detected in the supernatant. Since assays in which varying quantities of murine brain extract were utilized (data not shown) demonstrated that as little as 10% of the initial TCP activity was detectable using our assay, we can conclude that ≥90% of the TCP activity was sedimented with polymeric substrate in the assay. This result suggests that our TCP assay is accurately measuring the *total* TCP activity present in the cytosolic extracts.

Many other lysosomal and secreted carboxypeptidases and endoproteases might be expected to have been present in the cytosolic extracts in which we were attempting to measure TCP activity. Therefore, it was important to verify that the species released from α-tubulin was the appropriate moiety, [^{14}C]tyrosine, rather than one or more peptide fragments. One way in which we tested the product of TCP action was to perform western blots of our TCP substrate following digestion by the putative TCP (data not shown). The TCP substrate was immunoreactive with antibodies specific for either Tyr

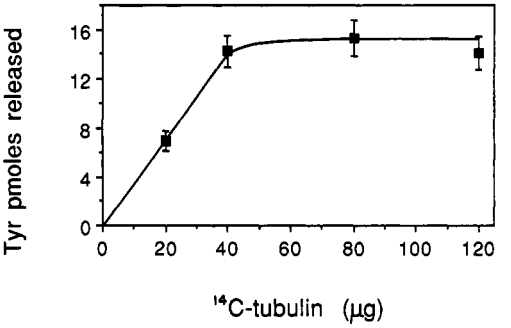

FIGURE 4: Substrate saturation of TCP activity in PC-12 extracts. A total of 100 μg of extract (+taxol) was assayed for 15 min using 20–120 μg of labeled substrate. Under these conditions TCP activity increased over the substrate concentration range from 20 to 40 μg and then reached a plateau, indicating that the [^{14}C]tyrosine was released by enzymatic activity which was saturated with 40–60 μg of substrate.

or Glu tubulin, and we observed no evidence of any immunoreactive proteolytic fragments. We also tested the product of the TCP assay by subjecting the TCA-soluble product to HPLC analysis; these results showed that all of the detectable radioactivity corresponded to [^{14}C]tyrosine or its oxidized derivative, with no radioactivity corresponding to dipeptides or peptides of higher molecular weight.

In order to ascertain that our TCP assay was specific for TCP and was not affected by the activities of other carboxypeptidases and endoproteases, we examined the effects of including several carboxypeptidase and protease inhibitors in the assay mixture. The inhibitors we tested were some of those reported to inhibit carboxypeptidase A-like enzymes, namely, potato carboxypeptidase A inhibitor (CPI), which inhibits both pancreatic and lysosomal CPA as well as CPB (Ryan et al., 1974; Kirschke & Barrett, 1987); EDTA, which inhibits pancreatic CPA, CPB, and CPE (Argarana et al., 1980; Folk, 1971; Fricker & Snyder, 1983); DTT, which inhibits CPA and CPB; and 8-hydroxyquinoline-5-sulfonic acid and PMSF, which inhibit CPB (Folk, 1971). Figure 5 documents the selective inhibition of carboxypeptidases A and B, and not of TCP partially purified from murine brain, which occurred in the presence of potato inhibitor, DTT, or 8-hydroxyquinoline-5-sulfonic acid. Since our measured TCP activity, both in extracts and in partially purified brain material, was minimally affected by the known carboxypeptidase inhibitors, we are confident that our assay is highly specific for TCP, even in the presence of other carboxypeptidases.

From the experiments in which inhibitors were tested we combined useful inhibitors into cocktails, which we used in each experiment to verify the specificity of the TCP assay. Table I shows the TCP activity measured in three types of cytosolic extracts and documents the effect of each inhibitor cocktail on the activity of TCP. It is clear that very little inhibition (0–20%) of TCP occurs when inhibitors of other enzymes are included in the polymeric assay. Although we do not yet know of a good diagnostic inhibitor of TCP, we made use of previous findings of Argarana et al. (1978), who reported that decreasing the pH of the assay buffer to 5.4 significantly inhibited TCP. Conveniently, lysosomal carboxypeptidase A and other lysosomal proteases are activated at this pH (Matsuda & Misaka, 1975); therefore, the *decrease* (of ~60–80%) rather than an *increase* in TCP activity measured when the assay was performed at pH 5.4 was a further indicator of the assay's specificity for TCP. The failure of CPI to further inhibit TCP at low pH indicated that lysosomal CPA contributed little, if any, toward the measured ^{14}C released. Moreover, when we used the monomeric tubulin assay to

