

Regulation of Microtubule Protein Levels during Cellular Morphogenesis in Nerve Growth Factor-treated PC12 Cells

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Abstract. Nerve growth factor induces neurite process formation in pheochromacytoma (PC12) cells and causes the parallel increase in levels of the microtubule-associated proteins, tau and MAP1, as well as increases in tubulin levels. Mechanisms to insure balanced accumulation of microtubule proteins and make their levels highly responsive to nerve growth factor were investigated. The effects on tau, MAP1, and tubulin are due to changes in protein synthesis rates, which for tau and tubulin we could show are due in part to changes in the mRNA levels. Whereas tubulin shows feedback regulation to modulate synthesis up or down, tau protein synthesis is not affected

in a straightforward way by microtubule polymerization and depolymerization. The degradation of tau, MAP1, and both tubulin polypeptides, however, are stimulated by microtubule depolymerization caused by colchicine, or nerve growth factor removal. Combined feedback on synthesis and stability make tubulin levels highly responsive to assembly states. In addition, the linkage of tau and MAP1 turnover with the state of microtubule polymerization amplifies any change in their rate of synthesis, since tau and MAP1 promote microtubule polymerization. This linkage lends itself to rapid changes in the state of the system in response to nerve growth factor.

NERVE process extension requires assembly of parallel arrays of microtubules that run longitudinally through both axons and dendrites. These microtubules are assembled from tubulin, the microtubule subunit protein, and from microtubule-associated proteins. The molecular events that occur during nerve process extension can be studied in a pheochromacytoma cell of neural crest origin called PC12 (15). When PC12 cells are cultured in nerve growth factor (NGF)¹ there is first a modest increase in tubulin pools (11). When neurite extension begins, the levels of two microtubule-associated proteins, tau protein and an isoform of MAP1, rise dramatically (11, 16). In vitro studies have shown that both tau protein and MAP1 promote microtubule assembly (6a, 17, 23) and it seems likely that they promote tubulin assembly in the growing neurite. Both proteins are bound to microtubules in PC12 cells (13). Microinjection of tau protein into fibroblast cells that do not contain tau shows that the injected protein can drive both the assembly of microtubules nucleated at the centrosome and the assembly of free microtubules and, additionally, can stabilize microtubules to drug-induced depolymerization (9). Therefore, it seems reasonable that tau protein in nerve processes can provide a driving force for microtubule assembly free of centro-

some nucleation sites and may also stabilize the resulting microtubules.

The changes induced by NGF in the morphology of PC12 cells, in the extent of microtubule assembly, and in microtubule protein levels are reversible. When NGF is removed, neurites are lost, microtubule assembly levels return to levels found in undifferentiated PC12 cells, and tubulin, tau, and MAP1 levels fall to undifferentiated levels (11). Little is known about the mechanisms that coordinate changes in microtubule assembly with changes in the levels of microtubule proteins during nerve cell morphogenesis.

To examine this question we have studied the regulation of microtubule protein levels in PC12 cells cultured in NGF. The rates of synthesis of tubulin, tau protein, and MAP1 all rise and fall in response to NGF addition and removal, respectively. For tubulin and tau protein, the changes in synthesis rates can be accounted for in part by changes in corresponding mRNA levels. However, additional levels of control were also observed. First, tubulin synthesis rates increase when the unassembled tubulin pool is depleted and decrease when the pool is increased. Decreases in tubulin pools have larger synthesis rate effects in undifferentiated cells than in cells cultured in NGF. Additionally, tubulin, tau, and MAP1 turnover rates all are higher when these proteins are unassembled. Turnover of all three proteins is also increased when differentiated PC12 cells are withdrawn from NGF and neurites retract. Possible implications of these regulatory mechanisms for nerve cell morphogenesis are discussed.

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1. *Abbreviation used in this paper:* NGF, nerve growth factor.

Materials and Methods

Cell Culture

Culture conditions for PC12 cells were as described in detail elsewhere (11) except that a 5% rather than a 12% CO₂ atmosphere was maintained.

[³⁵S]Methionine Labeling of Cells

To compare synthesis rates of microtubule proteins in cells subjected to different treatments, cells were rinsed with methionine-free medium and then labeled for 1.5 h in 0.2 mCi/ml of [³⁵S]methionine (Amersham Corp., Arlington Heights, IL) in medium that was otherwise methionine free.

For pulse-chase experiments, cells were labeled for 4 h in 0.4 mCi/ml of [³⁵S]methionine. The "pulse" extracts were prepared after a 1-h chase period because we found that during this period [³⁵S]methionine is still incorporated into proteins. The "chase" extracts were prepared after an 18-h chase (Figs. 5 and 6) or a 2-d chase (Fig. 7). For pulse-chase experiments, control, NGF-treated, and drug-treated cells were treated for 1 h with concanavalin A (20 μg/ml) before adding [³⁵S]methionine. Concanavalin A was also present in the labeling and chase media. (This treatment, recommended by F. Solomon, prevented detachment of cells from the culture dishes during the various manipulations to which they were subjected.)

Cell extracts were made as in Drubin et al. (11) except that 0.2 ml of lysis buffer pH 7.0 was used on a 100-mm plate rather than 0.5 ml.

Immunoabsorptions

3 μl of each extract was precipitated in 1 ml of 10% TCA and collected on a glass fiber filter. Filters were placed in Aquasol (New England Nuclear, Boston, MA) and the amount of [³⁵S]methionine incorporated was determined in a scintillation counter. The samples were normalized to contain the same [³⁵S]methionine activity and brought to 125 μl with lysis buffer (25 mM NaH₂PO₄ pH 7.0, 0.4 M NaCl, 0.5% SDS). To each sample 200 μl of 25 mM Tris, pH 7.4, 0.4 M NaCl, 0.1% deoxycholate, and 1% NP-40 was added to bring the total sample volume to 325 μl.

