Nerve Growth Factor Regulates Both the Phosphorylation and Steady-State Levels of Microtubule-associated Protein 1.2 (MAP1.2)

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Abstract. This study characterizes effects of nerve growth factor (NGF) on the steady-state level and phosphorylation of a high molecular mass microtubule-associated protein in PC12 rat pheochromocytoma cells. Past work showed that NGF significantly raises the relative levels of this phosphoprotein, designated MAP1.2, with a time course similar to that of neurite outgrowth. To study this in greater detail, MAP1.2 in PC12 cell lysates was resolved by SDS-PAGE in gels containing 3.25% acrylamide/4 M urea and identified by comigration with material immunoprecipitated from the lysates by MAP1 antibodies. Quantification by metabolic radiolabeling with [^{35}S]methionine or by silver staining revealed a 3.0–3.5-fold increase in MAP1.2 levels relative to total cell protein after NGF treatment for 2 wk or longer. A partial increase was detectable after 3 d, but not after 2 h of NGF exposure. As measured by incorporation of [^{32}P]phosphate, NGF had a dual effect on MAP1.2. Within 15 min to 2 h, NGF enhanced the incorporation of phosphate into MAP1.2 by two- to threefold relative to total cell phosphoproteins. This value slowly increased thereafter so that by 2 wk or more of NGF exposure, the average enhancement of phosphate incorporation per MAP1.2 molecule was over fourfold. The rapid action of NGF on MAP1.2 could not be mimicked by either epidermal growth factor, a permeant cAMP derivative, phorbol ester, or elevated K^+, each of which alters phosphorylation of other PC12 cell proteins. SDS-PAGE revealed multiple forms of MAP1.2 which, based on the effects of alkaline phosphatase on their electrophoretic mobilities, differ, at least in part, in extent of phosphorylation. Before NGF treatment, most PC12 cell MAP1.2 is in more rapidly migrating, relatively poorly phosphorylated forms. After long-term NGF exposure, most is in more slowly migrating, more highly phosphorylated forms. The effects of NGF on the rapid phosphorylation of MAP1.2 and on the long-term large increase in highly phosphorylated MAP1.2 forms could play major functional roles in NGF-mediated neuronal differentiation. Such roles may include effects on microtubule assembly, stability, and cross-linking and, possibly for the rapid effects, nuclear signaling.

THE most prominent characteristic of neuronal differentiation is the elaboration of extensive axonal and dendritic arbors. These structures are densely packed with microtubules which are required for the growth of neurites (9, 36). The biological rules governing the initiation and extension of these structures are not fully understood but are likely to involve accessory molecules known as microtubule-associated proteins (MAPs)[1] (for review see reference 28). Among these is the high molecular mass species, MAP1, the levels of which are developmentally up regulated in mammalian brain (2, 29). Although the distribution of MAP1 in the nervous system is widespread (5), recent immunological evidence (4) suggests that a MAP1 subspecies, MAP1B, may be more abundant than MAP1A in axons. At least in vitro, MAP1 promotes the assembly of microtubules (18) and forms filamentous arms on their surfaces (34). In addition, stabilization of microtubules and cross-linking of microtubules to one another or to other elements of the cytoskeleton are also among the possible functional roles of MAP1.

The role of MAPs in process outgrowth by intact neuronal cells has recently been studied using a model system, the PC12 clonal line of rat pheochromocytoma cells. In the presence of nerve growth factor (NGF), these cells acquire a neuronal phenotype (13) and they may therefore be used to correlate changes in composition with the onset and elongation of neurites. In particular, NGF has been found to greatly increase the relative levels of a phosphoprotein that is recognized by antisera prepared against high molecular mass brain

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1. *Abbreviations used in this paper*: EGF, epidermal growth factor; MAP, microtubule-associated protein; NGF, nerve growth factor.

MAPs, associates with microtubules, comigrates with a subspecies of rat brain MAP1 by SDS-PAGE analysis, and like MAP1, but unlike MAP2, precipitates upon boiling (15). It was unclear whether the influence of NGF on this species, designated MAP1.2 (15), is due either to an increase in the levels of this protein, to an enhancement of its phosphorylation, or to a combination of both of these effects. Subsequently, Drubin et al. (10) used a mAb in an attempt to quantify PC12 cell MAP1 and came to the conclusion that NGF induces an increase in the levels of this protein by ~20-fold. On the other hand, Lewis et al. (22) examined MAP1 mRNA levels in PC12 cells by means of a specific cDNA probe, and noted that NGF brought about at most only a severalfold relative increase in this message.

In view of the large NGF-induced increase in phosphorylated MAP1.2 levels and of the relatively modest effect of the factor on MAP1 mRNA, we have directly reexamined the actions of NGF on the steady-state levels and the extent of phosphorylation of this protein. This type of information will be important in understanding the relative roles of MAP1 synthesis and posttranslational modification in NGF-promoted acquisition and maintenance of neuronal morphology. Our results indicate that NGF brings about a rapid (minutes), specific increase in the incorporation of phosphate into PC12 cell MAP1.2, as well as a more slowly developing (days) increase in both the extent of phosphorylation and the relative levels of this protein. We also detect multiple forms of MAP1.2 that differ, at least in part, in degree of phosphorylation. The net long-term action of NGF, by virtue of its effects on MAP1.2 levels and phosphorylation, is to greatly enhance the cellular content of highly phosphorylated forms of MAP1.2.

Materials and Methods

Reagents

NGF was prepared from mouse salivary glands (Bio-Trol, Inc., Indianapolis, IN) by the procedure of Mobley et al. (26). Epidermal growth factor (EGF) was a gift from Dr. Fred Maxfield, Department of Pharmacology, NYU Medical Center, New York. Alkaline phosphatases from *Escherichia coli* or from bovine intestinal mucosa were purchased from Sigma Chemical Co., St. Louis, MO. 8(4-Chlorophenylthio)-cyclic-adenosine-3′:5′ monophosphate was from Boehringer Mannheim Biochemicals, Indianapolis, IN and 12-0-tetradecanoyl-phorbol-13-acetate from Calbiochem-Behring Corp., La Jolla, CA. Pyrophosphate tetrasodium and the protease inhibitors—pepstatin A, leupeptin, benzamidine, phenylmethylsulfonyl fluoride (PMSF), and trasylol—were all from Sigma Chemical Co. Reagents used for electrophoresis were obtained from either Sigma Chemical Co. or Bio-Rad Laboratories, Richmond, CA and Tris buffers from Sigma Chemical Co.

