Regulation of a High Molecular Weight Microtubule-associated Protein in PC12 Cells by Nerve Growth Factor

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ABSTRACT PC12 rat pheochromocytoma cells respond to nerve growth factor (NGF) protein by shifting from a chromaffin-cell-like phenotype to a neurite-bearing sympathetic-neuron-like phenotype. Comparison of the phosphoprotein patterns of the cells by SDS PAGE after various times of NGF treatment revealed a high molecular weight ($M_r \gtrsim 300{,}000$) band whose relative intensity progressively increased beyond 2 d of NGF exposure. This effect was blocked by inhibitors of RNA synthesis and did not require neurite outgrowth or substrate attachment. The enhancement by NGF occurred in serum-free medium and was not produced by exposure to epidermal growth factor, insulin, dibutyryl cAMP, or dexamethasone. Several different types of experiments indicated that this phosphoprotein corresponds to a high molecular weight (HMW) microtubule-associated protein (MAP). These included cross-reactivity with antiserum against brain HMW MAPs, co-cycling with microtubules and co-assembly with tubulin in the presence of taxol. The affected species also co-migrated in SDS PAGE gels with brain MAP1 and, unlike MAP2, precipitated upon boiling. Studies with [^{35}S]-methionine-labeled PC12 cells indicated that at least a significant proportion of this effect of NGF was due to increased levels of protein rather than to mere enhancement of phosphorylation. On the basis of the apparent effects of MAPs on the formation and stabilization of microtubules and of the importance of microtubules in production and maintenance of neurites, it is proposed that induction of a HMW MAP may be one of the steps in the mechanism whereby NGF promotes neurite outgrowth. Furthermore, these findings may lead to an understanding of the role of MAP1 in the nervous system.

One of the major actions of nerve growth factor protein (NGF) is the promotion of neurite outgrowth and regeneration (1). The mechanistic steps by which NGF-responsive as well as other neurons produce and maintain neurites are far from clarified. The rat PC12 pheochromocytoma cell line is a model system that has been employed to study neuronal differentiation and, in particular, NGF's mechanisms of action (2, 3). PC12 cells proliferate and express the phenotypic properties of adrenal chromaffin cells, their nonneoplastic counterparts. Exposure of these cells to NGF causes an alteration in their phenotype so that they take on (over a time course of days) numerous characteristics of sympathetic neurons, including outgrowth of long, branching neurites containing parallel arrays of microtubules (2, 4). A principal advantage of the PC12 line for examining NGF-promoted neurite outgrowth is that the cells respond to, but do not require the factor. Hence, unlike NGF-responsive neurons, which require NGF for survival and are difficult to study before first exposure to NGF, PC12 cells can be compared before and after various times of NGF treatment.

Studies with PC12 cells have revealed that neurite outgrowth in this system is blocked by inhibitors of transcription (5). However, one- and two-dimensional SDS PAGE analyses of proteins synthesized by the cells before and after NGF treatment indicate no qualitative and only a very limited number of quantitative changes (6–8). This has suggested that if transcription-dependent changes in protein synthesis are indeed causally related to neurite outgrowth, then such changes must occur among the less abundant class of cellular proteins (6).

In this study, we demonstrate that NGF selectively causes induction of a high molecular weight (HMW) phosphoprotein in PC12 cultures and present evidence that this protein corre-

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sponds to a subspecies of HMW microtubule-associated protein (MAP1) that has been previously identified in brain.

MATERIALS AND METHODS

Cell Culture

PC12 cells (passages 15–35) were grown as previously described (2) on collagen-coated tissue culture dishes in the presence or absence of 50 ng/ml NGF. For "suspension" cultures, the cells were maintained with or without NGF on plastic Petri dishes. The cells did not attach to these dishes, nor, when treated with NGF, did they grow neurites (9). Instead, they formed small clumps that floated in the medium or rested on the dish. All cultures were fed every 2–3 d.