To remove extract proteins that would nonspecifically bind to *Staphylococcus aureus* cells, the 325-μl extracts were used to resuspend *S. aureus* cell pellets prepared from 40 μl of a 10% suspension. After 10 min the *S. aureus* cells were removed by centrifugation. Next, antibodies were added to the supernatant. 2 μl of MAP1 mAb (7-I.1, kindly provided by Dr. David Asai; reference 1) was added first. After 30 min, 40 μl of a 10% suspension of *S. aureus* cells that had been preabsorbed in 10 mg/ml BSA in PBS (0.13 M NaCl, 8 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.2) and then washed in PBS was added. 10 min later the cells were collected by centrifugation. This procedure was repeated with the addition of 2 μl of β-tubulin monoclonal antibody (DMβ-1; reference 4) and was repeated lastly with 50 μl of affinity-purified tau antiserum (10, 21). The *S. aureus* cell pellets were washed twice in 25 mM Tris-Cl, pH 7.4, 0.4 M NaCl, 0.1% deoxycholate, 1% NP-40, and 0.5% SDS and once in 10 mM Tris-Cl, pH 7.4, 5 mM EDTA. The final *S. aureus* cell pellets were boiled in SDS sample buffer and loaded on SDS-PAGE gels.

To increase the signal-to-noise ratio for tau immunoabsorptions, we began two cycle immunoabsorptions halfway through the set of experiments described in this paper. For this procedure, the *S. aureus* cells from the first immunoabsorptions were washed once in 25 mM Tris-Cl, pH 7.4, 0.4 M NaCl, 0.1% deoxycholate, 1% NP-40, and 0.5% SDS and once in 10 mM Tris-Cl, pH 7.4, 5 mM EDTA. The cells were next resuspended and boiled in 30 μl PBS containing 2% SDS. The cells were removed by centrifugation while the samples were still hot and the supernatant was diluted by the addition of 105 μl of 25 mM Tris-Cl, pH 7.4, 0.4 M NaCl, 0.1% deoxycholate, 1% NP-40, and 50 μl PBS. The second immunoabsorption was performed exactly like the first one.

SDS Acrylamide Gels

Immunoabsorbed samples were analyzed on SDS polyacrylamide gels (18). Normalized total cell extract was also loaded on the gel to confirm that the samples were normalized properly. The gels were fluorographed with EN³HANCE (New England Nuclear). Fluorography was on Kodak X-O-Mat AR film. Quantitation of material in bands was done by densitometry.

RNA Hybridization

Five PC12 cultures, grown on 150-mm plates, were used to prepare each

RNA sample. The cells were trituated from culture plates in 37°C PBS and collected by centrifugation. 4 ml of 4°C extraction buffer (3 M LiCl, 6 M urea, 20 mM Tris 7.5, 10 mM MgCl₂, 1% β-mercaptoethanol) was added and the cells were immediately lysed by disruption with a Polytron homogenizer (top speed, 5 s). The extract was next gently agitated at room temperature for 15 min to completely solubilize the cells. The extract was incubated at 4°C overnight to allow precipitation of RNA. The precipitated RNA was collected by a 15-min centrifugation at 4,000 rpm and 4°C. Each RNA sample was resuspended in TE (10 mM Tris-Cl, pH 7.4, 1 mM EDTA) at room temperature, phenol extracted, and ethanol precipitated. The RNA pellet was resuspended in TE and the concentration was determined by spectrophotometry at O.D. 260. Normalized RNA samples were loaded on 0.9% agarose gels containing formaldehyde as described by Goldberg (14). RNA blots were prepared and probed as described previously.

Results

Microtubule Protein Synthesis Rates Increase in Response to NGF

When PC12 cells are cultured in NGF, neurites are extended, microtubule assembly is induced, and levels of the microtubule proteins tubulin, tau, and a MAP1 isoform increase. To determine if the increase in the levels of microtubule proteins is due to an increase in their rate of synthesis (rather than decreased degradation) the synthesis rates were compared in undifferentiated PC12 cells and PC12 cells treated with NGF for 5 d. The NGF treatment for 5 d was chosen because the response to NGF is maximal at this time. Also, after exposure of cells to NGF for 5 d, PC12 cells still have a high degree of plasticity; NGF removal causes, within 1–2 d, neurite loss and return of microtubule assembly levels and microtubule protein levels to those found in undifferentiated cells.

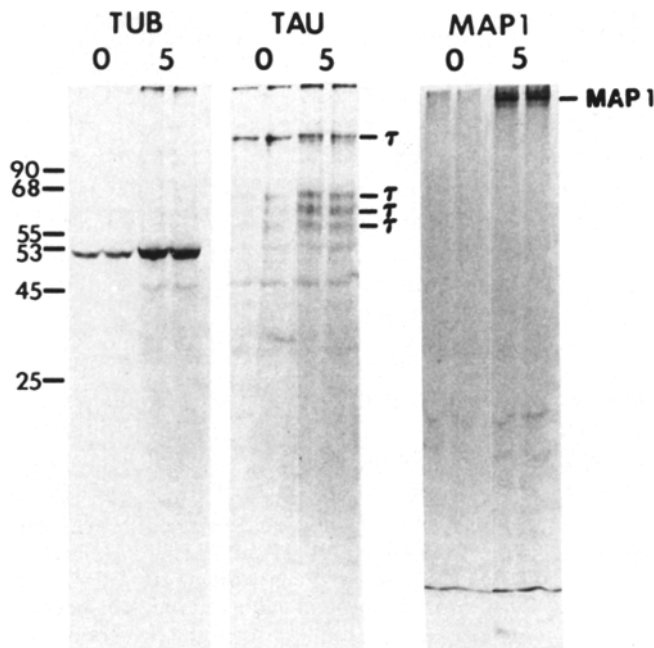


Figure 1. Microtubule protein synthesis changes in response to NGF. Duplicate cultures for untreated PC12 cells, or cells cultured for 5 d in NGF, were labeled for 1.5 h with [³⁵S]methionine and extracts were prepared. Tubulin, MAP1, and tau protein were sequentially immunoabsorbed from samples containing equivalent amounts of radioactivity. Fluorography of SDS-PAGE gel.