Protein determinations by the method of Bradford (6) were carried out with reagents supplied by Bio-Rad Laboratories, using BSA (fraction V; Sigma Chemical Co.) as the standard.

Antibody Preparations

A rabbit polyclonal antiserum, 3d2, was raised against a gel-purified fusion protein consisting of a portion of the *E. coli* trpE protein fused to a protein encoded by a partial mouse MAP1 cDNA clone (clone D, Figs. 2 and 3 in reference 22). This antiserum was shown to be specific for MAP1.2 on Western blots of purified mouse brain microtubules (Lewis, S. A., and N. J. Cowan, unpublished observation). Clone D detects an mRNA species of ~10 kb in PC12 cells and in rat brain (22). In addition, three previously described anti-MAP antibodies were used: a rabbit anti-bovine MAP1 polyclonal antiserum (23); a rabbit anti-bovine MAP polyclonal antiserum (32) provided by Dr. P. Sherline, Division of Endocrinology, Cornell Medical College, New York, NY; and a mouse monoclonal MAP1 antibody (10) provided by Dr. D. Asai, Dept. of Biological Sciences, Purdue University, Lafayette, IN.

Cell Culture and Radiolabeling of Proteins

PC12 cells were cultured with or without NGF as previously described (13). NGF-treated cells were maintained in 1% donor horse serum to maximize the density of neurite outgrowth with a minimum of cell clumping (see references 1 and 3). Several experiments performed on a substrate (matrigel; Collaborative Research, Inc., Waltham, MA) that minimizes cell clumping even further gave results simlar to those carried out on air-dried collagen.

Experiments requiring comparisons among cultures subjected to different experimental treatments were executed using sister cultures of cells plated and harvested in parallel. Metabolic radiolabeling of proteins was carried out using either 50 μCi of [^{35}S]methionine (1,000–1,200 Ci/mmol) in 1 ml of complete culture medium for 3 d, or 20–100 μCi/ml of [^{32}P]orthophosphate in a modified Krebs-Ringer solution (15) for 1.5–2 h. Both isotopes were purchased from New England Nuclear, Boston, MA. For NGF-treated cultures, NGF was also continuously present during exposure to the isotopes.

Immunoprecipitation and Immunoblotting of MAP1.2

For immunoprecipitations, cells were solubilized in a detergent-containing buffer (1) and then incubated with one of two different MAP1 antisera—3d2 (see above) or an anti-MAP1 polyclonal serum (23). Antigen-antibody complexes were then precipitated with protein A-Sepharose beads (Pharmacia Fine Chemicals, Piscataway, NJ), solubilized in SDS sample buffer (15), and subjected to electrophoresis on a one-dimensional slab gel as described below. Immunoprecipitated MAP1.2 was then located on the gel either by silver staining, or, when cellular proteins had been metabolically radiolabeled, by autoradiography. Immunoblotting was carried out by the method of Wiche et al. (35).

Phosphatase Experiments

Cells were rinsed while still attached to culture dishes three times with warm (37°C) PBS (11) and then harvested at 4°C in 100 mM Tris-HCl buffer (pH 8.2) containing 1–3 mM $MgCl_2$, 2 mM PMSF, 100 kIU/ml trasylol, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μM benzamidine. The suspension was homogenized with 20 strokes in a hand-held teflon glass mortar and pestle and allowed to stand on ice for 10 min. The supernatnat (produced by centrifugation in an Eppendorf microfuge for 10 min) was then aliquoted into tubes containing either diluent only, 10 U/ml *E. coli* alkaline phosphatase, or 5 or 10 μg/ml calf intestine alkaline phosphatase. Some tubes also contained 15–20 mM pyrophosphate tetrasodium. Incubation times were varied from 1–18 h at 35°C or 6–18 h at room temperature. The protocol which proved optimal for detection of the shift in MAP1.2 mobility by SDS-PAGE used the calf enzyme, 10 mg/ml, at room temperature for 18 h.

At the end of the incubation period, a fourfold concentrate of SDS sample buffer was added (1:3) to each reaction tube, mixed, and heated in a boiling water bath for 5 min. After separation by SDS-PAGE, proteins in the fixed gel were silver stained.

Gel Electrophoresis

Discontinuous SDS-PAGE (19) was performed using separating gels (16 cm) of the following composition: 4 M urea, 3.25% acrylamide, 375 mM Tris-HCl, pH 8.8. Stacking gels composed of 3% acrylamide and 125 mM Tris-HCl (pH 6.8) were used. Separating gels were fixed as previously described (1).

Proteins were detected either by silver staining (Gelcode; Pierce Chemical Co., Rockford, IL) or, when cultures had been metabolically radiolabeled, by autoradiography (XAR film; Eastman Kodak, Co., Rochester, NY). ^{32}P-labeled samples were equally loaded at ≥100,000 cpm/lane and ^{35}S-labeled samples at ≥250,000 cpm/lane. Films were preflashed and exposure times were typically 12–36 h at room temperature.

Some of the results obtained using the low percentage polyacrylamide–urea gel system were confirmed in experiments using 32-cm 6–12% or 5–15% acrylamide gradient gels.