Radiolabeling

[^{32}P]-ORTHOPHOSPHATE: The tissue culture medium was removed and the cell monolayers were washed two to three times with a phosphate-free, HEPES-buffered (pH 7.2) modified Krebs-Ringer saline (KRH;10). The KRH was then replaced with KRH supplemented with 50–100 μCi/ml of carrier-free [^{32}P]-orthophosphate (New England Nuclear, Boston, MA) and with or without 50 ng/ml NGF and the cultures were incubated for 2 h in a water-saturated 37°C atmosphere. At the end of incubation, the labeling saline was removed and the cultures were rapidly washed three times with phosphate-buffered saline (PBS;11) and the cells processed accordingly as described below. For cells grown in suspension, washing was carried out by low-speed centrifugation (200 *g* for 5 min). The cells were then suspended and incubated in KRH containing [^{32}P]-orthophosphate and ± NGF as above. At the end of incubation, the supernatant medium was removed and the cells were washed once with PBS, in both cases by low speed centrifugation as above.

[^{35}S]-METHIONINE: Cultures were labeled in complete growth medium ± 50 ng/ml NGF and with 100 μCi/ml of [^{35}S]-methionine (1,200 Ci/mmole; New England Nuclear) for 48–72 h. The medium was then removed, the cultures were rapidly washed three times with PBS and the cells were processed as described below.

Harvesting for SDS PAGE

For direct electrophoretic analysis, the cells were harvested by scraping in SDS-containing sample buffer (7), immediately boiled for 5 min, and then either analyzed the same day or stored frozen at −20°C until use the next day.

Preparation of Anti-MAP Antiserum

Microtubules were prepared from bovine brain by the method of Shelanski et al. (12) through two cycles of polymerization and depolymerization. The microtubule proteins were subsequently separated by SDS PAGE on 6% polyacrylamide slabs (13), and the area of the gel corresponding to the HMW MAP bands (14–16) was excised, and used for immunizations. ~0.5 mg of total MAP protein mixed with complete Freund's adjuvant were used for the first immunization in a rabbit. ~0.2 mg of protein mixed with incomplete Freund's adjuvant were used for each boost. The rabbit was bled one week after each boost and the titer of the antiserum tested by radioimmunoassay using [^{125}I]-labeled MAP protein (17). A titer of 1:500 was determined by this method after the third boost. This antiserum was further tested by immunoblotting (18) against whole microtubule protein to determine the specificity of the antiserum. For these experiments microtubule proteins prepared by one cycle of reassembly were run on SDS PAGE (6% acrylamide), and transferred to nitrocellulose paper using a Bio-Rad transblot apparatus (Bio-Rad Laboratories, Richmond, CA). The paper was subsequently incubated overnight with 5% bovine serum albumin to block nonspecific binding sites, followed by a 1-h incubation with antiserum (1:200 dilution in 5% BSA). The paper was washed five times with PBS, and incubated with biotinylated goat anti-rabbit (Vector Laboratories, Inc., Burlingame, CA) for 30 min. The paper was again washed five times with PBS, and incubated with avidin and biotinylated horse radish peroxidase for 30 min. After five more washes with PBS, the paper was incubated with 0.03% diaminobenzidine (Sigma Chemical Co., St. Louis, MO) and 0.005% H_2O_2 in 0.05 M Tris buffer, pH 7.8 to visualize the reaction. By this method, the antiserum recognized only the MAP1 and MAP2 proteins.