To compare synthesis rates, PC12 cells were labeled by incubation in [³⁵S]methionine for 1.5 h and protein extracts were prepared in SDS. Tubulin, MAP1, and tau protein were sequentially immunoprecipitated from each extract. The results of this experiment, performed in duplicate, are shown in Fig. 1. The β -tubulin synthesis rate increases threefold after correcting for total protein synthesis rates by normalizing the samples to total cpm. The rate of synthesis of the 68–70-kD tau complex increases 3.4-fold while the synthesis of the 120-kD tau-like protein (shown previously to be closely related to the 61–68-kD taus (Drubin [27]) does not change appreciably (1.03-fold) in response to NGF. Consistent with this observation, the 120-kD tau-like protein does not accumulate appreciably in response to NGF while the 61- and 68-kD proteins do (1). MAP1 synthesis is undetectable in undifferentiated cells and is prominent in differentiated cells. The MAP1 synthesis induction is most dramatic, consistent with observed changes in total MAP1 protein. Several MAP1 isoforms have been observed. Observations in this report are restricted to the single MAP1 isoform detected with the 7-1.1 antibody (1). This isoform has been shown by biochemical and immunocytochemical means to be localized on microtubules in PC12 cells (13).

When NGF is removed for 2 d from PC12 cells cultured in NGF for 5 d, the synthesis rates of all three microtubule proteins return to undifferentiated levels (not shown). Thus, continued induction of microtubule protein synthesis requires continued presence of NGF.

Tubulin and Tau mRNA Levels Increase in Response to NGF

Tubulin and tau cDNA clones were used as probes to determine if tubulin and tau mRNA levels change in response to NGF treatment. Fig. 2 shows Northern blots containing total RNA from undifferentiated PC12 cells, PC12 cells cultured in the presence of NGF for 5 d, and PC12 cells cultured for 5 d in NGF and then removed from NGF for 2 d.

When tau cDNA is used to probe PC12 cell mRNA, mRNAs of ~6 and 8 kb are detected. We believe that the RNA species migrating below the 6–8-kb doublet is a nonspecific RNA hybridization and does not represent a

form of tau mRNA for the following reasons. It is not found on poly A⁺ RNA, all tau translational activity resides in the 6–8-kb region of poly A⁺ RNAs, and the amount of the lower RNA species does not correlate with tau synthesis rates in any experiments. Since mRNA migrating at 6 kb contains all 61–68-kD tau translational activity in the brain (10), and since only the 8-kb mRNA is found in N115 cells, and these cells express only the 120-kD tau-like proteins (12), we believe that the 8-kb mRNA encodes the 120-kD tau-like protein. Consistent with this conclusion, levels of the 8-kb mRNA change only slightly when PC12 cells are treated with NGF, as do 120-kD synthesis rates. In contrast, the 6-kb mRNA increases dramatically in response to NGF, and decreases dramatically when NGF is withdrawn, as do 61- and 68-kD tau protein synthesis rates. In addition, undifferentiated N115 neuroblastoma cells contain undetectable tau mRNA and tau protein (12). After neurite extension, the 120-kD tau-like protein is expressed and the 8-kb mRNA species appears. In PC12 cells, tubulin mRNA levels increase 2.5-fold in response to NGF and decrease when NGF is removed (not shown).

Autoregulation of Microtubule Protein Synthesis

Previous studies using microtubule drugs (2, 7) or microinjected tubulin have shown that tubulin subunits feedback to regulate tubulin synthesis. Similar controls could play a role in the regulation of tubulin levels during PC12 cell neurite extension. This might suggest that increased tubulin synthesis is a result, rather than a cause, of the increased assembly into polymer. To test this possibility and to determine whether similar control mechanisms operate for tau protein and MAP1, PC12 cells were treated with various microtubule drugs for 4 h and then labeled for the next 1.5 h in [³⁵S]methionine in the continued presence of drug. Extracts were prepared and tubulin, MAP1, and tau protein were sequentially immunoprecipitated.

The results (*left panel* of Fig. 3 A) for experiments performed in duplicate show that tubulin synthesis rates in undifferentiated PC12 cells decrease in response to microtubule depolymerization caused by either colchicine (6.7-fold) or nocodazole (4-fold). In sharp contrast, taxol, a drug that promotes microtubule assembly, causes a very large (4.2-fold) increase in tubulin synthesis rates. This result was not observed previously in other cell lines. In NGF-differentiated PC12 cells (Fig. 3 B, *left panel*) taxol causes a smaller (1.9-fold) increase in tubulin synthesis rates, while colchicine and nocodazole cause similar dramatic reductions (6.0- and 3.5-fold) comparable to those seen in undifferentiated cells.

The effects of these microtubule drugs on tau protein synthesis are shown for undifferentiated (Fig. 3 A, *right panel*) and for differentiated PC12 cells (Fig. 3 B, *right panel*). First, as was previously reported (12), taxol, vinblastine, colchicine, and nocodazole all cause a mobility shift of all tau species suggesting a change in posttranslational modification, most likely phosphorylation. Second, we observe only minor changes (less than twofold) in the synthesis rate for tau protein in response to colchicine, taxol, or vinblastine. However, when nocodazole is used to depolymerize microtubules in both undifferentiated and differentiated PC12 cells, there is a 4.5-fold increase in 120-kD tau-like protein synthesis

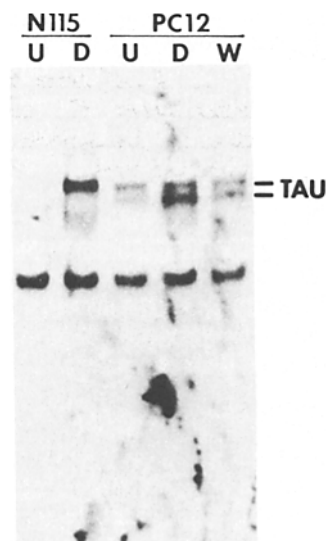


Figure 2. Tau mRNA level changes during N115 mouse neuroblastoma and PC12 cell differentiation. Equivalent amounts of total mRNA from undifferentiated (U), differentiated (D), or differentiated cells withdrawn from NGF for 2 d (W) were probed with a tau cDNA probe on a Northern blot. The band below the doublet labeled TAU is a polyA⁻ background band (see text).

