

Regulation of Microtubule Composition and Stability during Nerve Growth Factor-promoted Neurite Outgrowth

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Abstract. We have used the nerve growth factor (NGF)-responsive line of PC12 pheochromocytoma cells as a model system to study microtubule specializations associated with neurite outgrowth. PC12 cells treated with NGF cease proliferating and extend neurites. Long-term NGF treatment results in a two- to threefold increase in the proportion of total cellular tubulin that is polymerized in PC12 cells. The increase in this parameter first becomes apparent at 2-4 d with NGF and increases steadily thereafter. Several changes in microtubule-associated proteins (MAPs) of PC12 cells also occur after exposure to NGF. In immunoprecipitation assays, we observed the levels of MAP-2 to increase by at least several-fold after treatment with NGF. We also found that the compositions of three MAP classes with apparent M_r of 64K, 67K, and 80K are altered by NGF treatment. These MAPs, recently designated "chartins," are biochemically and immunologically distinct from the similarly-sized tau MAPs (Peng et al., 1985 *Brain Res.* 361: 200; Magendantz and Solomon, 1985 *Proc. Natl. Acad. Sci.* 82: 6581). In two-dimensional isoelectric focusing x SDS polyacrylamide gels, each chartin MAP class resolves into a set of proteins of similar apparent M_r but distinct pI. Peptide mapping analyses confirm that the isoelectric variants comprising each chartin MAP class are closely related in primary structure. Several

striking differences in the composition of the chartin MAPs of PC12 cells grown with or without NGF were consistently observed. In particular, following long-term NGF treatment, the abundances of the more acidic variants of each chartin MAP class were markedly enhanced relative to the more basic members. This occurs without substantial changes in the abundance of each MAP class as a whole relative to total cell protein. The combined results of in vivo phosphorylation and peptide mapping experiments indicate that the NGF-inducible chartin MAP species are not primary translation products, but are generated posttranslationally, apparently by differential phosphorylation of other chartin MAPs. These observations suggest that NGF treatment of PC12 cells leads to changes in the posttranslational processing of the chartin MAPs. The time course of these changes closely resembles that for the increase in the proportion of cellular tubulin that is polymerized and for neurite outgrowth.

One of the important events in the growth and stabilization of neurites appears to be the formation of microtubule bundles that extend from the cell body to the tips of the neurites. We propose that the biochemical changes in microtubules of PC12 cells induced by NGF are important for the formation of microtubule bundles and thereby for the formation of neurites.

ONE of the intracellular events that appears to be essential for neurite outgrowth is the formation of microtubule bundles (15, 16, 35, 45, 55). This is clearly illustrated with the PC12 line of nerve growth factor (NGF)-responsive pheochromocytoma cells (24, 35, 52).¹ In the absence of NGF, PC12 cells are rounded in shape and lack significant cytoplasmic extensions. They also lack parallel arrays of microtubules. After several days of NGF treatment, the cells extend long, branched neurites (26). In

conjunction with the formation of such neurites is the appearance of parallel arrays or bundles of microtubules that extend from the cell body to the tips of the neurites (35, 52). These microtubule bundles are the most prominent structural elements observed in electron micrographs of NGF-treated PC12 cells; intermediate filaments and microfilaments are present, but by comparison with microtubules they are quantitatively minor (4, 35, 52). Thus, these microtubule bundles provide the principal architectural framework of the neurites. The close temporal relationship between the appearance of microtubule bundles and the outgrowth of long neurites suggests a causal relationship between these events. This possibility is fully supported by pharmacological ex-

1. *Abbreviations used in this paper:* MAP, microtubule-associated protein; NGF, nerve growth factor; PC12(+) cells, PC12 cells grown with NGF for 3 wk; PC12(-) cells, PC12 cells grown without NGF.

periments which show that agents that prevent the formation of microtubule bundles in PC12 cells (24) and neurons (15, 16, 55, 56) also prevent neurite outgrowth.

We have been using the PC12 line as a model system to study microtubule specializations associated with neurite outgrowth. In previous studies, it has been shown that the stability of PC12 cell microtubules, as assessed by sensitivity to depolymerization induced by colchicine (4) or low temperature (6), increases after treatment with NGF, and that these stable microtubules are present in neurites (4). In the present report, we have measured the tubulin protomer-polymer ratio in PC12 cells grown without NGF or with NGF for varying times to obtain more detailed information on the NGF-promoted enhancement of microtubule stability. In addition we have also carried out further analyses of NGF-induced changes in microtubule-associated proteins (MAPs) of PC12 cells. MAPs are of particular interest with regard to NGF-promoted changes in microtubule stability and microtubule bundle formation because under in vitro conditions they enhance microtubule stability (30, 39, 49) and also influence the spacing between microtubules (10, 31, 54). Previous studies have shown that the relative abundances of MAP-1 species (18, 25) and tau (18) increase dramatically after long-term treatment of PC12 cells with NGF. Here we show that long-term NGF treatment, in addition to modulating the abundance of another MAP (MAP-2), also influences the posttranslational modification of several distinct MAPs (the "chartins" [36]) which are relatively abundant in PC12 cells grown with or without NGF. Preliminary accounts of this work have been published previously (6).

Materials and Methods

Cell Culture and Metabolic Labeling

The PC12 line of NGF-responsive pheochromocytoma cells was used in these experiments. Cells were grown as previously described in culture dishes coated with rat tail collagen and fed three times weekly with RPMI 1640 medium supplemented with 10% horse serum and 5% fetal calf serum (15% serum) (sera were obtained from KC Biological Inc., Lenexa, KS) (26). Exposure to NGF was in the same medium or in RPMI 1640 supplemented with 1% horse serum. NGF was prepared by the procedure of Mobley et al. (37), and used at a final concentration of 50 ng/ml.

To radiolabel PC12 cell proteins, NGF-treated or untreated cultures were incubated with 50–100 $\mu\text{Ci/ml}$ of [^{35}S]methionine (New England Nuclear, Boston, MA) for 22–28 h in medium containing 20–25% of the normal amount of methionine or for 3 d in complete medium. To specifically label phosphoproteins, cultures were labeled with 100 $\mu\text{Ci/ml}$ of [^{32}P]PO $_4$ (ICN K&K Laboratories, Inc., Plainview, NY, or New England Nuclear) in phosphate-free medium for 2 h as described previously (5, 25).

Drug Treatments

Some cultures grown without NGF or with NGF for 21 d were incubated with 2 $\mu\text{g/ml}$ of podophyllotoxin or nocodazole for 4 h before extraction (see below). The drugs were dissolved in dimethyl sulfoxide at a final concentration of 2 mg/ml, and appropriate volumes were added to the cultures.

Cell Extractions

Two extraction procedures were used in these experiments. One procedure (dish extraction procedure) was modified slightly from that of Black and Greene (4). Briefly, cells were rinsed twice with PBS, once with buffer 1 (0.1 M Pipes, pH 6.9, 1 mM MgSO $_4$, 2 M glycerol, 2 mM EGTA, aprotinin [0.02 trypsin inhibitory units per ml]), and then were incubated while remaining attached to the culture dish for two successive 10-min periods in buffer 2 (buffer 1 + 0.2% [wt/vol] Triton X-100) at room temperature. Triton X-100-insoluble residues were rinsed twice with buffer 3 (buffer 1 without

EGTA) and then suspended in a Ca $^{++}$ -containing buffer (0.1 M Pipes, pH 6.9, 1 mM MgSO $_4$, 5 mM CaCl $_2$, aprotinin), incubated on ice for 10–20 min, and then clarified by centrifugation (12,000 g for 10 min at 4°C) to obtain a Ca $^{++}$ -soluble fraction. In some experiments, extractions of attached cells were carried out by slightly different procedures as described by Burstein et al. (11). Comparable results were obtained with both methods of extraction and Ca $^{++}$ treatment. With these extraction conditions, unpolymerized tubulin is quantitatively solubilized by buffer 2, while microtubules remain with the Triton X-100-insoluble cell residue (3–5, 50). The Ca $^{++}$ -buffer depolymerizes the microtubules present in the Triton X-100-insoluble cell residues and thereby solubilizes their constituent proteins. Thus, the Ca $^{++}$ -soluble fractions contain most or all of the tubulin that was polymerized at the time of extraction (3–5, 50). Drug-treated cultures were extracted as above except that the drug was included in PBS, buffers 1 and 2 at 2 $\mu\text{g/ml}$. Ca $^{++}$ -soluble fractions from control as well as drug-treated cultures will be referred to as microtubule fractions.

The other extraction procedure, which was used to quantify the relative levels of polymerized tubulin in PC12 cells, was modified slightly from the homogenization extraction method described by Black and Kurdyla (5). Briefly, cells were rinsed twice with PBS, once with a microtubule-stabilizing buffer (0.1 M Pipes, pH 6.9, 4 M glycerol, 5% [vol/vol] dimethyl sulfoxide, 1 mM MgSO $_4$, 5 mM EGTA, aprotinin), and then they were scraped into stabilizing buffer containing 0.1% [wt/vol] Triton X-100. After homogenization with a Dounce homogenizer, the samples were centrifuged at 100,000 g for 45 min at room temperature to obtain Triton X-100-soluble and -insoluble fractions. The surface of the Triton X-100-insoluble pellet was gently rinsed twice with stabilizing buffer without EGTA. The resulting Triton X-100-soluble and -insoluble fractions, which contained unassembled and assembled tubulin, respectively, were assayed directly by gel electrophoresis.