Densitometric Quantification of MAP1.2 Levels and Phosphorylation

Individual lanes from autoradiograms or silver-stained gels were analyzed

with a scanning densitometer (model GS 300; Hoefer Scientific Instruments, San Francisco, CA). The electrical output from each scan was analyzed by a PC-operated software program (Macmillan Software Co., New York, NY) modified by Steven Drexler (Department of Pharmacology, NYU Medical Center) to perform plotting and integration of protein bands. The integrated values from autoradiograms of MAP1.2 fell within the linear range of the film's reciprocity as judged by a standard curve encompassing the 20-fold dilution of a ^{32}P-labeled source. A gel loaded with serial dilutions of a whole cell protein lysate was used to standardize the relationship between the density of the silver-stained MAP1.2 band and the amount of protein present. A linear association was found over a range of ~7.5-fold. Quantitative comparisons of control vs. NGF-treated cells were performed only with samples within this range.

Results

Identification and Detection of MAP1.2 in PC12 Cells

PC12 cells express a MAP1 subspecies designated MAP1.2 (15). Initial experiments to compare relative MAP1.2 protein levels in control (nontreated) and long-term NGF-treated PC12 cultures were performed by radioimmunoblotting analysis (Fig. 1). Four different antibody preparations were used (see Materials and Methods), two of which were monospecific for MAP1.2 and two of which were not. Three of the antibody preparations recognized MAP1.2 in both control and NGF-treated cultures and, when used on equal aliquots of the same PC12 lysates, indicated specific increases in MAP1.2 levels ranging from 2.1- to 3.3-fold in the NGF-treated cultures (Fig. 1, *A–C*). However, the fourth preparation (Fig. 1 *D*), a mAb, did not detect MAP1.2 in lysates of control cells, consequently indicating a much larger stimulation of levels by NGF.

Because of the variation in results with the antisera, we chose to quantify the relative levels of MAP1.2 present under different experimental conditions by direct electrophoretic comparison of whole cell lysates. To reliably resolve this high molecular mass protein, we used electrophoresis in low percentage polyacrylamide gels containing urea. Identification was confirmed by comigration with material specifically immunoprecipitated from the cells by MAP1 antibodies (Fig. 2). To verify that the band identified as MAP1.2 does not contain significant amounts of other proteins, cell extracts (labeled and unlabeled) were subjected to immunoprecipitation with excess 3d2 antiserum. This antiserum is monospecific for MAP1.2 (Fig. 1) and was prepared against fusion protein containing a sequence encoded by a partial mouse cDNA clone for MAP1 (see Materials and Methods). PAGE analysis of the protein remaining after immunoprecipitation of the control lysates revealed, both by silver staining and by fluorography of ^{35}S-labeled material, that at least 85% of the MAP1.2 band was cleared. In addition, the ^{32}P-labeled phosphoprotein identified as MAP1.2 in long-term NGF-treated cells was found to be 90% cleared by the antiserum.

Electrophoretic comparison of MAP1.2 in lysates of nontreated control cells with that in lysates of cells exposed to NGF for >2 wk reveals two major changes (Figs. 2–4). First, NGF brings about an increase in the intensity of MAP1.2 relative to other cell proteins. Second, there is a consistent decrease in the electrophoretic mobility of MAP1.2 from NGF-treated cells. These effects were observed in material labeled with either [^{35}S]methionine (for 3 d) or [^{35}P]orthophosphate (for 1.5–3 h) and detected by autoradiography (Fig. 2) as well as in material detected by silver staining (Fig. 3) or immunoblotting (Fig. 1, *A–C*).

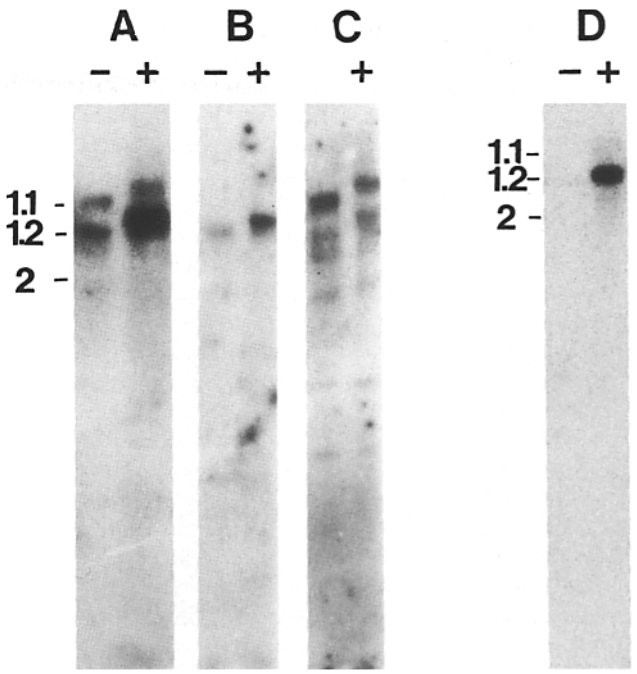

Figure 1. MAP1 in PC12 cells detected by four different antibody preparations. 30 μg of protein from the same cell extracts of untreated PC12 cells (−) or PC12 cells treated with NGF for 3.5 wk (+), was resolved on 3.3% polyacrylamide gels containing 4 M urea, blotted to nitrocellulose, and the blots reacted with (*A*) a rabbit polyclonal anti-bovine brain high molecular weight MAP antiserum (32); (*B*) 3d2, a rabbit polyclonal antiserum raised against MAP1 fusion protein (see Materials and Methods); (*C*) a rabbit anti-MAP1 antiserum (23); and (*D*) a mouse anti-MAP1 mAb (10). The bound antibodies were detected by ^{125}I-protein A autoradiography. Quantitative densitometry of the autoradiograms revealed NGF-induced increases in MAP1.2 of (*A*) 3.3-fold (*B*) 2.1-fold, and (*C*) 2.7-fold. No comparison could be made in *D* due to the low signal from control cells. Markers show the position of MAP1.1, 1.2, and 2 from rat brain microtubules. Note that MAP1.1 is detected by two of the preparations (*A* and *C*) and that its mobility is also shifted after NGF treatment.