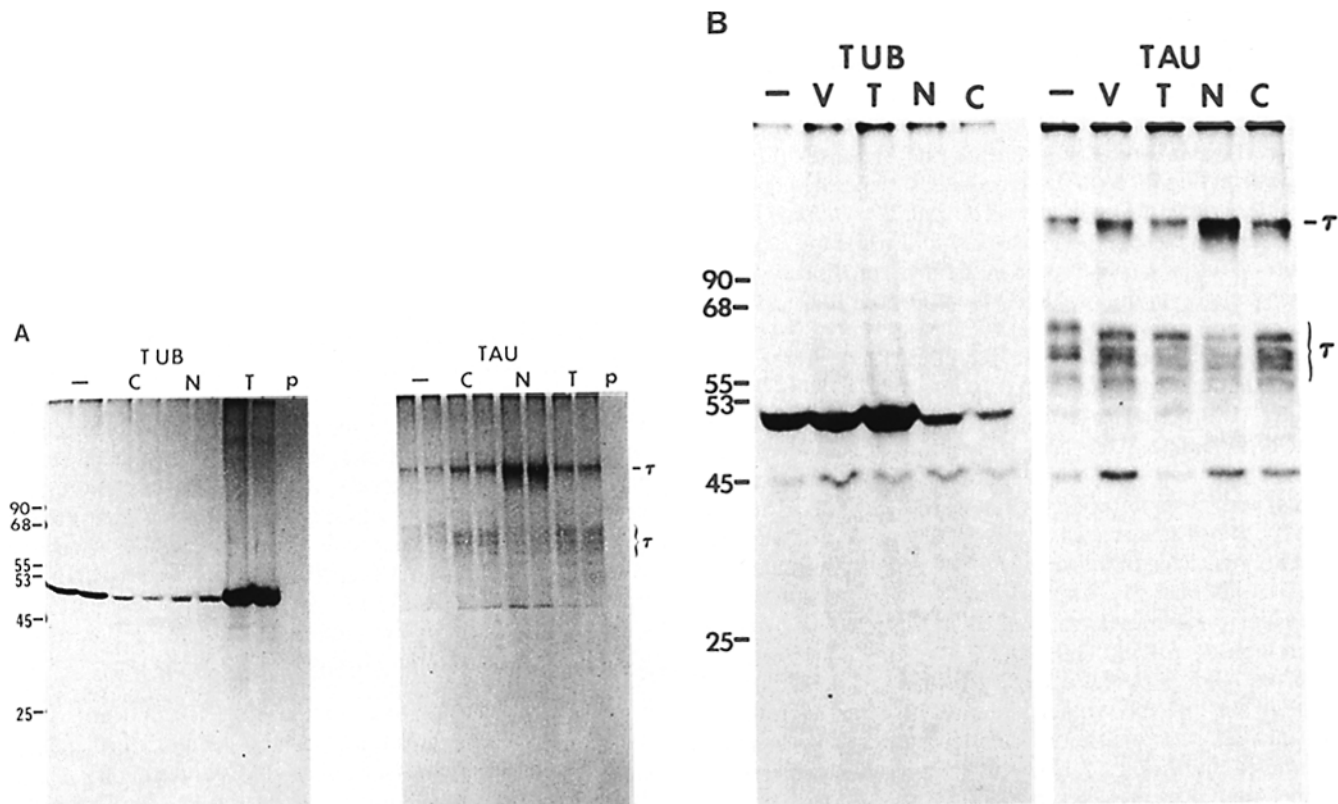


Figure 3. Autoregulation of microtubule protein synthesis in undifferentiated (*A*) and differentiated (*B*) PC12 cells. Cells were cultured for 4 h in the absence of drug (–), or the presence of 10 μ M colchicine (C), nocodazole (N), vinblastine (V), or taxol (T). Cells were labeled for the next 1.5 h with [35 S]methionine in the continued presence of drug. Proteins were immunoabsorbed as described in Fig. 1. *p* designates a (–) sample immunoabsorbed with nonimmune rabbit antiserum. Experiments in *A* were performed in duplicate.

with a concomitant 3.1-fold decrease in 61- and 68-kD tau protein synthesis rates. These nocodazole-induced changes in relative synthesis rates can be accounted for by changes in relative abundance of 6 and 8-kb tau mRNAs (Fig. 4). In undifferentiated cells, nocodazole causes an increase in 8-kb mRNA, thought to encode the 120-kD tau-like protein, and a slight decrease in the 6-kb tau mRNA, thought to encode the 61- and 68-kD taus. No consistent changes in MAP1 synthesis rates were observed in these experiments (not shown).

Microtubule Protein Turnover

Changes in the turnover of tubulin and associated proteins could contribute to the changes in the levels of these proteins during PC12 cell differentiation. Turnover rates in undifferentiated cells and in cells cultured for 5 d in NGF were compared by labeling for 4 h with [35 S]methionine, then chasing for 18 h. Tubulin, MAP1, and tau protein were sequentially immunoprecipitated from extracts prepared from duplicate cultures. Extracts were normalized to reflect initial [35 S]methionine incorporated in pulse samples and net incorporated [35 S]methionine remaining after the chase period. (Tau and MAP1 turnover in undifferentiated cells could not be observed due to insufficient sensitivity in these turnover experiments.) Tubulin turnover rates do not change appreciably in response to NGF (Fig. 5). In cells cultured for 5 d in NGF, tau protein and MAP1 turn over more quickly than tubulin (Fig. 5). In 18 h, approximately one-third of the tubulin remains whereas only one-tenth of the tau protein and MAP1 (data not shown) remains.