Immunoprecipitation

Immunoprecipitation procedures were as described previously (25) using monoclonal antibodies against brain MAP-2. The antibodies were provided by Dr. Lester Binder (University of Alabama, Birmingham, AL) and their properties are described in detail in reference 12.

Gel Electrophoresis and Peptide Mapping

One-dimensional (1-D) and two-dimensional (2-D) PAGE were after Laemmli (33) and O'Farrell (40), with the modifications described in references 3 and 44. Unless otherwise indicated, second dimension gels consisted of 7.5% acrylamide. pH gradients ranged from 4.8 to 7.2. Labeled proteins in 1-D and 2-D gels were visualized by autoradiography or fluorography (9). To quantify the distribution of labeled tubulin between unassembled and assembled fraction, the tubulin spots were excised from appropriate 2-D gels, dissolved in 30% H $_2$ O $_2$, and their radioactivity quantified by scintillation counting. The relative level of labeled tubulin that was polymerized was calculated as

$$\frac{\text{cpm tubulin in Triton X-100-insoluble fraction}}{\text{cpm tubulin in Triton X-100-soluble} + \text{-insoluble fractions}} \times 100.$$

One dimensional peptide mapping was performed as described by Cleveland et al. (13), using 15, 150, or 300 ng *Staphylococcus aureus* protease. All proteins for peptide mapping analyses were excised from 2-D gels. Proteolytic fragments were resolved on 1-D gels consisting of 15% acrylamide.

Results

Percentage of Tubulin that is Polymerized into Microtubules

Long-term NGF treatment increases the stability of PC12 cell microtubules to depolymerizing treatments such as colchicine or cold, thus suggesting a change in the tubulin protomer-polymer equilibrium. If this is correct, then long-term NGF treatment should lead to increases in the percentage of polymerized tubulin in PC12 cells. This prediction was tested by quantifying the relative level of polymerized tubulin in cells grown without NGF or with NGF for varying times. Fractions containing unassembled or assembled

tubulin (see Materials and Methods) were prepared from [³⁵S]methionine-labeled PC12 cells grown with or without NGF and then analyzed on 2-D gels. The assembled tubulin recovered by this method appears to correspond to microtubules. This was supported by the observations that only 4–6% of the total labeled tubulin was recovered in the assembled fraction when cultures were treated with 2 μg/ml of the microtubule-depolymerizing agent podophyllotoxin for 4 h before fractionation (these data are based on analyses of two cultures of cells grown without NGF and two cultures grown with NGF for 21 d), and that the assembled tubulin was solubilized by exposure to Ca⁺⁺-containing buffers.

Representative autoradiographs of assembled and unassembled fractions from cells grown without NGF [PC12(-) cells] or with NGF for 21 d [PC12(+)] are shown in Fig. 1. Two α-tubulins and two β-tubulins were detected in PC12 cells grown with or without NGF. The appearance of the α-tubulins in PC12(+) cells differs from that in PC12(-) cells (see also Fig. 3). This reflects a posttranslational acetylation that is induced by treatment with NGF (Black, M. M., manuscript in preparation). These data also indicate a substantial NGF-promoted shift in the proportion of tubulin that is polymerized. In a larger number of experiments, 23 ± 2% (mean ± SEM; n = 7) of the total labeled tubulin of PC12(-) cells incubated with [³⁵S]methionine for 24 h

was polymerized, while the corresponding value for PC12(+) cells was 54 ± 6% (n = 8). Increasing the labeling time from 24 to 72 h had little effect on the values obtained. The NGF-promoted increase in the relative levels of polymerized tubulin occurred to a similar extent in cultures exposed to NGF in medium containing 1 or 15% serum. Also, the impressions obtained from visual inspection of Coomassie Blue-stained gels were in accord with the quantitative data described above.

Fig. 2 shows the time course of the NGF-promoted increase in the relative levels of polymerized tubulin in PC12 cells. After an initial lag of 2 d, the value for this parameter increased steadily with time of NGF treatment. In these experiments, the cells began extending processes by 2–4 d with NGF, and by 10 d with NGF the vast majority of the cells had neurites. As time with NGF increased from 10 to 21 d, the neurite network of the cultures appeared increasingly dense. Thus, the NGF-induced increase in relative levels of polymerized tubulin occurs in conjunction with the appearance and continued growth of neurites. Quantitatively similar changes in the relative levels of polymerized tubulin have also been observed when cultured neuroblastoma cells are shifted from conditions that do not permit neurite outgrowth to conditions that do permit neurite outgrowth (41).

Drubin et al. (18) have reported that the levels of tubulin

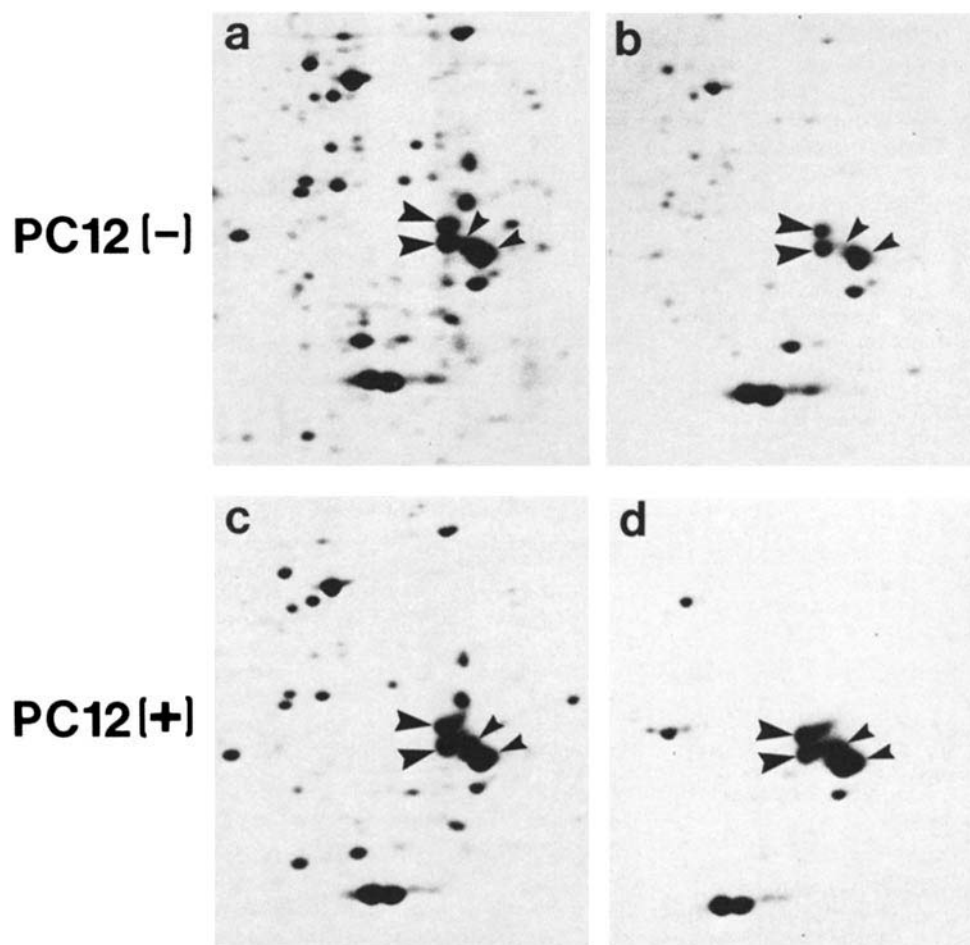


Figure 1. Partitioning of tubulin between Triton X-100-soluble and -insoluble fractions. PC12 cultures without NGF [PC12(-)] or with NGF for 21 d [PC12(+)] were labeled with [³⁵S]methionine for 24 h, fractionated into Triton X-100-soluble and -insoluble fractions as described in the Materials and Methods, and then analyzed on 2-D gels. The figure shows the tubulin-containing regions of autoradiographs of gels depicting the Triton X-100-soluble (a and c) and -insoluble (b and d) fractions prepared from PC12(-) and PC12(+) cultures. Equal proportions of the soluble and insoluble fractions were loaded onto the gels. The autoradiographs depicted in a and b represent 4-d exposures, while those in c and d represent 2-d exposures. In these and subsequent figures of 2-D gels, the acidic end of the gel is to the right. The PC12(+) cells were grown in 1% serum. The tubulin subunits in the various fractions are indicated by arrowheads (the large arrowheads identify the α-tubulins and the small arrowheads identify the β-tubulins). The

tubulin spots were confirmed as α and β-tubulins by peptide mapping after limited proteolysis, using [³⁵S]methionine-labeled tubulin obtained from cultured sympathetic neurons as a standard. Quantitatively similar images were obtained when the gels were stained with Coomassie Blue.