Time Course and Quantification of the NGF-induced Effects on MAP1.2

Past experiments (3, 10, 15) as well as those shown in Figs. 2 and 4 reveal a long-term NGF-dependent increase in the relative levels of phosphate-labeled MAP1.2 in PC12 cells. This might result from either an increase in the steady-state level of the protein or an increased incorporation of phosphate or both. We therefore determined the contribution of each of these parameters by labeling sister cultures either with [^{32}P]orthophosphate (for 1–3 h to measure relative phosphate incorporation) or with [^{35}S]methionine (for 3 d to quantify steady-state protein levels). Exposure to NGF was varied from 0, 15 min, 2 h, 3 d, to 2 wk or more. Whole cell lysates were prepared from each culture, equal amounts of TCA-precipitable radioactivity subjected to SDS-PAGE, and the resolved proteins visualized by autoradiography (Fig. 4). The autoradiographs were then scanned den-

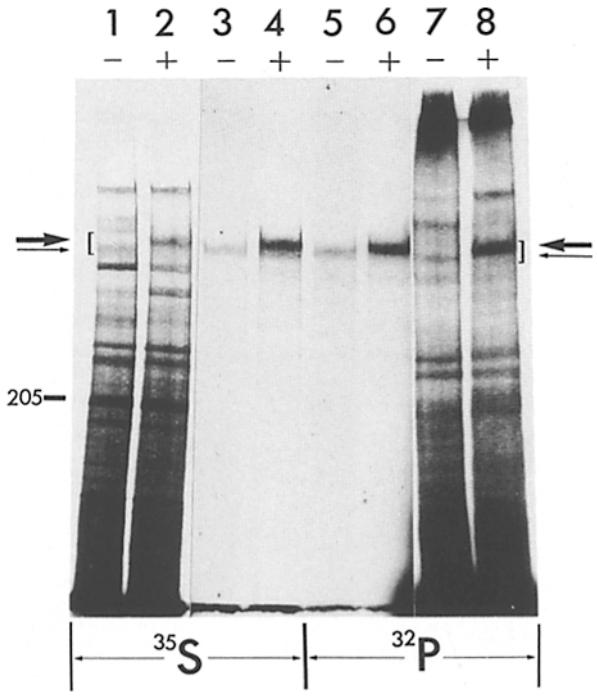

Figure 2. Resolution and identification of MAP1.2 by SDS-PAGE and autoradiography by virtue of its comigration with immunoprecipitated material. Sister cultures of PC12 cells maintained without NGF (−) or with NGF for 4 wk (+) were incubated with [^{35}S]methionine (3 d) or [^{32}P]orthophosphate (2 h). Lanes *1* and *2* and lanes *7* and *8* are from whole cell lysates of these cultures which were resolved by SDS-PAGE in a gel containing 3.25% acrylamide and 4 M urea. These lanes were loaded with 500,000 cpm/lane of TCA-precipitable ^{35}S and 250,000 cpm/lane of TCA-precipitable ^{32}P, respectively. The middle lanes (*3–6*) are from immunoprecipitations carried out in parallel with material from the same cultures. A MAP1 antiserum (3d2) generated against a fusion protein encoded by a MAP1 cDNA clone (22) was used as described in Materials and Methods to immunoprecipitate material from ^{32}P-labeled protein (lanes *5* and *6*) or ^{35}S-labeled protein (lanes *3* and *4*). A control experiment with samples incubated in the absence of the antiserum was clear of immunoprecipitable proteins (data not shown). Although in this experiment immunoprecipitation appears to show a disproportionately large (12-fold) increase in NGF-induced enhancement of ^{35}S-labeled MAP1.2, variable results have been obtained in six comparable independent immunoprecipitation experiments (ranging from 4–12-fold enhancement). For this reason, the immunoprecipitates are presented as indicating information about MAP1.2 electrophoretic migration rather than relative MAP1.2 levels. Brackets indicate the area to which MAP1.2 migrates on the gel. The thin arrow marks the migration distance of MAP1.2 characteristic of the non-NGF-treated cell while the thick arrow denotes the position of MAP1.2 from NGF-treated cells. Migration distance of the 205-kD myosin protein standard is indicated. All lanes are derived from the same gel.

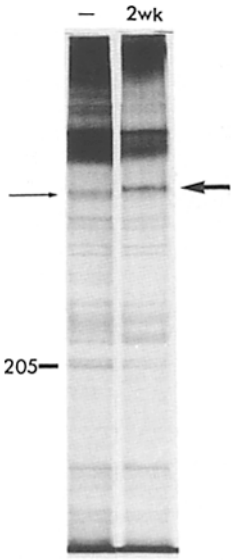

Figure 3. The mobility shift of MAP1.2 in whole cell lysates of long-term NGF-treated cells is detectable by silver staining. PC12 cells cultured without NGF (−) or with NGF for 2 wk (*2 wk*) were harvested in SDS sample buffer, immediately placed in a boiling water bath for 5 min, and approximately equal quantities of protein were then subjected to SDS-PAGE. (−) 20 μg; (*2 wk*) 18 μg protein. Arrows indicate the position of MAP1.2 as determined by silver staining of material specifically immunoprecipitated by anti-MAP 1 (data not shown; cf Fig. 1). Both samples were run simultaneously on the same gel.

sitometrically to quantify the relative levels of radioactivity incorporated into MAP1.2.

The values obtained from multiple experiments of the type described above are summarized in Table I. An increase in the relative steady-state level of MAP1.2 protein was detectable after 3 d, but not after 2 h, of NGF exposure. In long-term NGF-treated (>2 wk) cultures, this measurement averaged 3.5-fold that in control cultures. The magnitude of this relative increase was similar to that determined by scanning densitometry of silver-stained gels, or photographic negatives thereof, containing material from control and long-term NGF-treated cells. Four independent silver-stain experiments gave the following results: 3.3 and 5.0 for directly scanned gels, and 1.9 and 2.4 for the photographic negatives. The mean value was 3.1 ± 0.6 (SEM). The relative levels of MAP1.2 protein did not appear to increase further beyond 2 wk of NGF treatment.