In fibroblast and liver cells, it has been shown that assembled tubulin is degraded more slowly than unassembled tubulin (5). To see if the state of tubulin polymerization affects associated proteins as well as tubulin in PC12 cells, turnover of these proteins was compared in untreated PC12 cells and in cells treated with either colchicine to depolymerize microtubules or with taxol to promote assembly. The experiment shown in Fig. 6 is similar to that shown in Fig. 5 except that during chase periods cells were either treated with drug or no drug. As shown in the left panel of Fig. 6 *A*, taxol increases the stability of tubulin about twofold while colcemid decreases tubulin stability approximately sixfold. As shown in Fig. 6, *B* and *C*, Taxol has no effect on tau protein or MAP1 turnover (although the tau mobility shift discussed above occurs). This is consistent with the previous finding that essentially all tau protein and MAP1 are associated with microtubules in PC12 cells (13), and thus increased tubulin assembly does not promote additional tau and MAP1 binding. In contrast, disassembly of microtubules sharply decreases tau and MAP1 stability as shown in Fig. 6, *B* and *C*. Tau, tubulin, and MAP1 are therefore all more stable when they are assembled into a microtubule.

Stimulation of Microtubule Protein Turnover after NGF Withdrawal

To complete this analysis, it would be most satisfactory to compare the turnover rates of tau and MAP1 in undifferentiated and differentiated PC12 cells. However, the low levels of these proteins in the undifferentiated cells precludes

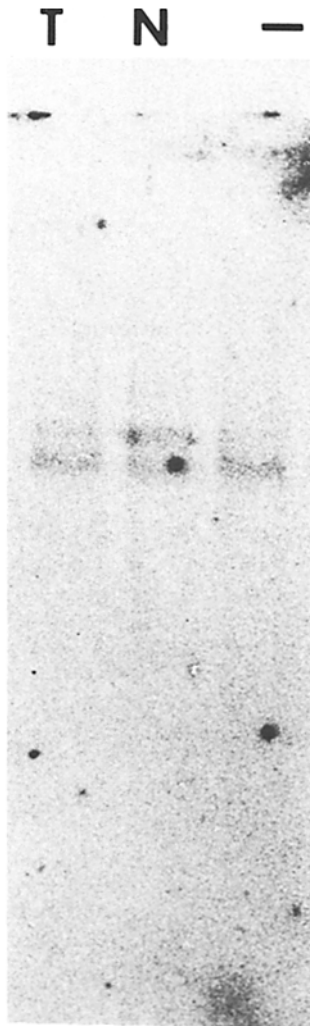


Figure 4. Tau mRNA levels in differentiated PC12 cells cultured in the absence of drug or in the presence of nocodazole (N) or taxol (T) for 6 h. Treatment and analysis of RNA is as described for Fig. 2.

pulse-chase experiments under these conditions. Instead, we asked whether changes occur in the turnover of microtubule proteins when NGF is withdrawn from differentiated PC12 cells. Removal of NGF from PC12 cells cultured for 5 d in the presence of NGF causes neurite retraction, microtubule disassembly, and reduction in tubulin, tau, and MAP1 protein levels (11). PC12 cells cultured for 5 d in NGF were incubated in [³⁵S]methionine during the fifth day in NGF. The cells were then removed from [³⁵S]methionine for 2 d in either the absence or continued presence of NGF. Removal of NGF for 2 d is sufficient to reverse all effects of NGF on PC12 cell morphology, microtubule assembly, and microtubule protein levels. As shown in Fig. 7, for experiments performed in duplicate; tubulin, tau protein, and MAP1 are all degraded more quickly when NGF is withdrawn from differentiated PC12 cells.

Discussion

Morphological changes that accompany cell differentiation make special demands on the biosynthetic activity of the cell. Changes must occur in the synthesis of several cytoskeletal proteins and these changes must be coordinated. It is likely that cells have mechanisms, therefore, for sensing the levels

of these proteins independently or there must be some logical regulatory circuitry that keeps these proteins in balance and allows appropriate changes in this balance during differentiation. The fact that cytoskeletal proteins exist in an insoluble form, presumably segregated from the biosynthetic machinery, raises new problems and suggests that novel mechanisms and strategies may exist for biosynthetic regulation. In fact, a rather intensive study has been made of tubulin feedback control (see below). Similarly other studies, for example those on RBC spectrin and ankyrin (20), have emphasized the important regulatory features of turnover to insure the proper stoichiometry of oligomeric proteins.

In this study, we used PC12 cells to explore regulatory mechanisms that coordinate the expression of several proteins of the microtubule cytoskeleton. PC12 cells are highly responsive to NGF which causes neurite extension, induction of microtubule assembly, increases in microtubule protein levels, and posttranslational modification of microtubule proteins (3, 16). In response to NGF, tubulin levels increase modestly while levels of tau protein and a MAP1 isoform increase more dramatically and parallel the outgrowth of neurite processes (11). In a related model system, tau protein is also induced when neuroblastoma cells extend neurites (12). The effects of NGF on PC12 cells are quickly reversed when NGF is withdrawn.

The studies presented here show that the primary mechanism for regulating the levels of tubulin, tau protein, and MAP1 is by increasing synthesis rates in response to NGF addition and decreasing the synthesis rates when NGF is removed. For tau protein and tubulin, where cDNA probes were available, it was shown that synthesis rate changes apparently reflect changes in mRNA levels. It is not known whether the mRNA levels are regulated by synthesis or degradation of the mRNA. The changes in synthesis rates are roughly proportional to changes in total microtubule protein levels observed by immunoblotting in an earlier study (11) and therefore synthesis rate changes largely can account for changes in absolute protein levels. Evidence was presented indicating that additional levels of control can operate to fine-tune microtubule protein levels and to make microtubule protein level changes more responsive to environmental signals.

Although formally in molecular biology one can write a hierarchy of causality consisting of transcription, translation, and protein stability, any of these could in fact be the primary locus of regulation. This possibility is most clearly seen for tubulin where it has been argued that tubulin mRNA levels are controlled by the level of the soluble tubulin polypeptide (7). Recent evidence suggests that this control is completely cytoplasmic and must be on the level of the stability of the tubulin mRNA (6, 22). By some unknown mechanism the free tubulin polypeptide must destabilize its own mRNA, perhaps by interfering with translation. In PC12 cells, we also find that increasing the tubulin subunit concentration by depolymerization of microtubules by specific drugs causes a reduction of the tubulin synthesis rate. Furthermore, taxol, a drug that promotes microtubule assembly, causes a sharp increase in tubulin synthesis rates in PC12 cells; interestingly this effect is much more dramatic in undifferentiated PC12 cells than in cells that have extended neurites.