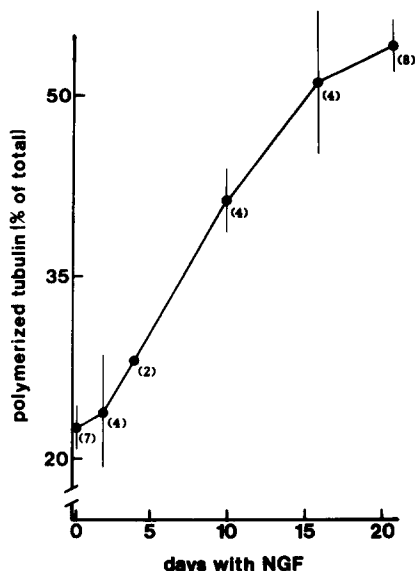


Figure 2. Time course of NGF-promoted changes in the relative levels of polymerized tubulin in PC12 cells. This figure shows quantitative data from experiments like those shown in Fig. 1. The values were obtained as described in Materials and Methods. The resulting values are graphed as percentage of total labeled tubulin which is polymerized versus time of treatment with NGF. The data shown are means \pm SEMs. Sample size for each data point is indicated in parentheses. NGF treatment was in 1% serum.

relative to total cell protein in PC12 cells increases two- to threefold after exposure to NGF. We also consistently observed an NGF-promoted increase in the relative levels of tubulin in PC12 cells. An example of this effect is shown in Fig. 3, which shows autoradiographs of 2-D gels of soluble extracts from PC12(-) and PC12(+) cells. The intensity of

the tubulin spots relative to the other spots is somewhat greater in PC12(+) cells than in PC12(-) cells. Such increases are apparently not accompanied by substantial changes in the transcription of the β -tubulin gene (22). Long-term exposure of PC12 cells to NGF leads to a threefold increase in total PC12 cell protein (26). Given the approximate doubling of the proportion of tubulin in the cells that is polymerized, the threefold increase in total cell protein, and the increase in tubulin relative to total protein (reference 18 and Fig. 3), the total content of tubulin polymer per cell increases by at least 12-fold after long-term NGF exposure. Drubin et al. (18) have also reported that long-term treatment of PC12 cells with NGF leads to a several-fold increase in the level of tubulin polymer per cell.

In light of the NGF-induced changes in microtubule stability and the tubulin protomer-polymer ratio, we carried out further experiments on MAPs in PC12 cells grown with or without NGF.

NGF Induction of MAP-2 in PC12 Cells

Using an immunoprecipitation assay, we detected MAP-2 in PC12(+) cells but not in PC12(-) cells (Fig. 4). In SDS gels, MAP-2 of PC12(+) cells ran as a doublet which closely resembled brain MAP-2 in electrophoretic mobility. This PC12 cell protein co-isolated with tubulin in taxol-based *in vitro* self-assembly assays (Fig. 4), and, as expected of MAP-2 (20), was heat stable (data not shown). As compared with other proteins, MAP-2 was present at relatively low levels in taxol microtubules prepared from PC12(+) cells (Fig. 4 B, lane 1). Since MAP-2 is usually quantitatively recovered with microtubules prepared from extracts treated with taxol (8), the latter observation suggests that MAP-2 is a relatively minor component of PC12(+) cell microtubules. Although we have not carried out detailed analyses of the time course of NGF effects on the abundance of MAP-2 in PC12 cells,

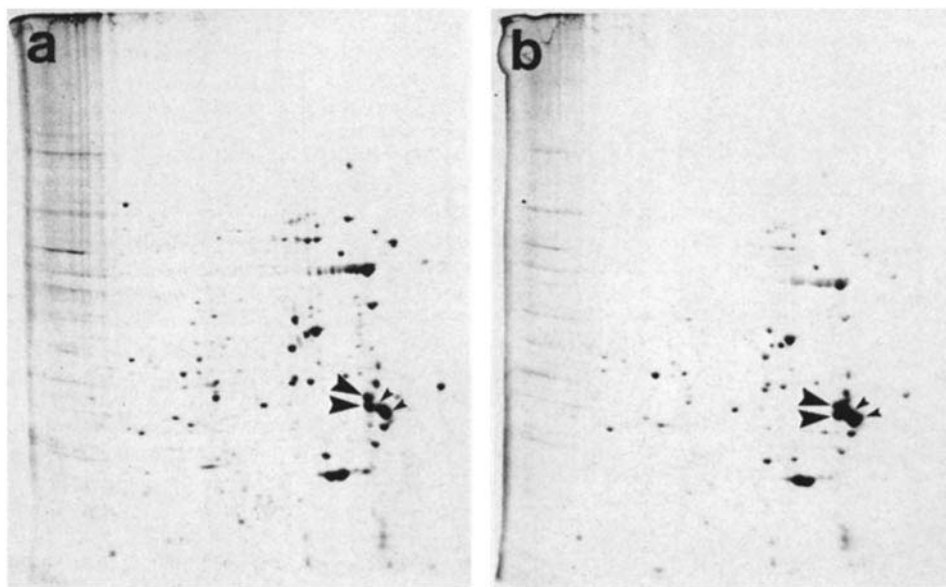


Figure 3. 2-D gel analysis of soluble proteins in PC12 cells grown without NGF (a) or with NGF for 21 d (b). Cultures labeled with [35 S]methionine for 24 h were extracted at 4°C with a buffer containing 0.1 M Pipes, pH 6.9, 1 mM MgSO₄, 2 mM EGTA, 0.2% (wt/vol) Triton X-100, protease inhibitors, clarified by centrifugation at 12,000 g for 10 min at 4°C, and the soluble material was analyzed by 2-D gel electrophoresis. The extraction conditions solubilized $\geq 90\%$ of the labeled tubulin in the cultures. Samples of PC12(-) and PC12(+) cell extracts containing equal methanol precipitable cpm were applied to the gels, and the resulting gels were incubated

without enhancing with x-ray film for 24 h. The tubulin subunits are indicated as in Fig. 1. The bars to the right indicate the position of molecular weight standards, which are, from top to bottom, 116,000, 94,000, 68,000, 55,000, and 43,000. Note that the intensity of the tubulin spots relative to other spots is greater in PC12(+) cells than in PC12(-) cells.

this protein was detectable after 2–3 d of NGF treatment, and at this early stage of NGF treatment, appeared as a doublet.

NGF-induced Changes in the 64–80K MAPs of PC12 Cells

In addition to MAP-1.2, MAP-2, and tau MAPs, three additional MAP classes have been observed in PC12 cells (6, 11, 42, 57). The M_r 's of these latter MAPs are 62–64kD, 64–67kD, and 76–80kD (and will be designated here as the 64K, 67K, and 80K MAPs, respectively). Pallas and Solomon (42) and Zieve and Solomon (57) originally identified these MAPs in PC12 cells grown without NGF (42, 57) and recently showed that they are immunologically distinct from tau MAPs (36). Additional evidence from our laboratories supports this conclusion (43; and see Discussion). These MAPs have been designated "chartins" to distinguish them from tau MAPs (36). In the present report, we show that MAPs resembling the chartins in mobility in 2-D gels are present in PC12 cells grown with or without NGF and that prolonged NGF treatment greatly alters the phosphorylation state of these MAPs, while having at most only a modest effect on their abundance relative to total cell protein.

Using a sequential extraction assay that enriches for proteins assembled into microtubules (see Materials and Methods, dish extraction procedure), the chartin MAPs were observed in both PC12(–) and PC12(+) cells that had been labeled with [³⁵S]methionine (Fig. 5). Consistent with their identification as MAPs, these proteins were absent or greatly diminished in microtubule fractions prepared from cultures depleted of microtubules by treatment with podophyllotoxin (Fig. 5) or nocodazole (1) before extraction and they also co-cycled with brain microtubule proteins through at least two assembly–disassembly cycles (data not shown). In 2-D gels, each chartin MAP consists of a set of proteins of slightly varying M_r 's and very different pI's (pI's for each chartin MAP class ranged from ~6.9 to ~6.3) (Fig. 5; references 5, 19, 42, 43). Under the electrophoretic conditions that we used, tau MAPs do not focus in the gels (43; Butler, M., M. L. Shelanski, L. A. Greene, unpublished observations). The assignment of the individual chartin MAPs to specific classes is based on peptide mapping analyses (42; and see below). Although the 2-D gel pattern of the chartin MAPs from PC12(–) cells generally resembles that from PC12(+) cells, several major differences were consistently observed. In particular, the abundances of the more acidic members of the 64K, 67K, and 80K chartin MAPs relative to their more basic members were considerably greater in PC12(+) cells than in PC12(–) cells. In fact, the 80K MAP class of PC12(+) cells contained several acidic members that were not detected in PC12(–) cells. Also, the 67K chartin MAP of PC12(+) cells contains one relatively basic member that was not detected in PC12(–) cells. Table I presents quantitative data for this shift with respect to the 64K chartin MAP. Fig. 6 summarizes the similarities and differences in the compositions of the 64K, 67K, and 80K chartin MAPs of PC12(–) and PC12(+) cells.