The increase in phosphorylation of MAP1.2, in contrast, commenced within 15 min of NGF exposure. Phosphorylation of this species continued to increase further throughout the time course studied. In agreement with previous studies (3, 15), the relative incorporation of phosphate into MAP1.2 increased by an average of 16-fold after >2 wk of NGF treatment.

Table I also shows the ratios of ^{32}P/^{35}S incorporation into MAP1.2 after various times of exposure to NGF. This provides an estimate of the relative average phosphate incorporation per molecule. After 2 h of NGF treatment, during which there was no detectable change in MAP1.2 protein levels, there was a threefold enhancement of relative phosphate incorporation into this species. In long-term NGF-treated cultures, normalization to the increase in MAP1.2 protein revealed an average enhancement of incorporation per molecule of over fourfold. These data thus show a rapid effect of NGF on MAP1.2 phosphorylation and that the large increase in phosphorylated MAP1.2 levels that occurs after long-term NGF treatment is due both to increased levels of MAP1.2 protein and to an enhanced average incorporation of phosphate per molecule.

The observed effects of NGF on MAP1.2 phosphorylation could arise from either enhanced phosphate turnover or increased steady-state phosphorylation. To distinguish between these possibilities, cultures were either exposed to [^{32}P]phosphate for 2 h in the presence or absence of NGF, prelabeled with [^{32}P]phosphate for 3 d and then exposed to NGF or control medium for 2 h, or pretreated with NGF for 2 wk and (in the continued presence of NGF) labeled with [^{32}P]phosphate for either 2 h or 3 d. For both the short- and

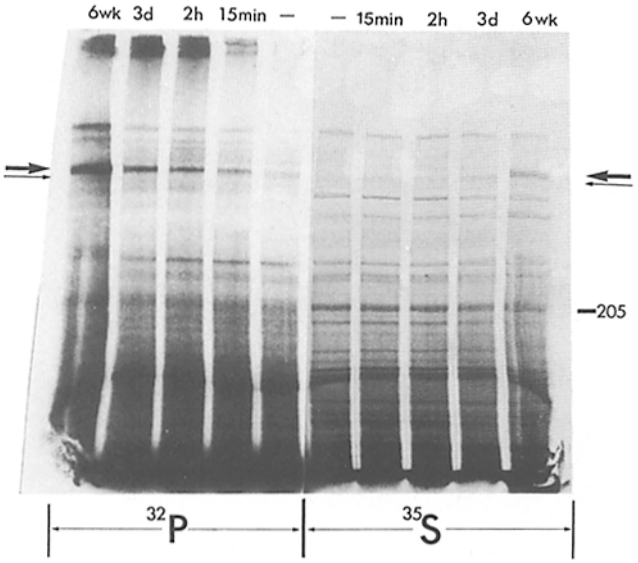

Figure 4. The time course of NGF-mediated changes in the relative phosphorylation and steady-state levels of MAP1.2. Cellular proteins from sister cultures of untreated PC12 cells (−) and PC12 cells treated with NGF for 15 min, 2 h, 3 d, or 6 wk were metabolically radiolabeled with [^{32}P]orthophosphate (*2 h*) or [^{35}S]methionine (*3 d*), solubilized, and analyzed by SDS-PAGE (on a 3.25% acrylamide gel containing 4 M urea) and autoradiography. Conditions for labeling and NGF treatment were as follows. *15 min*: NGF present during the last 15 min of the labeling period; *2 h*: NGF present during the entire incubation with phosphate and during the last 2 h of incubation with methionine; *3d*: phosphate present during the last 2 h of NGF treatment, methionine present throughout; *6 wk*: phosphate present during the final 2 h, methionine during the final 3 d. Thin arrows mark the migration distance of MAP1.2 from untreated cells. Thick arrows mark the migration distance of MAP1.2 from long-term NGF-treated cells. Each time point result is representative of three to five independent experiments. Position of the 205-kD standard is indicated. All lanes are derived from the same gel.

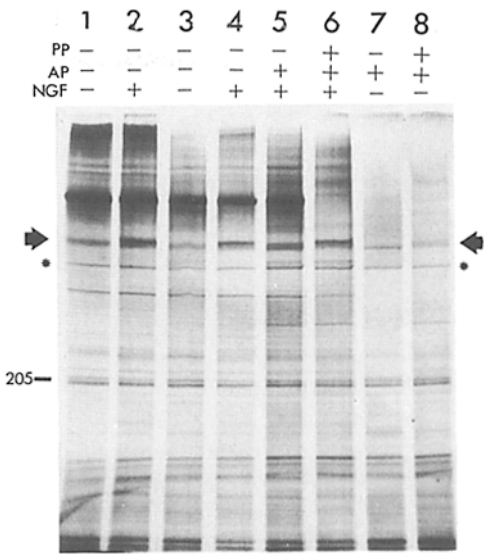

Figure 5. Alkaline phosphatase treatment of MAP1.2. Extracts of PC12 cells cultured without NGF (−) or with NGF for 8 wk (+) were each divided equally into four aliquots as described under Materials and Methods. Alkaline phosphatase (*AP*) from bovine intestinal mucosa, 10 μg/ml, was present in samples *5–8*. Pyrophosphate (*PP*), 16 mM, an inhibitor of this enzyme was added to samples *6* and *8*. Aliquots *1* and *2* were solubilized in 2% SDS sample buffer and heated in a boiling water bath immediately after preparation. Samples *3–8* were allowed to stand at room temperature for 18 h before undergoing the same solubilization and heating. All of the aliquots were then subjected to SDS-PAGE in a 3.25% acrylamide/4 M urea gel which was then fixed and silver stained. Arrows mark the position of MAP1.2. Asterisks identify an invariant band. Position of the 205-kD myosin protein standard is indicated. Each aliquot from − NGF cells contained 40 μg of protein at time zero and each from + NGF cells, 50 μg. These results are representative of three independent experiments.

long-term exposures to NGF, phosphorylation was similarly enhanced (as compared to controls) irrespective of whether labeling with phosphate was for 2 h or 3 d. These findings indicate that the observed changes were in steady-state phosphorylation and that this state is reached within 2 h.