Small increases in tubulin synthesis in response to taxol

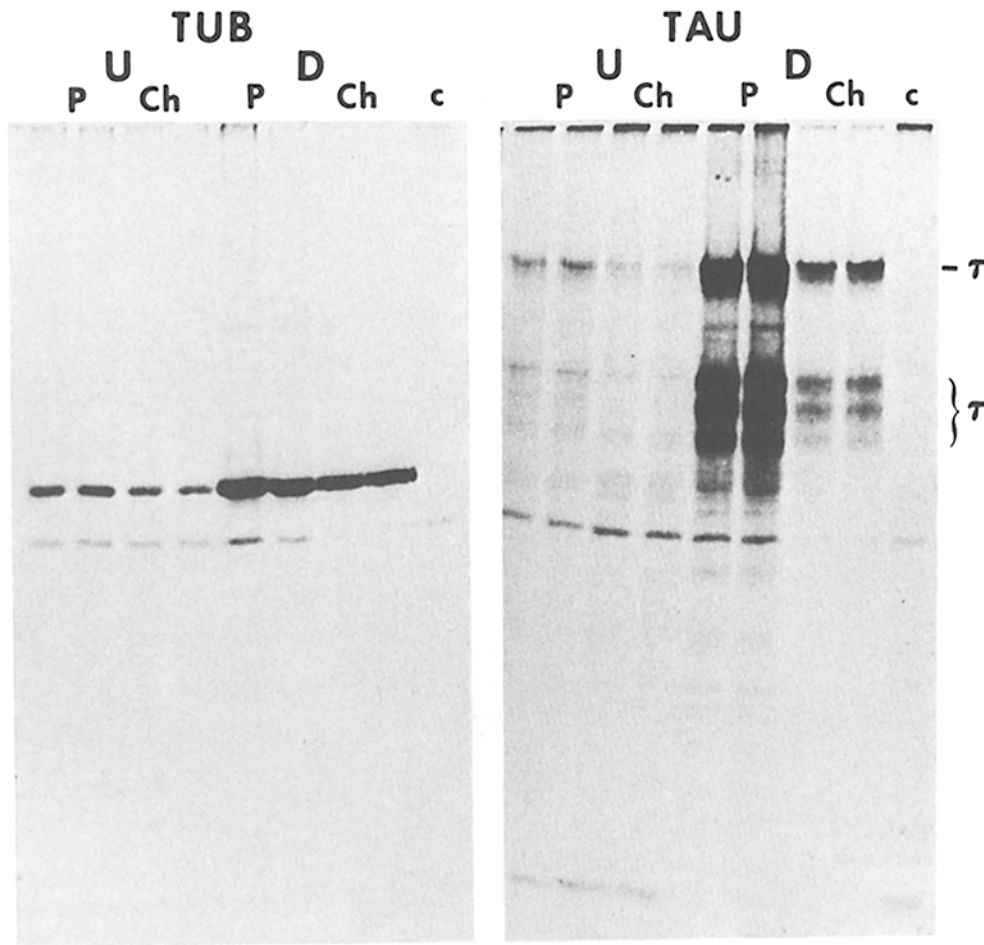


Figure 5. Comparison of tubulin, tau, and MAP1 turnover in undifferentiated PC12 cells (*U*) and in PC12 cells differentiated in NGF for 5 d (*D*). Pulse (*P*) samples were prepared after a 4-h labeling in [³⁵S]methionine. Chase (*Ch*) samples were prepared after an 18-h chase in excess unlabeled methionine. Immunoabsorptions were as described for Fig. 1. Duplicate cultures were used. *c* represents a control immunoabsorption.

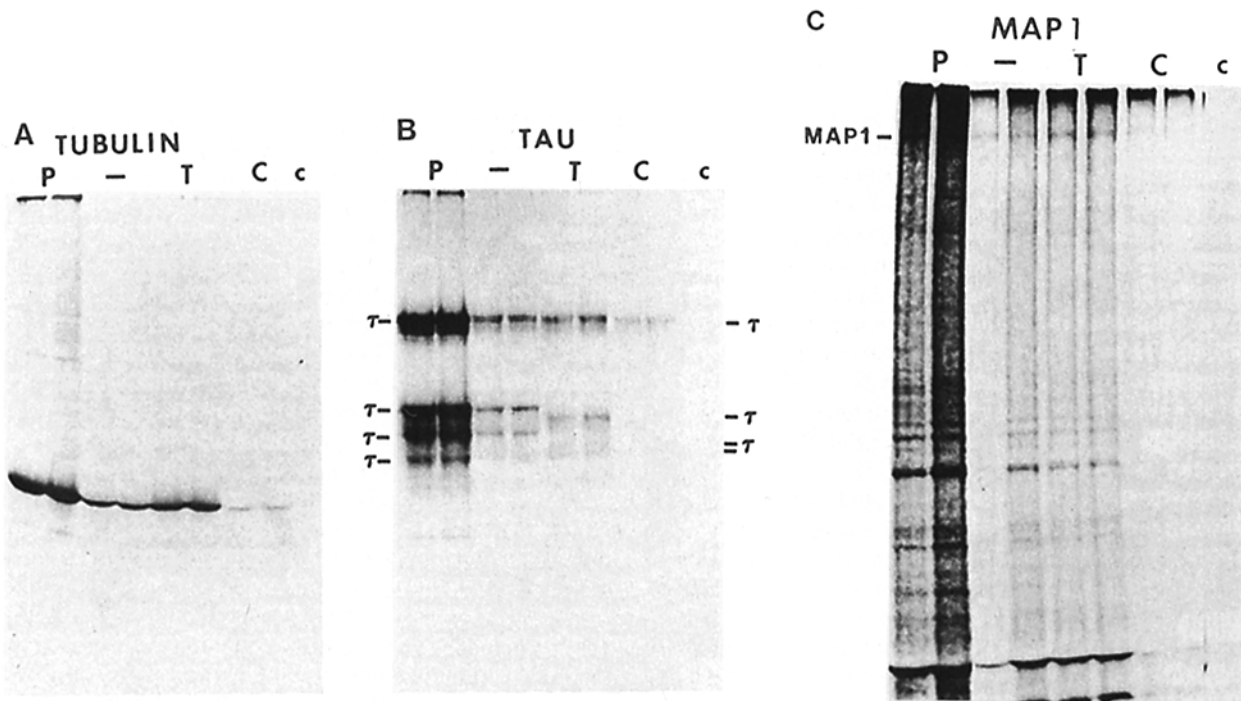


Figure 6. Effect of microtubule assembly state on tubulin, tau protein, and MAP1 turnover. This experiment, using differentiated PC12 cells, was performed exactly as the experiment in Fig. 5 except the 18-h chase was performed in the absence of drug (-), or in the presence of 10 μ M taxol (*T*) or 10 μ M colchicine (*C*). *P* represents pulse samples. *c* represents a control preimmune immunoabsorption. Duplicate cultures were used.