The above described results indicate that the relative abundances of individual chartin MAPs that are assembled into microtubules in PC12(+) cells differ from those in PC12(–) cells. However, the sequential extraction assay is relatively prolonged, and it is thus possible that the detailed composition of these MAPs is altered during preparation of the

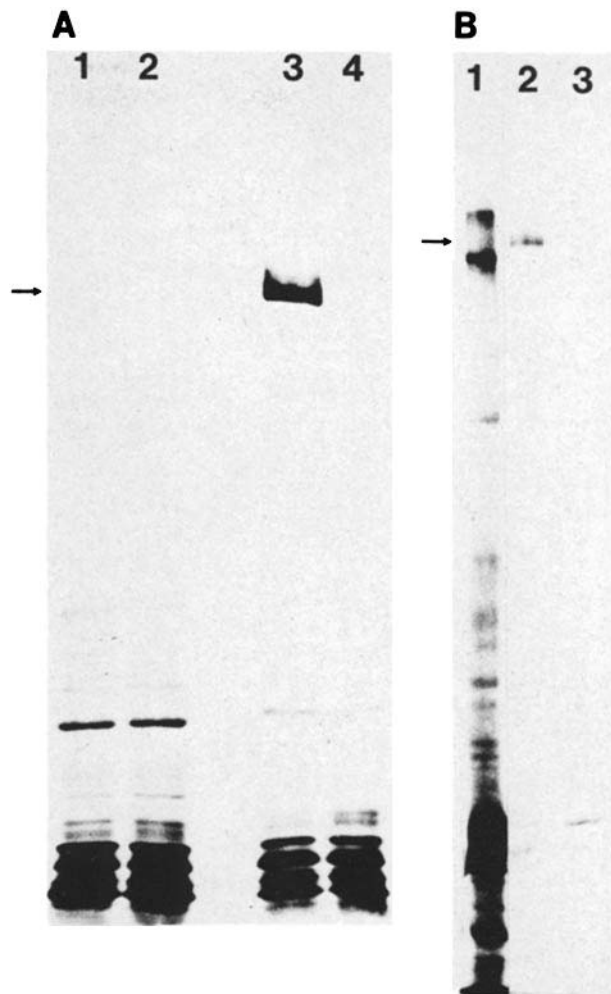


Figure 4. (A) [³⁵S]methionine-labeled extracts of PC12 cells grown without NGF or with NGF for 21 d containing equal TCA-precipitable cpm were assayed for MAP-2 by immunoprecipitation as described in Materials and Methods. The resulting precipitates were analyzed on a gel consisting of a 4–8% gradient of acrylamide. A shows a fluorograph from such a gel after an exposure time of 4 wk. (Lanes 1 and 2) Material precipitated from antibody-treated and control (without first antibody) samples of PC12(–) cells, respectively. (Lanes 3 and 4) Material precipitated from antibody-treated and control samples of PC12(+) cells, respectively. Comparable results were obtained with two different antibodies to brain MAP-2 (API3 and API4 in reference 12). In control experiments, we observed that combining extracts of unlabeled PC12(–) or PC12(+) cells with labeled extracts of cultured sympathetic neurons, which contain MAP-2 (43), had no detectable effect on the recovery of neuronal MAP-2 by immunoprecipitation (data not shown). (B) Microtubules were prepared from soluble extracts of [³⁵S]methionine-labeled PC12(+) cells using a taxol-based procedure (see Materials and Methods) and probed for MAP-2 by immunoprecipitation. Lane 1 shows the labeled material in the microtubule preparation; the arrow indicates the position of a MAP-2 standard. Lanes 2 and 3 show the precipitates from antibody-treated and control samples, respectively.

microtubule fractions. To evaluate this possibility, we compared the 2-D gel patterns of chartins in [³⁵S]methionine-labeled PC12(–) and PC12(+) cells dissolved directly into SDS-containing sample buffer. As shown in Fig. 7, the 2-D gel profile of the 64K chartin MAPs observed in whole cell

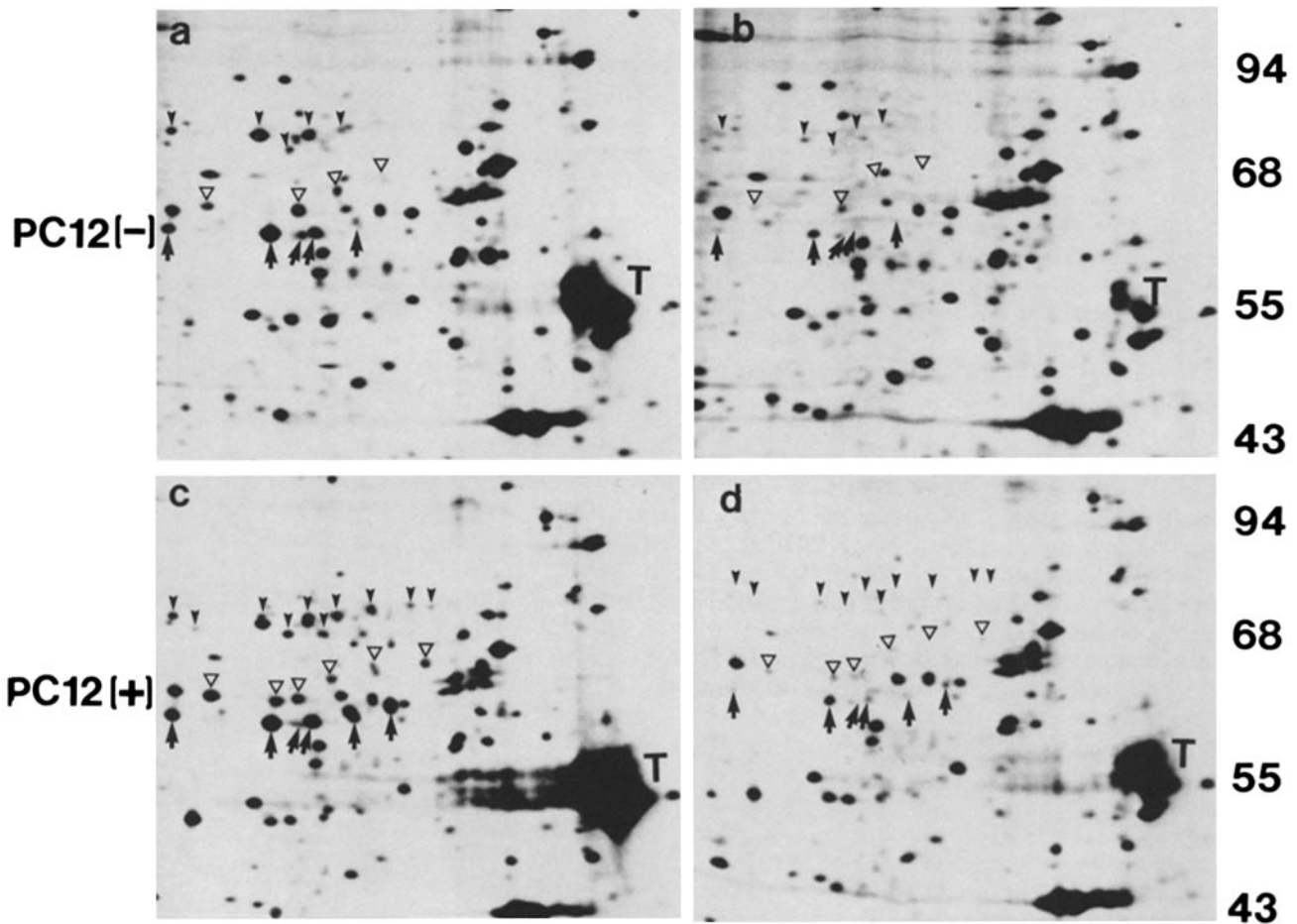


Figure 5. Identification of the 64K, 67K, and 80K MAPs in PC12(-) cells and PC12(+) cells. Fractions enriched in proteins assembled into microtubules (see Materials and Methods) were prepared from control and microtubule-depleted cultures labeled with [³⁵S]methionine for 24 h and then analyzed on 2-D gels. Cells were depleted of microtubules by treatment with 2 μg/ml podophyllotoxin for 4 h. The figure shows portions of fluorographs depicting the labeled proteins in fractions prepared from control (a and c) and podophyllotoxin-treated (b and d) cultures of PC12(-) (a and b) and PC12(+) (c and d) cells. The positions of the 64K, 67K, and 80K MAPs are indicated with arrows, open arrowheads, and filled arrowheads, respectively. Some of the MAPs of PC12(-) cells appeared as faint spots in the fluorographs, and did not reproduce well during photography. Note that tubulin (T) and most of the MAPs are readily detected in fractions enriched in assembled microtubule proteins from control cultures but are absent or greatly reduced in similarly prepared fractions from podophyllotoxin-treated cultures. In the latter cases, the arrows and arrowheads depict the expected positions of the MAPs, even when absent.

extracts of PC12(-) or PC12(+) cells closely resembles that seen in the microtubule fractions from the corresponding cells (see also Fig. 9). The 80K MAP species of PC12(-) and PC12(+) cells were not well visualized in 2-D gels of whole cell extracts of [³⁵S]methionine-labeled cultures. However, many of these MAPs are phosphorylated in situ,

and they can be readily detected in whole cell extracts as well as microtubule fractions of cultures labeled with [³²P]PO₄ (see Fig. 9). The patterns of phosphorylated 80K MAPs observed in whole cell extracts of PC12(-) or PC12(+) cells were very similar to those seen in microtubule fractions from the corresponding cells (see Fig. 9; unpublished obser-

Table I. Relative Abundance of 64K Chartin MAP Variants in PC12(-) and PC12(+) Cells.