Immunoprecipitation Experiments

PC12 cells were labeled with [^{35}S]-methionine or [^{32}P]-orthophosphate as described above, solubilized in 1% SDS containing 100 U/ml aprotinin and 0.5 mM phenylmethanesulfonyl fluoride (PMSF; Sigma Chemical Co.), boiled for 5 min, and sonicated to reduce viscosity. The numbers of TCA-precipitable counts in the preparations were determined and immunoprecipitation was carried out by modifications of previously described procedures (19). Equal numbers of counts for the NGF-treated and -untreated cells were incubated overnight at 4°C with anti-MAP antiserum (1:20 dilution) in a 50 mM Tris buffer, pH 7.4 containing 0.19 M NaCl, 6 mM EDTA, 2.5% Triton X-100, 0.1% SDS, 100 U/ml aprotinin, and 0.5 mM PMSF (solutionA). In some experiments, excess brain MAP2 (1 mg/ml) was also present during incubation. The latter was prepared by the procedure of Herzog and Weber (20). For controls, preimmune serum was used. After incubation with the primary antibody, 0.1 ml of protein A-Sepharose solution (prepared by allowing 100 mg of protein A-Sepharose to swell in 1 ml of solution A for about 2 h, and resuspending the beads in 1 ml solution A after three to four washes) was added, and the mixtures were incubated at room temperature for 2 h. The incubation mixtures were centrifuged in a microfuge for 5 min, and washed three times with solution A. For the last wash Triton X-100 was eliminated from this solution. The samples were then placed in sample buffer, and separated on a 6% polyacrylamide gel, containing 0.1% SDS.

Co-assembly with Tubulin

Labeled cultures were harvested by scraping in ice-cold reassembly buffer (RB: 0.1 M MES, pH 6.8; 1 mM EGTA; 0.5 mM $MgCl_2$; reference 12)) containing 100 U/ml aprotinin (Sigma Chemical Co.) 0.5 mM PMSF and 1 mg/ml of disassembled three-times cycled bovine brain microtubules (12). For cultures labeled with [^{32}P]-orthophosphate, this and all subsequent solutions also contained 20 mM nitrilotriacetic acid (NTA) to inhibit phosphatase activity (21). The cell harvests were homogenized by 10 strokes in a glass-glass homogenizer at 4°C and then incubated for 30 min on ice to promote microtubule disassembly. The homogenate was then centrifuged for 30 min at 120,000 *g* in a Beckman airfuge (Beckman Instruments, Inc., Fullerton, CA) at 4°C. Essentially all of the labeled material co-migrating with MAP1 was found in the supernatant. The supernatant fraction was then adjusted to contain 1 mM GTP and 4 M glycerol and incubated at 37°C for 30 min to promote assembly of microtubules (12). This material was centrifuged as above for 30 min at 37°C, the supernatant was removed and the "microtubule" pellet was taken up in RB supplemented with inhibitors as above. After incubation on ice for 30 min to promote dissassembly, this material was centrifuged as above at 4°C and the supernatant fraction collected. An aliquot of this "cycle 1" material was removed, mixed with an equal volume of 2× sample buffer, boiled and analyzed by SDS PAGE as described below. The remaining part of the supernatant was carried through a second cycle of assembly by the procedure described above and the resulting microtubule pellet dissolved in sample buffer, boiled, and analyzed by SDS PAGE.

Taxol-facilitated Assembly

Labeled cultures were harvested in RB containing 100 U/ml aprotinin, 0.5 mM PMSF, 20 mM NTA, 0.5 percent Triton X-100, and 2 mg/ml of bovine brain tubulin which had been freed of MAPs by chromatography on phosphocellulose (16). After homogenization, incubation on ice, and centrifugation at 4°C, all as described above, the MAP-containing supernatant fraction was adjusted to contain 1 mM GTP and 12 μM taxol (22, 23) and incubated for 30 min at room temperature (23). This mixture was then centrifuged at 4°C as above. The supernatant was removed and processed for SDS PAGE and the pellet was washed once in the above buffer without tubulin and Triton and recentrifuged. The pellet was then suspended in 0.35 M NaCl in the above buffer without Triton but with taxol to solubilize MAPs as described by Vallee (23). This material was recentrifuged and the resulting supernatant and pellet fractions were processed for SDS PAGE.

Thermostability

Stability to boiling was determined by modifications of previously described procedures (20, 24). Labeled cultures were harvested in reassembly buffer containing 10 mM dithiothreitol, 40 mM NaCl, 100 U/ml aprotinin, 5 mM PMSF and 20 mM NTA and with or without ~1 mg/ml of carrier three-times cycled disassembled bovine brain microtubules. The cellular material was homogenized as above and incubated for 10 min at 4°C to promote microtubule disassembly. The homogenate was then centrifuged at 120,000 *g* as above at 4°C and the supernatant fraction was transferred to a preheated glass tube in boiling water. After exactly 5 min, the boiled fraction was rapidly cooled on ice and then centrifuged at 120,000 *g* at 4°C for 30 min. Aliquots of all fractions were processed for SDS PAGE.