Role of Phosphorylation in the NGF-dependent Mobility Shift of MAP1.2

One possible contribution to the NGF-promoted shift of MAP1.2 to a more slowly migrating position by SDS-PAGE analysis is elevated phosphorylation. If this is the case, then removal of phosphate groups should increase the electrophoretic mobility of MAP1.2 in NGF-treated cell material. As illustrated in Figs. 5 and 6 *A*, incubation of extracts

Table I. The Effect of NGF on the Relative Radiolabeling of MAP1.2

	Relative densitometric levels of MAP1.2/duration of NGF treatment			
	15 min	2 h	3 d	≥2 wk
^{32}P-labeled MAP1.2	2.6 ± 0.3* (n = 3)	3.0 ± 0.4 (n = 3)	5.9 ± 1.1 (n = 4)	16.0 ± 1.9 (n = 16)
^{35}S-labeled MAP1.2	1.3 ± 0.4 (n = 3)	1.0 ± 0.1 (n = 3)	1.6 ± 0.3 (n = 4)	3.5 ± 0.4 (n = 5)
^{32}P/^{35}S ratio	2.0	3.0	3.7	4.6

The relative levels of ^{32}P- and ^{35}S-labeled MAP 1.2 in whole PC12 cells treated with NGF for 15 min, 2 h, 3 d, or ≥2 wk were quantified densitometrically as described in Materials and Methods. Levels are expressed here relative to those in non-NGF-treated control cells plated and harvested in parallel with the experimental cells (i.e., normalized to total incorporated TCA-precipitable cpm of ^{32}P or ^{35}S). Sister cultures were labeled with [^{32}P]orthophosphate or [^{35}S]methionine in three independent trials at the 15-min and 2-h points, four trials at 3 d of treatment, and five trials at the ≥2 wk time point. 11 additional experiments with ^{32}P-labeled MAP 1.2 from long-term NGF-treated cells are included using 5–15% or 6–12% gradient gels. The average ^{32}P/^{35}S ratio for all 16 experiments is quite similar to that obtained from the five experiments matched with ^{35}S-labeled sister cultures (4.6 vs. 4.1, respectively). n, number of experiments.
* SEM.

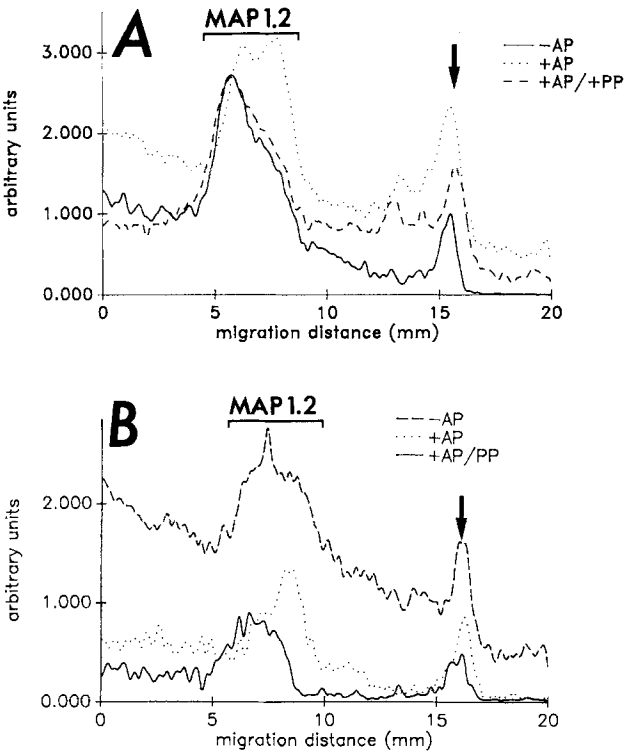

Figure 6. Densitometric scans of MAP1.2 from PC12 cells incubated with alkaline phosphatase. (*A*) Lanes *4–6* and (*B*) lanes *3*, *7*, and *8* of Fig. 5 were scanned over 20 mm encompassing the area in which MAP1.2 migrates. *A* shows the scans from long-term NGF-treated cells. The solid line is the trace generated from an aliquot without alkaline phosphatase; the dotted line, with alkaline phosphatase; and the dashed line with both the enzyme and 16 mM pyrophosphate, an inhibitor of the phosphatase. *B* depicts the scans from untreated cells. Here, the dashed line is the scan from the sample without alkaline phosphatase; the dotted line, with phosphatase; and the solid line, with the enzyme and the inhibitor, 16 mM. The MAP1.2 peak is marked by the bracket. The arrow points to a protein peak, marked by the asterisks in Fig. 5, whose position was invariant across all treatments.

from NGF-treated cells with alkaline phosphatase converted the MAP1.2 to a form with electrophoretic mobility characteristic of that in nontreated control cells. Similar treatment of extracts from nontreated control cells also affected MAP1.2 so that it ran as a sharper band at the position of the front of the typically broad MAP1.2 band present in controls (Figs. 5 and 6 *B*). Prelabeling of cells with [^{32}P]orthophosphate confirmed the loss of phosphate during enzymatic treatment. Pyrophosphate, an inhibitor of alkaline phosphatase (27), blocked these effects of enzymatic treatment. This indicates that the enzyme-induced shift was due to changes in phosphorylation rather than to proteolysis by a contaminant in the preparation.