	MAP (relative abundance)				
	a	b	c	d	e
PC12(-) cells	0.10 ± 0.02	0.79 ± 0.02	0.09 ± 0.02	0.01 ± 0.01	0.02 ± 0.02
PC12(+) cells	0.13 ± 0.01	0.44 ± 0.02	0.18 ± 0.02	0.14 ± 0.01	0.13 ± 0.02

The relative abundance of several isoelectric variants of the 64K chartin MAP of PC12(-) and PC12(+) cells was quantified. The specific MAP species analyzed are designated by letters (a, b, c, d, and e) in Fig. 6. Fractions enriched in assembled MAPs from control cultures of PC12(-) and PC12(+) cells were analyzed by 2-D gels. The five spots corresponding to the five major species of 64K chartin MAPs of PC12(-) and PC12(+) cells were excised from the gels and their radioactivity was quantified. The gels were re-fluorographed after cutting out the spots, and in all cases, each MAP was completely removed without detectable contamination by nearby non-MAP proteins. For each preparation, the radioactivity associated with each MAP was expressed relative to the sum of the radioactivity associated with all five MAPs. The data shown are mean ± SEM (n = 4 for each set of data).

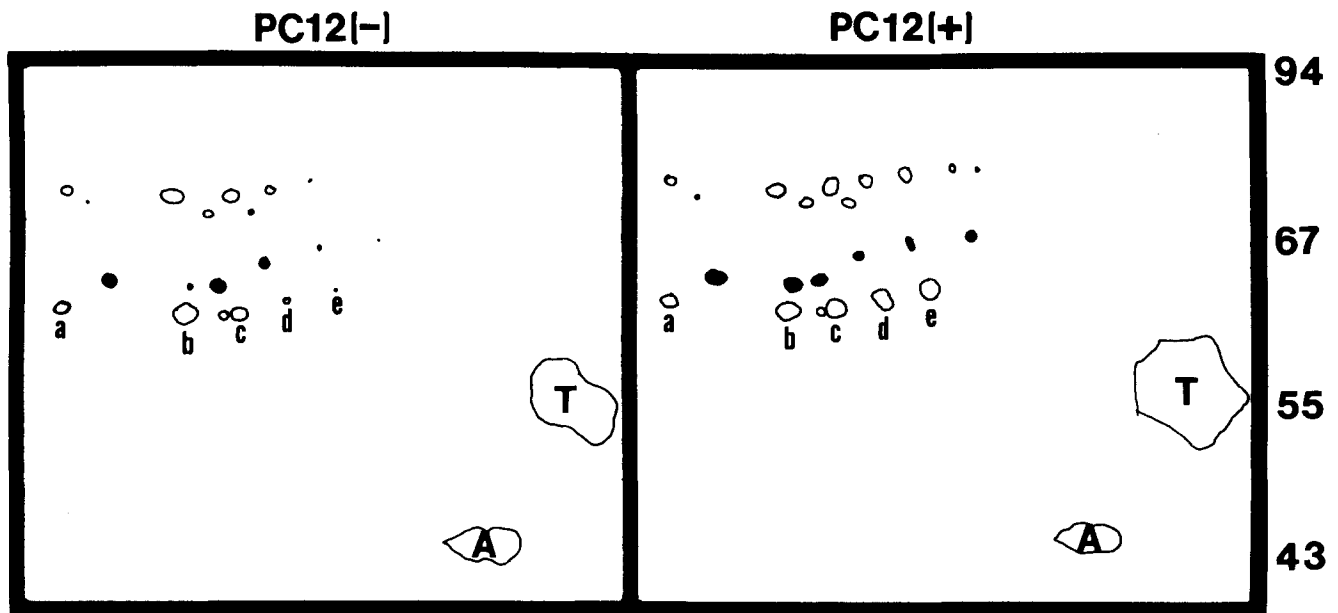


Figure 6. Comparison of the 64–80K MAPs of PC12(–) and PC12(+) cells. Tracings of 2-D fluorographs from Fig. 5, *a* and *c*, showing the positions and relative abundance of the 64K (lower set of open circles), 67K (filled circles), and 80K (upper set of open circles) MAPs from PC12(–) and PC12(+) cells. These tracings are representative of the patterns obtained in eight experiments with PC12(–) cells and 10 experiments with PC12(+) cells. The relative abundance of each MAP is reflected in its size. The dots in the tracing of PC12(–) cell MAPs identify MAPs that were barely detectable in heavily exposed fluorographs. Note that the spot size for the 2–3 most basic members of the 64K and 80K MAP classes (basic is to the left) of PC12(–) and PC12(+) cells are similar in size, while spots of the more acidic members from PC12(+) cells are considerably greater in size than the corresponding MAPs from PC12(–) cells. The relative abundance of the 64K MAPs (labeled *a*–*e*) have been quantified (see Table I). *A* and *T* indicate the positions and size of the actin and tubulin spots, respectively.

variations²). These results show that the compositions of the 64K and 80K chartin MAPs are indeed altered by treatment with NGF, and that these changes are accurately reflected in analyses of chartin MAPs in microtubule fractions. With respect to the 67K chartin MAP, the relatively basic variant present in the assembled MAP fraction of PC12(+) cells (see Fig. 5 and 6) was not detectable in the corresponding whole cell extracts of these cells (see Figs. 7 and 9). We therefore cannot presently rule out the possibility that this particular variant arises artifactually during extraction. However, NGF-promoted enhancement of the relative abundance of the most acidic 67K chartin MAP that is apparent in microtubule fractions is also seen in whole cell extracts (compare Fig. 7 with Figs. 5 and 9).

The NGF-induced changes in the chartin MAPs occurred in medium containing 1% (Figs. 5 and 8) as well as 15% serum (see, for example, Figs. 7 and 9). Also, these changes were fully reversible upon withdrawal of NGF; within 5 d of NGF removal, the pattern of these MAPs resembled that in PC12(–) cells (data not shown). Under these conditions, the cells also lose their neurites (26).

Peptide mapping analyses by Pallas and Solomon (42) indicate that the 64K, 67K, and 80K classes of chartin MAPs of PC12(–) cells are each comprised of structurally related

proteins. Our own analyses confirm this for the 64K and 67K chartin MAPs of PC12(–) cells (Fig. 8) and show that this is also true for PC12(+) cells (Fig. 8). In additional peptide mapping analyses, we have shown that the five principal members of the 64K chartin MAP (designated *a*–*e* in Fig. 6) of PC12(+) cells are closely related in primary sequence and that this is also true for the six major species of the 67K chartin MAP (data not shown). We have not carried out detailed analyses on the 80K MAP.

The data of Figs. 5 and 7 show that long-term exposure of PC12 cells to NGF results in the selective enhancement in the levels of specific variants of each chartin MAP class. From the results shown in Fig. 7, it appears that relative to total cell protein, the total levels of each chartin MAP class (i.e., the amount defined by summing the levels across all variants of each MAP class) is not substantially influenced by treatment with NGF. These observations thus indicate that the selective enhancement of specific chartin MAP variants by NGF is not secondary to changes in the total levels of these MAP classes. Evidence is presented below that supports the hypothesis that the NGF-promoted changes in the chartins are due to effects of the factor on their posttranslational modification, namely phosphorylation.

Phosphorylation of the 64–80K MAPs, and Effects of NGF

Exposure of PC12(–) and PC12(+) cells to [³²P]PO₄ for 2 h established that the chartin MAPs are phosphorylated in situ (Fig. 9 and references 11 and 42). The 2-D gel patterns of the [³²P]PO₄-labeled chartin MAPs from PC12(–) and PC12(+) cells are quite distinct from one another, and the

2. While the correspondence between the phosphorylated 80K chartin MAPs in whole cell extracts and in microtubule fractions can be somewhat appreciated in Fig. 9, it is very clear in comparisons with more heavily exposed autoradiographs of whole cell extracts. We have not presented such autoradiographs because the appearance of the 64K chartin MAP becomes obscured by the relatively high levels of phosphoproteins in this region of the gel.