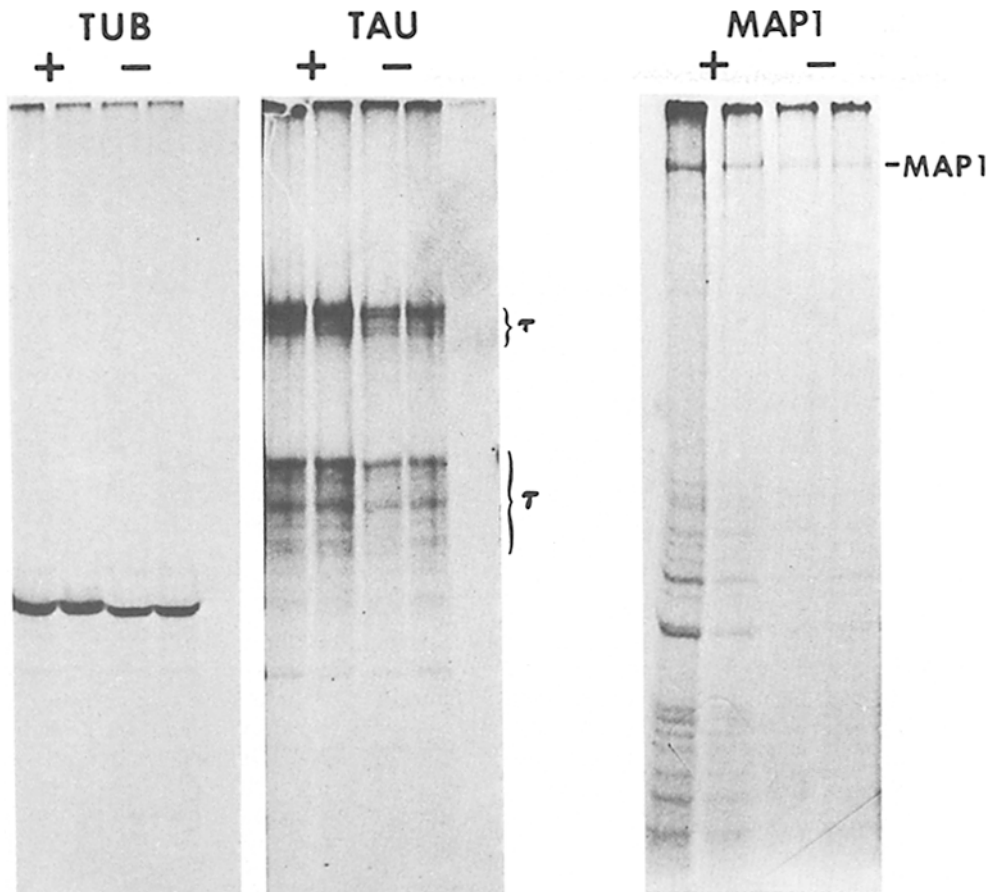


Figure 7. Microtubule protein turnover in response to NGF withdrawal from differentiated cultures. PC12 cells were cultured in NGF for 5 d and then labeled 4 h with [³⁵S]methionine. The cultures were next chased with unlabeled methionine for 2 d in the continued presence (+) or in the absence (-) of NGF. Tubulin, tau protein, and MAP1 were immunoprecipitated as described for Fig. 1.

have been seen previously but never as dramatic as those seen in undifferentiated PC12 cells. In all cases the effects of depressing synthesis with colchicine or nocodazole have been larger than the increases in synthesis rates with taxol. The explanation for the exceptionally large stimulation in undifferentiated PC12 cells may be due to an exceptionally large unpolymerized tubulin pool. The ratio of unassembled to assembled tubulin varies in different cell types and decreases in PC12 cells after NGF addition (3, 11). Recently Caron et al. (6) showed that the rate of synthesis in fibroblasts is more or less inversely proportional to this ratio, and in cells like hepatocytes, where the unassembled pool is very small, there may be little scope for increasing the synthesis rate. Taxol could have a much smaller effect in differentiated PC12 cells because these cells have a small pool of unassembled tubulin like hepatocytes, and because tubulin is synthesized at close to the maximal rate appropriate for differentiated cells (Fig. 1). Taxol treatment of undifferentiated PC12 cells would, however, lower the tubulin pool and in a sense trick the cell into thinking it is forming a neurite, thereby inducing near maximal rates of tubulin synthesis. This suggests that a major locus regulating the tubulin mRNA level may be the control of the monomer level that is in turn controlled by other factors such as the level of MAP1 and tau. Colchicine and nocodazole do not cause larger tubulin synthesis decreases in differentiated cells with higher microtubule assembly levels than undifferentiated cells perhaps due to higher baseline synthesis rates in the presence of NGF.