Table I: TCP Activity in Proliferating and Differentiated PC-12 Cells and Murine Brain Extracts[a]

	polymeric assay						monomeric assay	
	PC-12, prolif		PC-12, diff		brain extract		brain extract	
inhibitors	(4)	%	(3)	%	(2)	%	(3)	%
none	8.1 ± 1.4	100	18.0 ± 0.2	100	15.9 ± 1.7	100	3.1 ± 0.5	100
CPI/EDTA/DTT	7.7 ± 1.4	95	17.6 ± 0.8	98	13.2 ± 0.1	83	1.4 ± 0.1	45
8-OH/PMSF	8.7 ± 2.2	107	17.6 ± 0.7	98	12.7 ± 0	80	1.0 ± 0.5	32
pH 5.4	1.7 ± 0.4	21	3.1 ± 0.8	18	6.9 ± 0.4	43	0	0
CPI, pH 5.4	1.7 ± 0.2	21	3.5 ± 0.9	19				

[a] TCP activity was measured in extracts prepared from either proliferating PC-12 cells ("prolif"; undifferentiated), PC-12 cells that were differentiated for 5–7 days with NGF ("diff"; differentiated), or adult murine brain tissue. For the brain extracts, the results from the polymeric and monomeric assays were compared. For each extract, enzyme activity was measured over 30 min either in the presence or in the absence of one of several inhibitor cocktails, which were designed to inhibit contaminating CPA, CPB, CPE, or other protease activity. In addition, the extracts were incubated at lower pH (±CPI) to assess the level of contaminating lysosomal CPA activity. Values represent specific activity measured as pmol/(min·mg) (±SD) and the percent activity of control values after inhibitor treatment. Numbers in parentheses represent the number of experiments that were performed on each type of extract. CPI = carboxypeptidase A inhibitor from potato, 8-OH = 8-hydroxyquinoline-5-sulfonic acid, and PMSF = phenylmethanesulfonyl fluoride.

Table II: Comparison of TCP Specific Activity, Endogenous Tyr Tubulin Levels, and Contribution of Extract Tyr Tubulin to the Total Tubulin in TCP·MT Complexes in Extracts from Differentiating Cells[a]

	PC-12				L_6			
	prolif	diff	Δ (%)	ref	prolif	diff	Δ (%)	ref
TCP activity [pmol/(min·mg)]	8.1	18.0	+220	*c*	2.2	1.6	−27	*c*
Tyr tubulin in extract (%)	100	137	+37	*c*	100	110	+10	*b*
extract Tyr in TCP·MT pellet (%)	12	16	+33	*c*	43	0.3	−43	*c*

[a] Values for TCP specific activity, the level of Tyr tubulin in the PC-12 extracts, and the content of Tyr tubulin in the TCP·MT complexes, before and after cellular differentiation, were calculated as described in Materials and Methods. The values for the level of Tyr tubulin in differentiating L_6 myoblasts were obtained from an earlier study. The level of Tyr tubulin within differentiated cell extracts is expressed as the percent increase over levels found in proliferating cells (100%). The values for the level of extract Tyr tubulin sedimenting in the TCP·MT complex are expressed as the percentage of total Tyr tubulin in the complex. The changes (Δ) are expressed as either an increase (+) or decrease (−) during differentiation. [b] Gundersen et al. (1989). [c] This study.