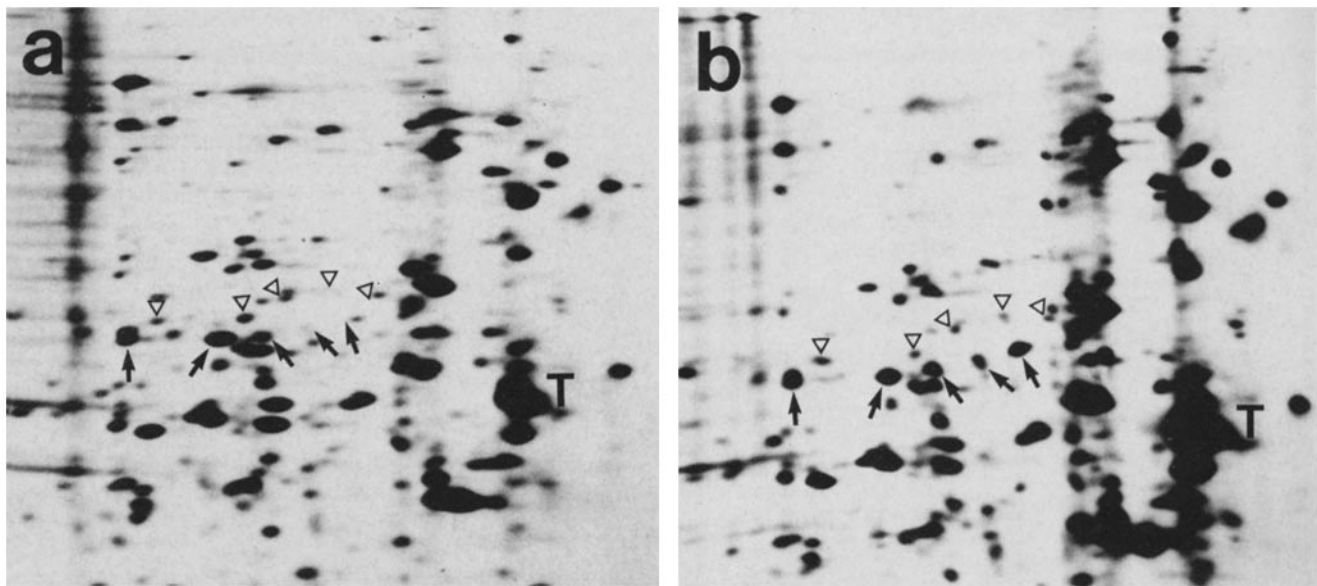


Figure 7. 64K (\leftrightarrow) and 67K (∇) chartin MAPs in 2-D gels of whole cell SDS extracts of PC12(-) (a) and PC12(+) (in 15% serum) (b) cells. Cultures labeled with [35 S]methionine for 3 d were dissolved in a solution containing 5% 2-mercaptoethanol, 8 M urea, and 2% SDS, and then analyzed by 2-D gel electrophoresis and fluorography. Equal methanol precipitable cpm from the SDS extracts of the PC12(-) and PC12(+) cells were applied to the gels, and the fluorographic exposures were for 2 d. The 64K and 67K chartin MAPs are indicated as in Fig. 5.

differences are, with the one exception mentioned above, equally apparent in total cell extracts as well as in fractions enriched in microtubule proteins (Fig. 9). For example, much more phosphate is incorporated into the 64K chartin MAPs in PC12(+) cells than in PC12(-) cells (Fig. 9, a and b, and Fig. 10 A) and this additional phosphate is concentrated in the more acidic members of this MAP class (particularly those labeled *d* and *e* in Fig. 6). Similar considerations also apply to the 67K and 80K chartin MAPs. These differences in the 2-D gel patterns of the [32 P]PO $_4$ -labeled chartins from PC12(-) and PC12(+) cells closely mirror the differences observed when [35 S]methionine-labeled MAPs from these cells were compared with each other (compare Fig. 9 with Figs. 5, 7, and 8).

These experiments also revealed a protein with an M_r of 54 kD that was phosphorylated in PC12(+) cells to a much greater extent than PC12(-) cells (see also reference 11). This protein has a mobility in 2-D gels that is very similar to that of β -tubulin and can be precipitated by a monoclonal antibody to β -tubulin (Butler, M., L. A. Greene, and M. L. Shelanski, unpublished observations). Enhanced phosphorylation of β -tubulin after NGF treatment of PC12 cells is consistent with the observations that β -tubulin phosphorylation is coupled to its assembly into microtubules in murine neuroblastoma cells (21) and that NGF treatment of PC12 cells results in a substantial increase in the levels of polymerized tubulin per cell (see above).

Time Course of NGF-promoted Increase in Phosphate Incorporation into PC12 Cell Microtubule Proteins

The enhanced phosphorylation of the 64K chartin MAP as well as β -tubulin and MAP-1.2 is readily observed in 1-D gels of total cell SDS extracts of NGF-treated PC12 cells (reference 11 and Fig. 10 A). The identity of the 64K MAP and β -tubulin is indicated by their enrichment in microtubule

fractions following sequential extraction as described in the Materials and Methods, by their absence from such fractions when cells are treated with podophyllotoxin or nocodazole before extraction, and by their insolubility after boiling under conditions in which tau MAPs remain soluble (data not shown). The identity of MAP-1.2 has been previously established (25). We have used 1-D gels to quantify the time course by which NGF brings about enhanced phosphorylation of the 64K chartin MAP, and to compare this with those for MAP-1.2 and β -tubulin (Fig. 10 B). The relative labeling of the phosphorylated 64K chartin MAP remains low for the first 2-4 d of NGF treatment, and then increases steadily thereafter, with the most dramatic increases occurring between 4 and 10 d of NGF treatment. The time course of the increase in labeling of this MAP closely resembles those observed for MAP-1.2 and β -tubulin (Fig. 10 B), and also those observed for the increase in relative levels of polymerized tubulin (compare Fig. 10 B with Fig. 2) and for the initiation and continued growth of neurites (reference 26 and see above).

Discussion

The present results show that NGF treatment of PC12 cells results in an increase in the levels of tubulin relative to total cell protein, a doubling in the proportion of cellular tubulin that is polymerized, an increase in the relative abundance of MAP-2, and enhanced phosphorylation of the 64K, 67K, and 80K chartin MAPs. NGF effects on PC12 cells fall into two general categories, short-term effects, which become manifest within seconds to minutes of exposure to NGF, and long-term effects, which become apparent only after several hours to days of NGF treatment (23). All of the NGF-promoted changes described in the present report occur gradually over several days, and thus belong to the class of long-term effects of NGF on PC12 cells.