SDS PAGE and Detection of Labeled Proteins

Samples were collected and boiled in sample buffer as described above. Electrophoresis was carried out in the discontinuous system described by Laemmli (13) on slabs (25 or 9 cm) of either 6½–13% gradients of acrylamide or 5, 5½, or

6% acrylamide. The latter concentrations were particularly useful for resolving high molecular weight MAPs. Samples of twice cycled microtubules (to reveal the position of MAPs) and of molecular weight standards (myosin, M_r 200,000; β-galactosidase, M_r 117,000; phosphorylase-b, M_r 94,000; albumin, M_r 68,000; ovalbumin, M_r 43,000; carbonic anhydrase, M_r 30,000; soybean trypsin inhibitor, M_r = 20,000; lysozyme, M_r = 14,000; purchased from Bio-Rad Laboratories and Pharmacia, Inc., Piscataway, NJ) were also loaded on each gel. After electrophoresis, the gels were fixed, stained, and destained as previously described (7). For samples containing [^{32}P], the gels were dried and incubated with Kodak XAR-5 X-ray film. For samples containing [^{35}S]-methionine, the slabs were impregnated with salicylate before drying (25), and then fluorographed with preflashed (26) XAR-5 film. Because the salicylate impregnation greatly obscured the patterns of stained proteins, the R_f values of markers were determined before treatment.

Indirect Immunofluorescent Staining

PC12 cells were cultured on collagen-coated coverslips, either with or without NGF. In the former case, the cells were pretreated with NGF for two wk and then passaged onto the coverslips and maintained for an additional 6 d with NGF. Cultures were prepared for staining by several procedures: (*a*) fixation for 30 min at room temperature with 4% paraformaldehyde in PBS; (*b*) extraction to prepare stabilized cytoskeletons (27) followed by fixation as above; (*c*) pretreatment with 5 μg/ml taxol for 1 h followed by extraction and fixation as in (*b*). After being washed free of fixative, the cultures were incubated for 1 h with PBS containing 0.1% (vol/vol) Triton X-100 and 1% normal goat serum and then incubated for 1 h with either anti-HMW-MAP antiserum (1:50 and 1:200) or a monoclonal antibody to α-tubulin (1:1,000; kindly provided by Dr. S. Blose, Cold Spring Harbor). The coverslips were washed and then incubated with FITC-goat anti-rabbit or anti-mouse antiserum (1:200; Cappel Laboratories Inc., Cochranville, PA), washed and observed with a fluorescence microscope. All antibody dilution and washes were carried out using PBS containing Triton X-100 and normal goat serum as above. In some cases the anti-HMW-MAP antiserum was supplemented with excess MAP2 (~0.2 mg/ml) prepared by the method of Herzog and Weber (20). Parallel control cultures were incubated with the same dilution of preimmune serum.

MATERIALS: NGF was prepared as described by Mobley et al. (28), EGF was prepared by the method of Savage and Cohen (29) and was the generous gift of Dr. Fred Maxfield (Department of Pharmacology, New York University Medical Center), insulin was from Eli Lilly Chemical Co. (Indianapolis, IN), dibutyryl cAMP was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN), and taxol and camptothecin were supplied by the National Cancer Institute. The phosphocellulose-chromatographed tubulin and bovine brain microtubules were kindly provided by Dr. J.-F. Letterier, Department of Pharmacology, New York University Medical Center.