Table III: TCP Activity in Proliferating and Differentiated L_6 Myoblasts[a]

	polymeric assay				monomeric assay	
	prolif		diff		prolif	
inhibitors	(4)	%	(5)	%	(4)	%
none	2.2 ± 0.3	100	1.6 ± 0.2	100	1.4 ± 0.2	100
CPI/EDTA/DTT	2.0 ± 0.3	90	1.5 ± 0.2	94	0.3 ± 0.2	19
8-OH/PMSF	2.1 ± 0.3	93	1.6 ± 0.2	98	0.5 ± 0.1	35
pH 5.4	0.4 ± 0.1	18	0.2 ± 0.1	15	4.2 ± 0.7	292
CPI, pH 5.4	0.3 ± 0.1	15	0.2 ± 0	12	3.6 ± 1.1	250

[a] Proliferating and differentiated (11 days) rat L_6 myoblasts were processed to measure TCP activity for a standard 30 min assay. The inhibitor concentrations and applications were identical to those in Table I. Specific activity is expressed as pmol/(min·mg) (±SD). Numbers in parentheses indicate the number of experiments performed. The difference in specific activities between the control groups ("none") before and after differentiation, using the polymeric assay, is significant ($P < 0.005$).

monitor TCP activity in a cytosolic extract, we found quite a different situation (see Table I, right-hand columns); here the inhibitor cocktails reduced activity dramatically, suggesting that the monomeric assay is not very specific for TCP, but is measuring other enzymatic activities as well. Table I also documents the increased sensitivity of our polymeric TCP assay, as compared to the assay utilizing monomeric tubulin, since more TCP activity can be detected in mouse brain extracts by our polymeric assay than can be detected with the monomeric assay. In fact, using dilute extracts of L_6 cells, we have found that activities as low as ~10 pmol/mg of extract protein can be measured in a standard 30-min assay, with radioactivity that is significantly higher than background levels. We estimate that the sensitivity of the assay could be further increased; the release of as few as 3–4 pmol of tyrosine could be readily detected if substrate of even higher specific activity were used. From Table I and from other assays which compared the polymeric and monomeric substrates (not shown) we calculate that our TCP assay is at least 10-fold more sensitive than previously described protocols (Hallak et al., 1977; Flavin & Murofushi, 1984) utilizing monomeric tubulin.

TCP Activity during Neural and Myogenic Differentiation. We applied the newly devised TCP assay to the measurement of its activity during two morphogenetic events: neurite outgrowth in PC-12 cells and myoblast fusion into myotubes in L_6 cells. Table I reveals an ~2.2-fold increase in the specific activity of TCP over 5–7 days of neurite extension in P-12 cells, corresponding to an increase of ~37% in the level of Tyr tubulin (expressed as an increase in the percentage of total extract protein after differentiation; Table II). Further, the level of polymerizable extract Tyr tubulin measured in the TCP·MT complex rose by a similar amount (~33%, Table II). In both the proliferating and the differentiated cell extracts the specific activity of TCP was unaffected when the inhibitor cocktails were included in the assay buffer, while activity in both cases was dramatically inhibited by the shift to the lower pH. Thus, the measured activity of PC-12 cell extracts, and its increase during NGF-induced differentiation, was attributable to TCP.

We also measured TCP activity in a differentiating rat myoblast cell line (L_6, Table III). The specific activity of TCP in extracts from these non-neuronal cells was determined to be lower than in either PC-12 cell or brain tissue extracts.