Although tubulin synthesis rates (and tubulin mRNA lev-

els) could be linked in part to the overall level of assembly, tau and MAP1 do not respond simply to these perturbations. Increased microtubule assembly apparently does not cause changes in tau protein and MAP1 synthesis rates, since taxol has no effect on the synthesis of these proteins. This should not be surprising since tau protein and MAP1, in contrast to tubulin, are mostly bound to microtubules and are not in a freely diffusible state; additional microtubule assembly should not affect tau and MAP1 pools. Decreases in microtubule assembly caused by colchicine or nocodazole should sharply increase the levels of tau protein and MAP1 not bound to microtubules. However, no reproducible effects on MAP1 synthesis were observed. For tau protein, in both NGF-treated and untreated cells, nocodazole caused a decrease in the synthesis of the 61- and 68-kD protein and an increase in the synthesis of the 120-kD protein. This effect is apparently mediated by changes in tau mRNA levels, since nocodazole causes a decrease in the 6-kb tau mRNA and an increase in the 8-kb species. Colchicine, which by other biochemical (see Figs. 3 and 6) and morphological criteria has a similar effect to nocodazole in causing microtubule disassembly, has no effect on tau protein synthesis. The different response to colchicine and nocodazole could reflect differences in the interaction of a colchicine-tubulin complex vs. a nocodazole-tubulin complex, with whatever regulatory apparatus regulates the rate of a tau synthesis. Further studies are needed to better understand these interesting effects on tau synthesis rates.

Tubulin, tau protein, and MAP1 levels are also regulated

by protein turnover. These proteins seem to be more stable when they are associated in a microtubule than in a soluble state, since colchicine and nocodazole increase their rates of degradation. Increased degradation of tubulin in response to colchicine was observed previously, but with tubulin it is possible that increased degradation rates are merely due to recognition of drug-tubulin complexes as targets for proteolytic enzymes. Since colchicine and nocodazole do not bind to tau protein or MAP1, it is unlikely that these proteins could be seen as abnormal and targeted for degradation. Instead, it is more likely that all three proteins are less stable when they are not assembled. This mechanism could help to insure that microtubule protein levels are maintained in the proper stoichiometry, as is the case with spectrin and ankyrin (20). Excess proteins that fail to assemble would be degraded. Changes in microtubule protein stability coupled to assembly and disassembly could play a role in the accumulation of these proteins in response to NGF and the disappearance of microtubule proteins after NGF removal.

Induction of microtubule assembly by taxol causes a slight increase in tubulin stability. If NGF causes a decrease in un-assembled tubulin pools when assembly is induced, a slight increase in tubulin stability would be expected. No effect on tubulin degradation rate, however, was observed when PC12 cells were cultured in NGF. An important difference between microtubule assembly induced by taxol vs. NGF is that NGF increases total tubulin levels when assembly is induced and the cell may maintain the same steady-state concentration of un-assembled tubulin, hence the same rate of degradation. Taxol, on the other hand, causes assembly of virtually all tubulin. The modest increase in tubulin stability observed in response to taxol would be hardly detectable in NGF-treated cells due to the more modest change in the proportion of assembled tubulin.

The interrelationships between the state of assembly and biosynthesis imposes important features on cell differentiation. The addition of NGF to PC12 cells causes an increase in microtubule assembly and a dramatic change in the composition of associated proteins. The coordinated changes in the biosynthesis and degradation render the entire process sensitive to the NGF signal. The levels of these proteins are not regulated independently and are not independent of total polymer level. The logic of the regulatory interactions leads to a system that is metastable and rapidly responds to NGF rather than producing a system that is stable and buffered in either the differentiated or undifferentiated state as described below.

Tubulin synthesis rates do not in themselves drive neurite outgrowth. Modest increases in tubulin synthesis occur in response to NGF but are not temporally correlated with neurite outgrowth (11). In either the differentiated or undifferentiated state, tubulin synthesis rates are buffered by the total pool of unpolymerized tubulin as has been discussed previously. However, the tubulin pools in undifferentiated PC12 cells seem to be higher so that in the presence of NGF, tubulin synthesis rates increase despite the autoregulatory restrictions imposed by the large un-assembled tubulin pool. Tubulin begins to accumulate in response to NGF before neurite extension. Then, when neurites sprout, tubulin synthesis rates can be potentiated in response to a decreased pool of free tubulin, regulated most likely by proteins like tau and MAP1 that drive polymerization.

A major difference between the undifferentiated and differentiated state of PC12 cells is the level of tau and MAP1 protein, which in turn are regulated by the level of mRNA in the case of tau, and perhaps to a lesser extent in the case of MAP1 (19). The rates of synthesis of these proteins are not strictly tied to the tubulin monomer pool and it seems most likely that they are regulated as part of the differentiation process mediated by NGF. The regulatory responses studied here suggest that increases in tau and MAP1 levels should increase microtubule polymerization. Tau protein is present in differentiated PC12 cells at near stoichiometric levels on microtubules (11). Excess production of tau should lead to free tau protein, which would most likely be degraded, or incorporated into new microtubules. Increased tau and MAP1 should decrease the free tubulin, which in turn should stimulate tubulin synthesis. Thus the coordinated assembly of associated proteins and tubulin can be controlled by the feedback on tubulin synthesis and the rapid degradation of unincorporated associated proteins.

Maintenance of differentiated state is completely dependent on NGF, and the logic of the biosynthetic regulatory machinery enforces this metastability. Removal of NGF causes dramatic decreases in the rates of synthesis of tau and MAP1. Both of these proteins have shorter half lives than tubulin, making the system responsive without requiring the degradation of tubulin, the major structural protein. As disassembly proceeds, the increased tubulin pool would cause a decrease in the synthesis of 61- and 68-kD tau forms, an increase in the synthesis of the 120-kD form, and increased degradation of tau protein and MAP1. These changes may further increase the rates of disassembly. Tubulin levels would decline much more slowly (11). Thus, though the overall levels of tubulin, tau, and MAP1 may be regulated in part at the level of transcription or mRNA, the autoregulatory mechanisms involving mRNA stability and the changes in expression and protein stability are clearly responding to the pools of the proteins themselves. These both coordinate the differentiation response of the microtubule cytoskeleton to NGF and lead to the characteristic metastability of the system.

We thank Stuart Feinstein for helpful discussions and for supplying PC12 cells. We are grateful to Louise Evans for help with the figures and Cynthia Cunningham-Hernandez for her assistance in preparing the manuscript.

This research was supported by the National Institute of General Medical Sciences.

Received for publication 5 November 1987, and in revised form 11 January 1988.

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