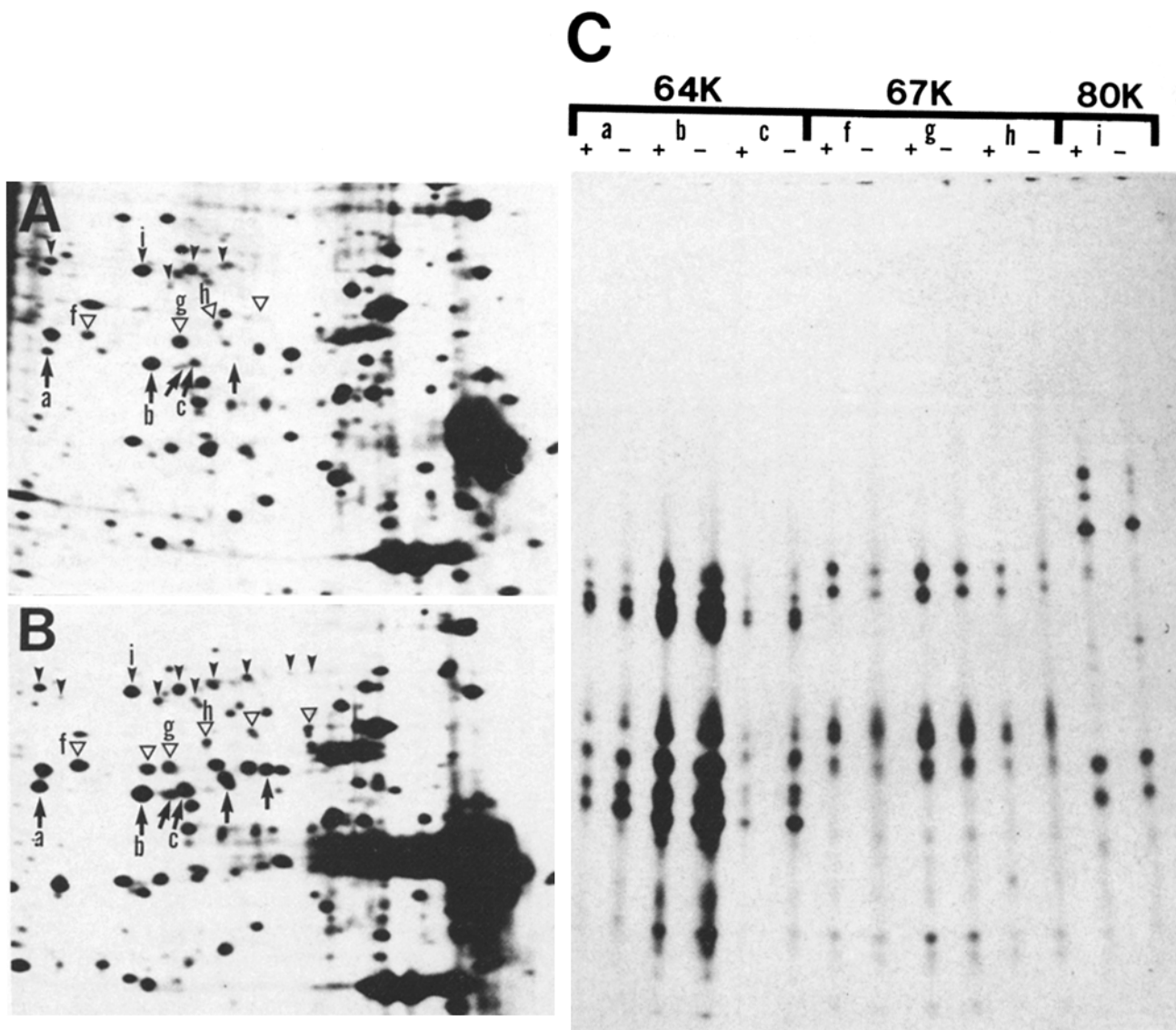


Figure 8 Peptide mapping analyses of several species of the 64–80K MAPs in PC12(–) and PC12(+) (21 d with NGF) cells. Fractions enriched in assembled MAPs were prepared from PC12(–) and PC12(+) cells and analyzed on 2-D gels. Portions of autoradiographs of the gels of the PC12(–) and PC12(+) samples are shown in *A* and *B*, respectively. The various MAPs are indicated as in Fig. 5. Several of these MAPs have been given a letter designation; MAPs of PC12(–) cells with the same letter designation as MAPs of PC12(+) cells have the same relative mobility in 2-D gels. The MAPs with the letter designations were excised from the gels and peptide mapped (13). The resulting peptide maps are shown in *C*. +, peptide maps of PC12(+) cell MAPs; –, peptide maps of PC12(–) cell MAPs. Note that the members of individual MAP classes give rise to very similar fragment patterns after limited proteolysis, indicating that these proteins are related in primary structure.

NGF-promoted Increase in Microtubule Stability

In previous studies with PC12 cells (4, 6), we have shown that microtubules in long-term NGF-treated cells are more stable to the depolymerization-promoting action of colchicine and low temperature than microtubules in cells grown without NGF, suggesting that NGF treatment alters the tubulin protomer–polymer equilibrium in favor of microtubules. The present studies have confirmed this possibility by showing that the proportion of polymerized tubulin doubles after 3 wk of treatment with the factor (Figs. 1 and 2). Drubin et al. (18) have also reported that NGF treatment leads to an increase in the relative levels of polymerized tubulin in PC12 cells. This increase coincides with the appearance and con-

tinued growth of neurites, suggesting a causal relationship between these events.

One of the essential events in the initiation and continued growth of neurites is the formation and elongation of microtubule bundles (15, 16, 24, 35, 55, 56). In untreated PC12 cells, microtubules occur singly in the cytoplasm of interphase cells (35, 52). Coincident with the appearance of long neurites, microtubules become organized into compact bundles that extend from the cell body to the neurite tips (35, 52). The formation of these bundles is apparently essential for neurite outgrowth in that its prevention by appropriate pharmacological treatments also prevents neurite extension (24). Microtubules in bundles frequently show enhanced stability relative to unbundled microtubules (1, 51). Thus, the

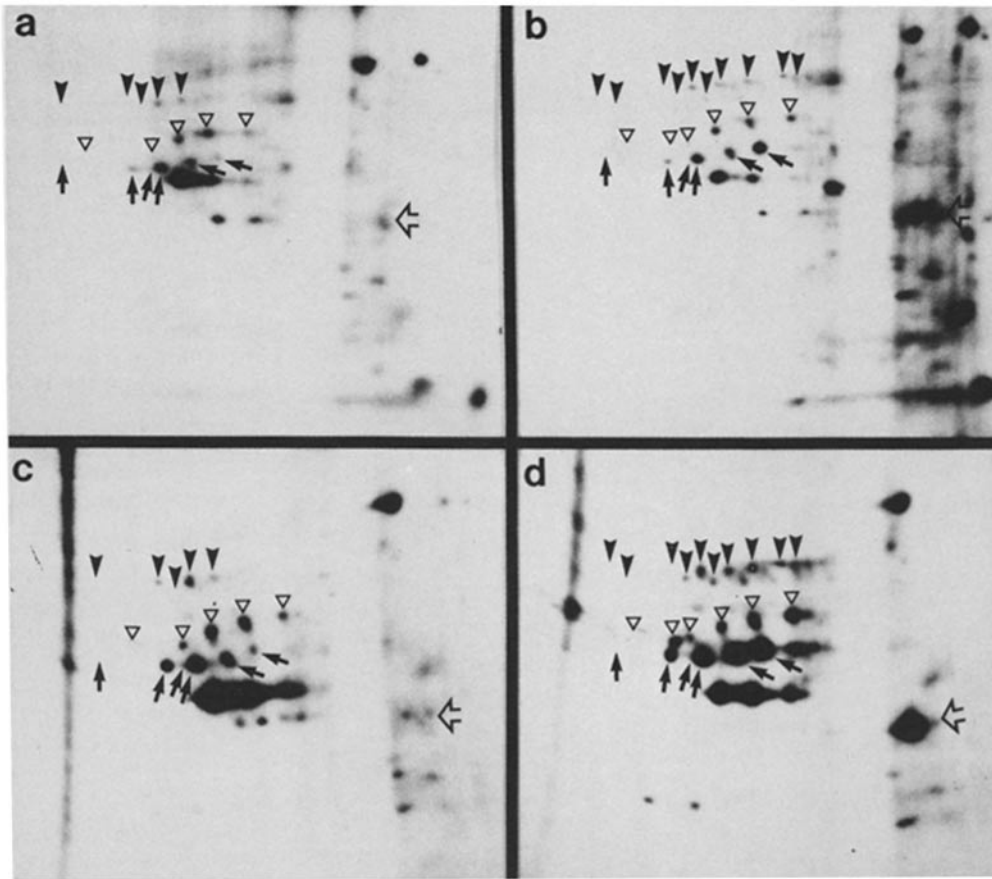


Figure 9. Phosphorylation of the 64–80K MAPs in intact PC12(–) and PC12(+) cells. Cultures labeled with [32 P] PO $_4$ were either processed as whole cell extracts by solubilization in a solution containing 5% 2-mercaptoethanol, 8 M urea, and 2% SDS (BUS) (*a* and *b*) or were sequentially extracted to prepare fractions enriched in assembled MAPs (*c* and *d*). The resulting material was analyzed on 2-D gels [equal precipitable cpm from the BUS extracts of PC12(–) and PC12(+) cells were applied to the gels]. The figure shows portions of autoradiographs of BUS extracts from PC12(–) (*a*) and PC12(+) (*b*) cells (the autoradiographs were exposed for 4 d without an intensifying screen) and assembled MAP fractions from PC12(–) (*c*) and PC12(+) (*d*) cells. The positions of the 64–80K chartin MAPs are indicated as in Fig. 5. The open arrow indicates the approximate position of β -tubulin. NGF treatment was in 15% serum.

NGF-promoted shift in tubulin protomer-polymer equilibrium may reflect a specialization that is important for generating and/or stabilizing the microtubule bundles that provide the structural framework of the neurite.

NGF-induced Alterations in the Chartin MAPs

The present work has demonstrated effects of long-term NGF treatment on a group of PC12 cell MAPs with M_r 's of 64–80 kD. These MAPs exhibit electrophoretic and biochemical properties similar to MAPs originally observed in PC12 cells grown without NGF (42, 57) and which have been designated as chartins (36). Although chartins overlap somewhat with tau MAPs in M_r , a number of observations clearly demonstrate that the two MAP families are distinct from each other. For instance, (*a*) chartin and tau MAPs do not cross-react immunologically (36, 43); (*b*) as reported here (for the 64K MAP) and elsewhere (5), chartin MAPs are insoluble under conditions of boiling in which tau MAPs remain soluble; (*c*) tau MAPs are more basic than chartins and in our 2-D gels, which display the chartin MAPs very nicely, the tau MAPs are not resolved (43); (*d*) as examined in neurons, chartins differ from tau MAPs in the efficiency with which they co-assemble with tubulin in the presence of taxol (43)³ and in their distribution between cell bodies and neurites (44); (*e*) the present studies found little if any effect of NGF on the relative total levels of chartins in PC12 cells

while, in contrast, Drubin et al. (18) reported a substantial increase in this parameter for tau MAPs.