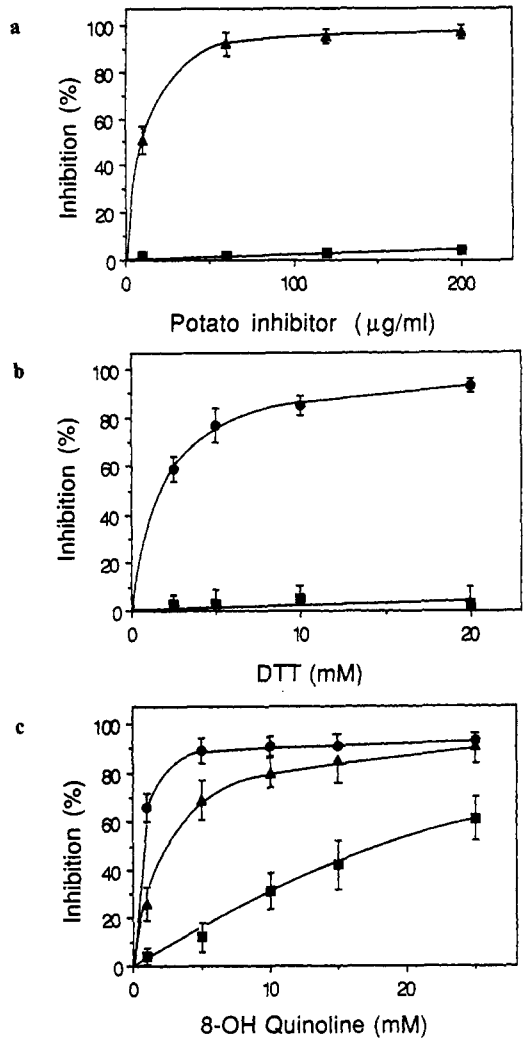

FIGURE 5: TCP activity is not diminished in the presence of known inhibitors of carboxypeptidase A or B. In (a), TCP activity (■) was measured in the presence of increasing concentrations of carboxypeptidase inhibitor from potato (CPI; 10–200 μg/mL), a potent inhibitor of CPA (▲). TCP activity remained unchanged at CPI concentrations that inhibited pure preparations of CPA by >90%. In (b), the effect of DTT on TCP and CPB (●) activities was measured. Again, TCP remained unaffected at DTT concentrations (5–20 mM) that inhibited pure CPB by 85–90%. (c) TCP, CPA, and CPB activities were measured in the presence of increasing concentrations of 8-hydroxyquinoline-5-sulfonic acid (8-OH). While moderate inhibition of TCP was noted at higher concentrations of inhibitor (10–25 mM), TCP activity remained uninhibited at levels (2–5 mM) that greatly inhibited CPB (65–90%) and CPA (up to 70%) activities. 0.12 mg/mL CPI, 10 mM DTT, and 2 mM 8-OH were routinely used to assay for the presence of these activities in brain and cell extracts. Standard deviations not shown here were well within the heights of the symbols.

Accordingly, we increased the specific activity of the [^{14}C]-tyrosine in the substrate (~3-fold) as well as the amount of extract we added to the substrate (to 500 μg). These conditions allowed us to reliably measure TCP activity in these extracts.

Although considerable [^{14}C]tyrosine release was measured using the monomeric tubulin substrate, most of this activity was found to be both inhibited by either of our cocktails and stimulated by the shift to the lower pH, indicating that little of the activity was due to TCP but instead contributed by other cytoplasmic or lysosomal enzymes. Comparison of the values obtained at low pH using the polymeric versus the monomeric substrate revealed the large contribution made by lysosomal enzymes toward the total activity measured in the monomeric assay and emphasized the ability of the polymeric assay to selectively measure TCP activity, even when this activity might account for only a small proportion of the total enzymatic activity. In contrast, Flavin and Murofushi (1984) stated that the monomeric assay they used was not capable of reliably detecting any TCP activity in bovine brain extracts.

The specific activity of TCP declined somewhat during myogenesis (~27%, Table III). During differentiation, the contribution of endogenous Tyr tubulin to the activity measurement changed. In proliferating L_6 cells, due to the large amount (500 μg) of undifferentiated myoblast extract required to measure TCP activity, a significant amount of Tyr tubulin (~20 μg) was sedimented, along with the 60 μg of substrate tubulin (containing ~30 μg of Tyr tubulin), during the assay. However, after 11 days of differentiation, the level of Tyr tubulin measured in the pellet closely matched the amount of the labeled substrate tubulin present (Table II), indicating that no significant amount of Tyr tubulin from the extract was found in the pellet under these conditions. Specifically, 43% (n = 4) of the Tyr tubulin in the pellet was contributed by the extract from proliferating cells, while <1% (n = 4) of the Tyr tubulin was contributed by the extracts from differentiated cells. Although the level of Tyr tubulin in the differentiated cell extracts remained constant, the level of polymerizable Tyr tubulin dropped to negligible levels, resulting in the decline in the specific activity of TCP. Thus, the determination of the *total* amount of Tyr tubulin in the TCP·MT complex, and not just in the extract, was critical for the accurate calculation of the specific activity of TCP.

The correlation we have found between changes in TCP specific activity and the level of assembly-competent Tyr tubulin in the extract suggest that TCP may be regulated by the level of its substrate, polymerized Tyr tubulin, during cellular differentiative events. Further, our results suggest that our TCP assay is sensitive enough to be of widespread use in assaying cytosolic TCP activity during other important cellular processes involving morphogenesis and differentiation.