RESULTS

Effect of NGF on Phosphoproteins

To identify changes in phosphoproteins caused by NGF, PC12 cultures were treated with the factor for various times (from several hours to several weeks) and then exposed to [^{32}P]-orthophosphate for 2 h. NGF was present during phosphorylation except for control, non-NGF-treated cultures. The labeled phosphoproteins were then resolved by SDS PAGE and visualized by radioautography. The results of one such experiment are shown in Fig. 1. A marked increase in phosphorylation of a band at about $M_r \simeq 60{,}000$ was noted by 2 h. After a week of NGF treatment, this band was substantially less prominent. A second band at $M_r \simeq 56{,}000$ also showed a transient increase, while a minor band at $M_r \simeq 64{,}000$ was enhanced at 2 h of NGF exposure and remained elevated even after a week of NGF treatment. An additional faint band at $M_r \simeq 72{,}000$ showed enhancement only after 2–3 d of NGF exposure. The most striking change, however, occurred in a very high molecular weight ($M_r \gtrsim 300{,}000$) band that showed an increased labeling by about 2 d of NGF treatment and a progressive enhancement of labeling thereafter.

Because of the magnitude of the latter change, the high molecular weight (HMW) phosphoprotein was chosen for further study. To facilitate resolution of the high molecular weight range, gels with a lower percentage of acrylamide (5–6%) were employed.

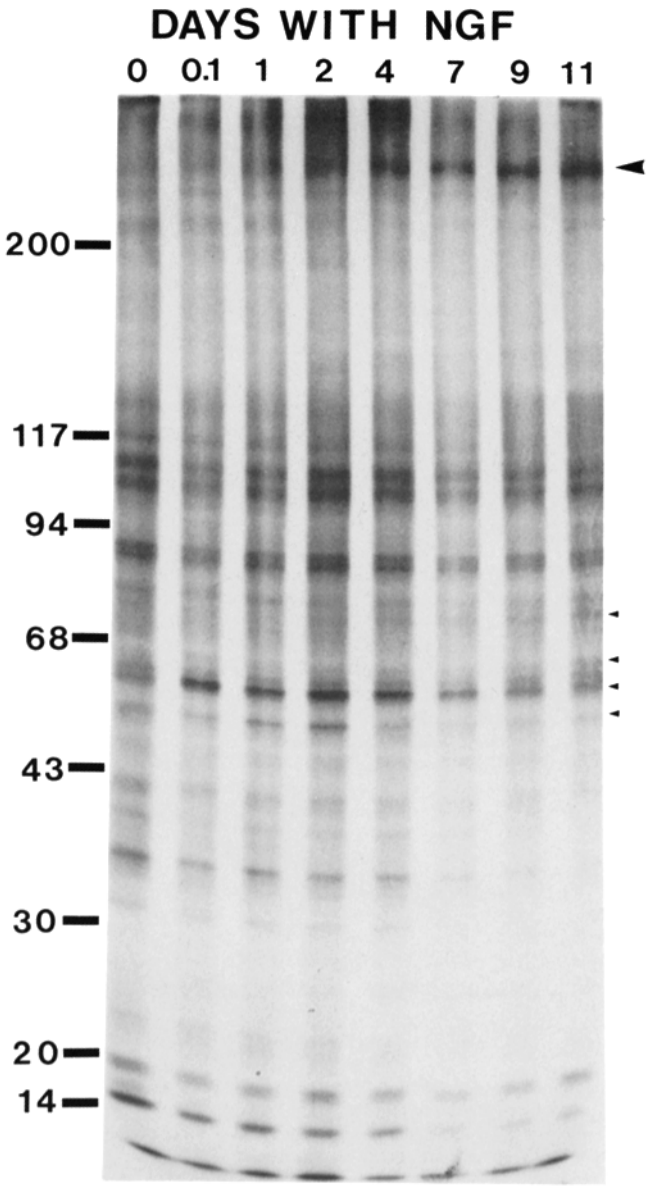

FIGURE 1 Pattern of phosphoproteins in PC12 cells as a function of the time of exposure to NGF. Cultures were incubated with [^{32}P]-orthophosphate for 2 h either without NGF pretreatment (0 and 0.1 d) or after pretreatment as indicated. Except for the 0 time point, NGF was present during the incubation. At the end of this time of incubation, the cultures were prepared and resolved by SDS PAGE as described in Materials and Methods. Resolution was on 6½–13% acrylamide gel (25 cm in length); 100,000 TCA-precipitable cpm were loaded in each lane. Radioautography was for 2 d. Arrowheads indicate the positions of bands whose relative intensities are altered in response to NGF treatment. Numbers on the left-hand side indicate the position of molecular weight markers (mol wt × 10^{-3}).