As demonstrated here, the chartin MAPs are present in PC12 cells grown with or without NGF (Fig. 6). Each chartin MAP consists of a set of structurally related proteins that vary substantially in pI and, to a lesser extent, M_r (references 42 and 57 and Fig. 6). Although the patterns of chartin MAPs in PC12(–) cells show many similarities to those in PC12(+) cells, several differences were consistently observed (Fig. 6 and 7). For example, the two most acidic members of the 64K chartin MAP (*d* and *e* in Fig. 6) are only trace components in PC12 cells grown without NGF, while they are relatively prominent components in long-term NGF-treated PC12 cells (Figs. 6 and 7 and Table I). Although these specific variants of the 64K chartin MAP are much more abundant in PC12(+) cells compared to PC12(–) cells, the total levels of this MAP (i.e., the amount defined by all of its variants) relative to total cell protein does not appear to be substantially enhanced by treatment with NGF (Fig. 7). These observations indicate that NGF treatment leads to a selective enhancement of specific members of the 64K class of chartin MAPs. The *in vivo* phosphorylation experiments (Fig. 9) indicate that these highly acidic variants are extensively phosphorylated, and also suggest that they are the most heavily phosphorylated (on a mol/mol basis) members of the 64K chartins (reference 42 and Figs. 5 and 9). These considerations suggest that the NGF-promoted enhancement of these highly acidic variants of the 64K MAP class is due to an effect of NGF on their phosphorylation state. This effect may be enhanced phosphorylation and/or reduced dephos-

3. Comparable results have also been obtained with PC12 cells (Black, M. M., unpublished observations).

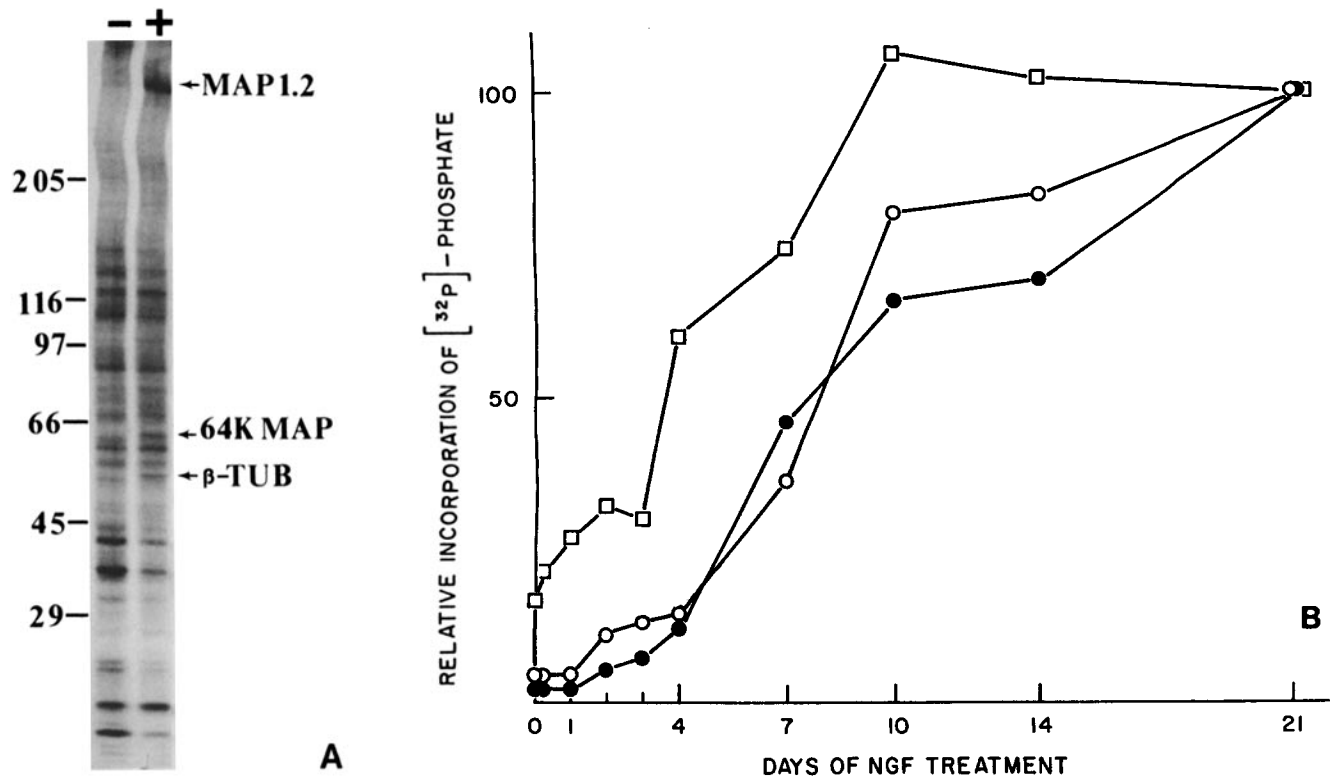


Figure 10. Time course of the NGF-promoted change in the relative levels of phosphorylated microtubule proteins in PC12 cells. Cells were cultured without NGF or with NGF (in 1% serum) for the indicated times and then labeled with [32 P]PO $_4$ for 2 h. The cells were then dissolved in an SDS-containing sample buffer and equal numbers of TCA-precipitable cpm (100,000) were loaded onto a 6–12% acrylamide gel (32 cm in length). After electrophoresis, the gel was dried and autoradiographed. **A** shows representative 1-D gel profiles of [32 P]PO $_4$ -labeled proteins from cells grown without NGF (–) or with NGF for 21 d (+). The position of MAP-1.2, the 64K chartin MAP, and β -tubulin are indicated on the right, while those of molecular weight standards ($\times 10^{-3}$) are indicated on the left. **B** shows a quantitative analysis in which the autoradiograph was examined with a scanning densitometer and the relative areas beneath the indicated MAP-1.2 (□), 64K chartin MAP (●), and β -tubulin (○) were quantified. The area of each peak at 21 d of NGF treatment was arbitrarily set at 100 and all other values were normalized to this value.

phorylation. Similar considerations may also explain the selective enhancement of the acidic members of the 67K and 80K chartin MAPs in PC12 cells treated with NGF.

MAPs comparable to the 64–80K chartin MAPs of PC12 cells have been identified in neuroblastoma cells (42) and neurons (5). In neuroblastoma cells, the more highly phosphorylated members of these MAP classes preferentially associate with microtubules (42), and similar findings have also been obtained with neurons (5) and PC12 cells (42; Black, M. M., unpublished observations). Thus, enhancement of the highly phosphorylated variants of the chartins in NGF-treated PC12 cells is specifically reflected in the composition of their microtubules.

Functional Implications of the NGF-promoted Changes in PC12 Cell MAPs

There are two aspects of the response of PC12 cells to NGF in which changes in microtubules and MAPs might be functionally involved. One is the cessation of proliferation that occurs within the first week of NGF treatment (26). However, since the levels of chartin MAP phosphorylation and microtubule polymerization continue to increase over at least 3 wk, it seems unlikely that these changes are due entirely to withdrawal from the cell cycle.

A second response to NGF is the elaboration of neurites.

As discussed above, there is a good reason to believe that the NGF-promoted shift in the tubulin protomer-polymer ratio is an important step in the generation and stabilization of neurites. One of the determinants of the tubulin protomer-polymer equilibrium is the concentration of reactants involved in polymer formation (32, 39, 48). Tubulin and MAPs are major reactants in this process and the results presented here and in other studies (18, 24) indicate that the relative abundances of these components in PC12 cells increase after treatment with NGF. The NGF-promoted increase in tubulin levels is temporally out of phase from the NGF-promoted enhancement of microtubule stability (18), suggesting that these events are not causally related. In contrast, the NGF-promoted increases in the abundances of MAP-1 species (18, 25), MAP-2 (Fig. 4), tau MAPs (18), and highly phosphorylated chartins (Figs. 5–10) occur with time courses that closely parallel the NGF-induced increase in microtubule stability and neurite outgrowth. In vitro studies with purified MAPs from brain or cultured cells indicate that these proteins enhance microtubule stability (14, 30, 39, 49). Furthermore, there is evidence that MAPs may influence the spacing between microtubules (10, 31, 49, 53, 54) and are capable of cross-linking microtubules with other cytoskeletal components (27, 28, 34, 46, 47). These considerations therefore suggest that the changes in MAPs brought about by NGF

contribute to the observed NGF-promoted increase in microtubule stability and formation of microtubule bundles.

An additional set of recent findings (11) are of particular interest with respect to the possible functional role of chartin MAP phosphorylation in neurite outgrowth. It was observed that treatment with Li⁺ inhibits NGF-induced neurite outgrowth from PC12 cells as well as regeneration of neurites by sympathetic neurons and NGF-primed PC12 cells. One of the molecular correlates of Li⁺ treatment was a specific and dramatic suppression of the phosphorylation of the chartin MAPs; the phosphorylation of other phosphoproteins, including tubulin and MAP-1.2, was unaffected (11).

Finally, further support for the hypothesis that the NGF-promoted changes in PC12 cell MAPs is involved in neurite outgrowth is provided by comparing the profile of MAPs in PC12(-) and PC12(+) cells with that in neurons. Neurons contain a variety of MAPs (43), of which MAP-1 species, MAP-2, tau MAPs, and the chartins, especially their highly phosphorylated variants, are quite prominent (5, 7, 14, 19, 38, 43, 48). On the basis of biochemical and immunological criteria, some of the MAP-1 species, as well as MAP-2, tau, and the highly phosphorylated chartins are relatively specific for neurons (2, 5, 8, 12, 17, 19, 29, 42, 44). PC12(-) cells have relatively low levels of these neuron-type MAPs and lack neurites. Treatment with NGF induces a neuron-like profile of MAPs in PC12 cells as well as a neuron-like morphology. The apparent parallels between the inductions by NGF of both neurites and MAP composition is consistent with a causal relationship between these events.

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