Activators of Known Protein Kinases Do Not Mimic the NGF-mediated Rapid Increase in MAP1.2 Phosphorylation

Agents known to promote phosphorylation reactions via activation of known protein kinases were added to PC12 cultures during metabolic radiolabeling with [^{32}P]orthophosphate to better understand the mechanism through which

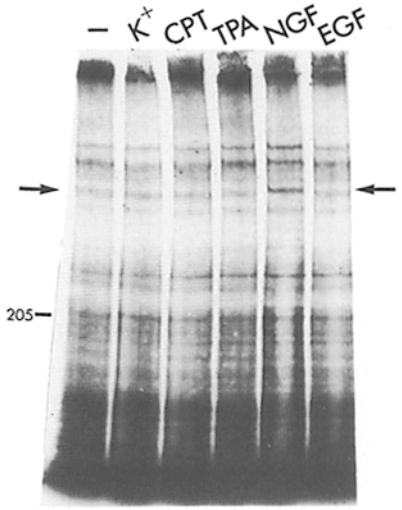

Figure 7. Specificity of the rapid phosphorylation of MAP1.2. Sister cultures were metabolically labeled for 2.5 h with [^{32}P]orthophosphate either in the absence of any added agents (−), or in the presence of 44 mM KCl (K^+), 1 mM 8(4-chlorophenylthio)-cAMP (*CPT*), 200 nM tetradecanoyl-phorbol-13-acetate (*TPA*), 50 ng/ml NGF, or 5 ng/ml EGF. Equal numbers of TCA-precipitable cpm (100,000) from each cell culture were subjected to SDS-PAGE and autoradiography as described in Materials and Methods. The arrows mark the position of MAP1.2 and the solid bar marks the 205-kD standard.

NGF acts to enhance the phosphorylation of MAP1.2. 8-(4-Chlorophenylthio)-cyclic adenosine monophosphate, a permeant analog of cAMP and activator of protein kinase A (25); 12-O-tetradecanoyl-phorbol-13-acetate, which activates protein kinase C (8); or 44 mM KCl, which leads to activation of Ca^{++}-dependent protein kinases via the opening of voltage-dependent Ca^{++} channels, were used. Each of these treatments rapidly produces elevated phosphorylation of at least several PC12 cell proteins (15, 16, 20, 24). This was confirmed during our studies in which these treatments lead to enhanced phosphorylation of at least one other cytoskeletal protein in both NGF-treated and non–NGF-treated cultures (Aletta, J. M., and L. A. Greene, unpublished observations). It is noteworthy, however, that these agents do not promote neurite outgrowth in PC12 cell cultures. EGF was also tested. This trophic polypeptide produces several effects on PC12 cells similar to NGF, but not neurite outgrowth (17). EGF also triggers several rapid changes in PC12 cell protein phosphorylation (16).

As shown in Fig. 7, none of the above treatments mimicked the rapid effect of NGF on MAP1.2 phosphorylation. In addition, the phosphorylation of MAP1.2 in cultures exposed to NGF for 2 wk or more was not enhanced further by 2 h of treatment with any of these agents (data not shown).

Discussion

Recognition of MAP1.2 on Low Percentage Polyacrylamide/Urea Gels

We have identified the high molecular mass protein, MAP1.2, in PC12 cells before and after NGF treatment and quantified both its levels and phosphorylation. Our analysis

has relied on the mobility of the protein in one-dimensional gels. The possibility that significant quantities of other proteins of equivalent electrophoretic mobility are present in the gels seems remote for several reasons. First, after immunoprecipitation with a specific antibody, only a small amount of protein of electrophoretic mobility similar to MAP1.2 remains in the lysates of both control and NGF-treated cells (see Results). In addition, in response to long-term exposure to NGF, there is a shift in the mobility of MAP1.2 (by all methods of detection) such that little protein remains at the location in the gel corresponding to the position of MAP1.2 from untreated cells. This mobility shift can be reversed by phosphatase treatment. Finally, our immunoblotting results, with the exception of that in Fig. 1 *D*, corroborate our quantitative data based on scanning densitometry (cf. Fig. 1).

NGF-specific Phosphorylation of MAP1.2

Previous observations have revealed that NGF produces a striking long-term increase in the relative incorporation of phosphate into PC12 cell MAP1.2 (3, 10, 15). We show here that this is in part due to a dual effect on phosphorylation. There is a rapid effect of NGF on phosphate incorporation into this protein followed by a slower increase in this parameter. We have estimated that by two wk or more of treatment, the average NGF-induced increase in phosphate groups is about fourfold per molecule. Our data indicate that this reflects changes in steady-state levels rather than turnover.

Our results indicate that the rapid enhancement of phosphorylation is NGF specific and cannot be reproduced by EGF or a variety of agents that lead to activation of several well-characterized protein kinases. EGF, insulin, and cAMP derivatives have also been shown to be unable to mimic the long-term effect of NGF on MAP1.2 (15). Each of the agents used, in contrast, stimulates phosphorylation changes of other PC12 cell proteins (16, 20, 24). This suggests that the NGF-enhanced phosphorylation of MAP1.2 is mediated either via a novel protein kinase or via inactivation of a rather specific phosphoprotein phosphatase.

The present findings may be compared with those concerning other microtubule elements. With long-term NGF treatment of PC12 cells, beta-tubulin and chartin MAPs also undergo enhanced phosphorylation (1, 3, 7). In the latter case, this is due to a large increase in the levels of more highly phosphorylated isoforms (3). Phosphorylation of chartin MAPs is rapidly inhibited by application of Li^+ (7), activators of adenylate cyclase (14), or agents that lead to microtubule disassembly (1). In contrast, the phosphorylations of MAP1.2 and beta-tubulin are not significantly affected by such treatments.

Multiple Forms of MAP1.2 in PC12 Cells

The resolution of high molecular mass proteins provided by the low percentage acrylamide/4 M urea gels used here has revealed multiple forms of PC12 cell MAP1.2 with slight, but consistently detectable, differences in mobility. As indicated by our experiments with alkaline phosphatase, these differences are due, at least in part, to phosphorylation, with the least phosphorylated forms having the greatest mobility.

Our study also revealed that treatment with NGF brings about a shift in the proportion of MAP1.2 that migrates with slower electrophoretic mobility. This is consistent both with the influence of phosphorylation on the mobility of MAP1.2 in gels and with the effect of NGF on MAP1.2 phosphorylation. The observation that most of the MAP1.2 protein (as revealed by immunoblotting, silver staining, and by labeling with [^{35}S]methionine) undergoes this shift in long-term, NGF-treated cultures is consistent with the finding that NGF increases the average number of phosphates per molecule, rather than merely increasing phosphate turnover. This also appears to be the case with short-term NGF exposure; the MAP1.2 detected by phosphate label in such cases showed a shift to lower mobility.