Specificity of and Requirements for the HMW Phosphoprotein Response

In addition to NGF, several other agents have been reported to elicit responses from PC12 cells. These agents include epidermal growth factor (EGF;30), insulin (31), dibutyryl cAMP (32, 33), and dexamethasone (34). Although such compounds

do not mimic many of the actions of NGF (e.g., promotion of neurite outgrowth) they do possess some actions in common with NGF. To test the specificity of the response of the HMW phosphoprotein, PC12 cultures were phosphorylated either during 2 h or following 7–10 d of treatment with the above agents (EGF, 1 and 10ng/ml; insulin 100 ng/ml; dB cAMP, 1 mM; dexamethasone, 1 and 10 μM; NGF, 50 ng/ml). Phosphorylation of the $M_r \gtrsim 300{,}000$ band was changed only by NGF. An example of an experiment on cells treated for 8 d with and without several of these compounds is shown in Fig. 2. Also, when given in combination with NGF, the above agents did not alter NGF's long term effect on the HMW band.

Because PC12 cells can be maintained in the absence of serum if NGF is present (35), it was possible to test whether serum components in complete medium were required for enhanced labeling of the HMW band. As illustrated in Fig. 3, the action of NGF occurred in serum-free medium.

A number of responses of PC12 cells to NGF appear to require transcription since they become apparent only after several days of treatment and are blocked by low concentrations of RNA synthesis inhibitors (3, 5). The slow time course (Fig. 1) of the effect of NGF on the HMW phosphoprotein suggested this might also be so in this instance. In agreement with this possibility, enhancement of the HMW band by NGF

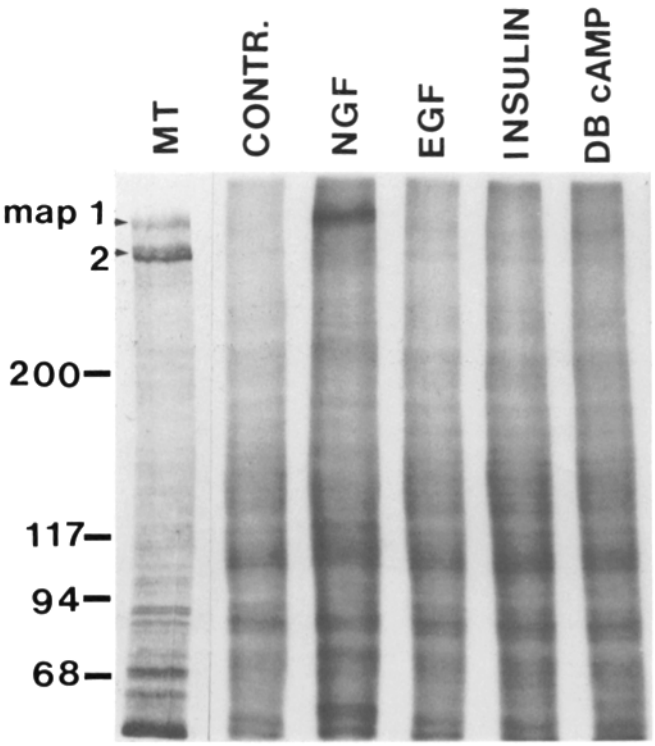

FIGURE 2 Specificity of effect of NGF on the HMW phosphoprotein. PC12 cultures were grown for 8 d in the presence of no additive (*contr.*), *NGF* (50 ng/ml), epidermal growth factor (*EGF*; 10 ng/ml); *insulin* (100 ng/ml) or dibutyryl cAMP (*DB cAMP*; 1 mM) and then exposed to [^{32}P]-orthophosphate in the presence of the same compounds. The cells were then processed and resolved by SDS PAGE (6% acrylamide) as described in Materials and Methods; 100,000 TCA-precipitable counts were loaded per lane. Radioautography was for 1 d. The left-hand lane was loaded on the same gel with bovine brain microtubule proteins (*MT*) and stained with Coomassie Blue. The left-hand side indicates the positions of MAP 1 and MAP 2 as well as the positions of molecular weight markers (mol wt × 10^{-3}).