Discussion

In this study we have measured the activity of TCP during two differentiative events, using a sensitive and specific MT-based assay. Previous attempts to measure TCP activity reliably in cell and tissue extracts were unsuccessful, due in large part to the use of an assay that lacked adequate sensitivity and specificity (Flavin & Murofushi, 1984). The lack of a suitable assay had not only hindered attempts to study the tyrosination cycle during cellular differentiation, but had also stymied attempts to purify the enzyme (Argarana et al., 1980). Since polymerized cytoplasmic tubulin coexists at steady state with an unpolymerized pool (Johnson & Borisy, 1977) and would thus complicate the interpretation of TCP acting on both types of substrates (Arce & Barra, 1985), in most previous investigations the TCP substrate utilized was tubulin kept unpolymerized by dilution below the steady-state concentration for assembly. We reasoned that [^{14}C]-labeled tubulin assembled with the aid of taxol would, instead, provide a stable polymeric substrate for the enzyme. In addition to imposing a more stringent binding requirement on the enzyme, the taxol-stabilized MTs could be pelleted and then resuspended in a suitable buffer, freeing the assay solution from contaminating enzymes that could mimic TCP activity but would not sediment with MTs. Activity of the tyrosinating enzyme, TTL, if present in the cytosolic extracts, would not be expected to contribute significantly to the total tyrosine released in our assay, for several reasons: First, the TTL does not work significantly on polymerized tubulin (Wehland & Weber, 1987b), and therefore, would not be expected to sediment with

the TCP·MT complex. Second, the buffers used to resuspend the TCP·MT pellet and subsequently measure TCP activity are not permissive for TTL activity, which requires ATP. Third, the concentration of free tyrosine generated by the TCP during a routine assay (10–20 pmol of tyrosine released using 100 μg of PC-12 extract = 0.2 μM) is at least 2 orders of magnitude below the reported K_m of TTL for tyrosine (25 μM; Schroder et al., 1985). Finally, the detyrosinating activity of the TTL (generated by reversing its normal activity) requires ADP and P_i (Rodriguez et al., 1973), suggesting that there could be no significant contribution of TTL activity (acting either in the forward or the reverse direction) toward the total amount of tyrosine released during a routine assay.

We further refined the TCP assay by accurately measuring the amount of Tyr tubulin that sedimented and was thus available to the enzyme, in its complex with MTs, during the assay. For example, in order for us to detect the slight decline in TCP specific activity that occurred during myoblast differentiation, it was necessary to measure the *total* amount of Tyr tubulin that sedimented in the TCP·MT complex. Although the total amount of [^{14}C]tyrosine released was similar both before and after differentiation, the amount of unlabeled Tyr tubulin that was contributed to the TCP·MT pellet by the differentiated cell extracts was much less than that contributed by the extracts from proliferating cells. This decrease in polymerizable Tyr tubulin resulted in a lesser dilution of the [^{14}C]tubulin substrate and was thus reflected in the calculations of TCP specific activity. Therefore, this additional step can assume great importance in the measurements of TCP activity during morphogenetic events such as myogenesis, events that significantly alter the level of endogenous, assembly-competent, Tyr tubulin.

The use of our assay, employing a novel polymeric substrate, has allowed us to determine the level of TCP activity not only in extracts of adult brain, but also in extracts of cell lines and non-neuronal tissues, and to monitor TCP activity during cellular differentiation. Neurite outgrowth involves the increased synthesis of tubulin and microtubule-associated proteins [MAP 1 and τ, Drubin et al. (1985); MAP 2, Fischer et al. (1991)] and the assembly and stabilization of MTs (Black & Greene, 1982; Drubin et al., 1985), which also accumulate elevated levels of Glu tubulin subunits (Lim et al., 1989; Baas & Black, 1990; Bulinski & Gundersen, 1991). As a first step in defining the role of α-tubulin detyrosination by TCP in neurite outgrowth, we measured the level of TCP activity both before and after differentiation. PC-12 cells that are allowed to differentiate for 5–7 days show a 2–2.5-fold increase in the level of total tubulin and a 10–20-fold increase in the levels of MAP 1 and τ, as measured by densitometry of immunoblots (Drubin et al., 1985). Although the level of total tubulin increases over this interval, the proportion of polymerized tubulin decreases, thus making it accessible to the TTL and possibly accounting for the moderate increase in Tyr tubulin that we observed during PC-12 differentiation. It may be noteworthy that there is a broad correlation between the increase in the specific activity of TCP ($\sim$2.2-fold) during differentiation and the increase in the level of Tyr tubulin (and perhaps more specifically, Tyr in polymer), in contrast to the 10–20-fold increase in protein observed for other brain MAPs.