Although we are presently unable to reliably estimate the proportions of the various forms of MAP1.2, it is clear that very little of the MAP1.2 in non-NGF-treated PC12 cultures is in the most slowly migrating, highly phosphorylated form and, as noted above, that in contrast, this form is the most prominent in long-term, NGF-treated cultures. Thus, while the long-term effects of NGF on overall MAP1.2 levels and phosphorylation are each reasonably modest, it is important to note that the factor appears to bring about very substantial changes in the levels of individual forms of this protein.

A presently unresolved issue is the effect of NGF treatment on the distribution of the multiple forms of MAP1.2 at early times. That is, by phosphate label, the short-term effect of NGF is to enhance phosphorylation of MAP1.2 and to retard its electrophoretic mobility, whereas, in contrast, by methionine label, little or no mobility shift is seen (cf. Fig. 4) under such conditions. One interpretation of these data is that at short times of treatment, NGF brings about a very large increase in phosphate incorporation into a relatively small pool of MAP1. Thus, it may be that the early action of NGF is directed at a subfraction of MAP1.2 while the long-term actions affect most of the cellular content of this protein. As noted below, this could be relevant to different functional roles of the highly phosphorylated forms.

Regulation of MAP1.2 Protein Levels by NGF

In the initial description of NGF's long-term influence on MAP1.2 levels in PC12 cultures, immunoprecipitation of [^{35}S]methionine-labeled material suggested an increase in the relative levels of MAP1.2 protein (15). Subsequently, Drubin et al. (10) reported an NGF-promoted 20-fold relative increase in PC12 cell MAP1.2 levels based on quantitative immunoblotting using a MAP1 mAb. Lewis et al. (22) measured MAP1 mRNA in PC12 cells by means of a specific cDNA probe and found only a modest (<2–3-fold) increase in the relative amounts of this message. In the present work, we have used SDS-PAGE to directly compare the relative steady-state levels of MAP1.2 protein present in whole cell extracts of nontreated and long-term, NGF-treated PC12 cells. This was achieved either by silver staining or by analysis of material labeled with [^{35}S]methionine for 3 d. Both methods indicated a 3.0- to 3.5-fold increase in the relative levels of MAP1.2 protein. This, together with the ~fourfold NGF-promoted increase in phosphorylation per molecule, accounts for the overall 16-fold relative increase in incorporation of phosphate into MAP1.2 in NGF-treated cultures.

It is clear that there is a discrepancy between the present results and those of Drubin et al. (10) regarding the extent to which NGF increases MAP1.2 protein levels. One possible explanation is that the mAb used by Drubin et al. has a

greater affinity for a phosphorylated epitope of MAP1.2. This could lead to an underestimation of MAP1.2 protein levels in non-NGF-treated cells, since, as our study indicates, this species contains relatively little phosphate. This possibility is consistent with the results we obtained by immunoblotting with the same mAb used by Drubin et al. (Fig. 1 *D*). That is, in contrast to several polyclonal antisera, this antibody showed essentially no recognition of MAP1.2 in non-NGF-treated cells. It may be significant in this light that our estimation of the overall NGF-promoted relative increase of phosphate incorporation into MAP1.2 (16-fold) is similar to the 20-fold increase in MAP1.2 protein reported by Drubin et al. Another potential source of the difference in results could lie in the culture conditions. Compared with the present work, Drubin et al. (10) used PC12 cells propagated under different conditions and grown on a different substrate in a different medium.

Possible Functional Roles for MAP1.2 Regulation by NGF

There are several possible ways in which NGF-dependent enhancement of MAP1.2 phosphorylation and steady-state protein levels may be of functional significance. One evident role is in neurite outgrowth. The initiation of neurites requires the presence of microtubules. In parallel with its promotion of neurite outgrowth in PC12 cultures, NGF increases the proportion of cellular tubulin that is assembled (3, 10) and promotes the appearance of arrays of bundled microtubules (13). There is evidence that MAP1 may be involved in the assembly and stabilization of microtubules and/or in their cross-linking with one another or with other cellular elements (28). Thus, by means of its interactions with microtubules, MAP1 may play a necessary role in the formation and growth of neurites. NGF's effects on both the levels and phosphorylation state of MAP1.2 may be important in this regard. In particular, the enrichment of phosphorylated MAP1.2 in PC12 cell neurites vs. cell bodies (Aletta, J. M., and L. A. Greene, unpublished observations) presents some interesting possibilities. For instance, the more highly phosphorylated forms of MAP1.2, which are greatly enhanced in level by long-term NGF treatment, could be more functionally active in their interactions with microtubules. Also supportive of a potential role for MAP1.2 regulation in neurite outgrowth is the observation that fibroblast growth factor, the only other agent known to induce the neuronal differentiation of PC12 cells (33), promotes long-term effects similar to NGF on levels of highly phosphorylated MAP1.2 (30).

In addition to such a possible structural role for the long-term effects of NGF on MAP1.2, based on its rapidity and NGF selectivity, the early increase in MAP1.2 phosphorylation could conceivably serve a signaling function. Interestingly, in this regard, a phosphorylated MAP1-like molecule has been localized at putative sites of mRNA maturation within the nuclei of fibroblasts (31). In such cells, the phosphoprotein appears in nuclei after growth stimulation. In PC12 cells, along with its promotion of neuronal differentiation, NGF regulates the expression of a variety of genes (21). Perhaps, a phosphorylated form of MAP1.2 in PC12 cell nuclei serves some function during the NGF-mediated regulation of gene expression. In agreement with this proposition, fibroblast growth factor, the only other known agent to mimic NGF's pattern of gene regulation (21), also elicits the rapid phosphorylation of MAP1.2 (Rydel, R. E., and L. A. Greene, unpublished observations). It may also be relevant in this regard that some experimental evidence has been obtained that another cytoskeletal protein, actin, may function as an initiation factor within the nucleus (12).

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