We also monitored TCP activity during another MT-dependent differentiation event, myogenesis. Myoblasts induced to differentiate and form myotubes also accumulate stable Glu MTs as an early event in the myogenic program (Gundersen et al., 1989), corresponding to a 4-fold increase over undifferentiated cells in the total level of Glu tubulin subunits. However, the specific activity of TCP decreased slightly ($\sim$ 27%) but significantly ($P < 0.005$) during this process. Since the level of TCP's product (Glu tubulin) rose without a corresponding increase in TCP activity, we suggest that the amount of available substrate (stable Tyr MTs) may be the most significant factor in determining the posttranslational modification state of tubulin during myogenic differentiation. In this regard, it should be noted that during myogenesis in L_6 cells, the total level of Tyr tubulin remains relatively constant (Table II). Since detyrosination is a polymer-dependent event (Gundersen et al., 1987), the rise in Glu tubulin in MTs is probably much higher than 4-fold, inferring a substantial decrease in the level of substrate Tyr polymer present in these extracts and possibly accounting for the slight drop in TCP specific activity that we observed. This possibility is corroborated by our quantification of the amount of Tyr polymer contributed by the differentiated cell extracts that sedimented with the TCP·MT complex. In conclusion, the modest increase (in PC-12 neurite outgrowth) or slight decrease (in L_6 myogenesis) in TCP activity during these events indicates that it is changes in MT dynamics, and not in enzymatic activities, that are responsible for the increase in the level of Glu tubulin in MTs that accompanies these differentiative events.

In summary, we have characterized TCP activity during two types of cellular differentiation. For this work, it was necessary to devise a useful new assay for TCP, whose specificity and sensitivity we have documented in extracts of both brain tissue and differentiated cells. Our TCP assay, which utilizes the MT-binding property of TCP (using MTs to sediment and concentrate the enzyme in a single step), should permit the further measurement of TCP in differentiating cells and in tissues in which tubulin posttranslational modification is occurring, as well as the elucidation of the mechanisms that regulate TCP activity. Our assay and the information we now have regarding TCP activity and its characterization and regulation during differentiative events should assist us in the eventual purification of the enzyme.

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Cutoff Size Does Strongly Influence Molecular Dynamics Results on Solvated Polypeptides†

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ABSTRACT: The behavior of a 17-residue model peptide is analyzed by means of molecular dynamics simulations including explicitly more than a thousand water molecules. On the basis of the charge-group concept, Coulomb interactions are truncated for three values of the cutoff radius: 0.6, 1.0, and 1.4 nm. It is found that the stability of an α-helix, which acts as a common starting configuration, is a function of the cutoff size. While the overall stability of the helix is conserved in a simulation using a cutoff of 1.0 nm, it is lost within a very short period of 100 ps when the cutoff is increased to 1.4 nm. This demonstrates that the commonly used cutoff size of 1.0 nm is inappropriate because it does not ensure the convergence of Coulomb interactions. In order to permit an independent judgment, we have performed a 225-ps simulation using the Ewald summation technique, which is more elaborate but circumvents the problem to find an appropriate cutoff value. In contrast to the 1.4-nm cutoff trajectory, the Ewald technique simulation conserves the helical character of the peptide conformation. This demonstrates that even 1.4 nm is too short a cutoff. Due to the fundamental uncertainty introduced by the use of a simple cutoff, this truncation scheme seems questionable for molecular dynamics simulations of solvated biomolecules.

It is no matter of debate that the inclusion of solvent effects is essential for a realistic description of structural and dynamical features of biomolecules. From a conceptional point of view, molecular dynamics (MD) simulations taking into account explicitly solvent (usually water) molecules are the most direct way to meet this goal (Brooks et al., 1988; Karplus & Petsko, 1990; van Gunsteren & Berendsen, 1990). However, the high numerical effort has long prohibited extensive simulations of that kind.

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