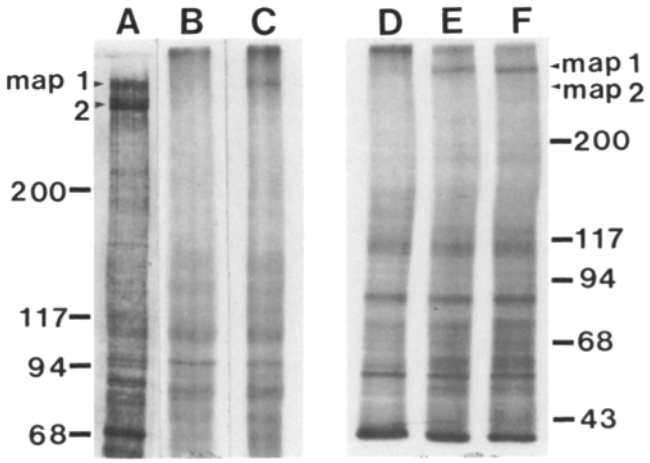

FIGURE 3 Induction by NGF of the HMW phosphoprotein in suspension culture (*B* and *C*) and in defined, serum-free medium (*D*, *E*, and *F*). For the suspension experiment, PC12 cells were grown for 12 d in suspension with (*C*) or without (*B*) 50 ng/ml NGF. For the serum-free experiment, PC12 cells were grown for 8 d either in complete medium without (*D*) or with (*E*) 50 ng/ml NGF or in serum-free RPMI 1640 medium with 50 ng/ml NGF (*F*). All cultures were exposed to [^{32}P]-orthophosphate for 2 h with the continued presence or absence of NGF and then processed and resolved by SDS PAGE in 6% polyacrylamide gels. ~100,000 TCA-precipitable cpm were loaded per lane; autoradiography was for 1 d. Lane *A* was loaded on the same gel as *B* and *C* with bovine brain microtubule proteins and stained with Coomassie Blue. The left- and right-hand sides indicate the positions of MAP 1 and MAP 2 as well as of molecular weight markers (mol wt × 10^{-3}) all loaded on the same gels.

was inhibited by 0.2 μg/ml of camptothecin and 0.01 μg/ml of actinomycin-D.

Enhancement of the HMW phosphoprotein in PC12 cultures occurs with about the same time course as promotion of neurite outgrowth. Might the former then be a consequence of the latter? To test this, PC12 cells were maintained with or without NGF in suspension culture for 10–21 d and then labeled with [^{32}P]-orthophosphate as described in the Materials and Methods section. Under these circumstances, the suspended NGF-treated cells do not form neurites (9), but nevertheless appear to undergo the metabolic and transcriptional changes required for neurite production. This is illustrated by their ability to commence neurite outgrowth almost immediately upon being plated on an adhesive substrate, even if their RNA synthesis is blocked (9). As illustrated in Fig. 3, enhancement of the HMW phosophoprotein also occurred under these circumstances.

Cross-reactivity of HMW Phosphoprotein with Brain HMW MAPs

Due to its apparent size and phosphorylation, the possibility arose that the HMW phosphoprotein might correspond to a HMW microtubule-associated protein (or HMW MAP). HMW MAPs are phosphoproteins that co-purify with brain tubulin through repeated cycles of assembly and disassembly and which have electrophoretic mobilities by SDS PAGE of $M_r \gtrsim 300{,}000$ (cf. references 14–16, 20, 23). The HMW MAPs have been subclassified as MAP1 and MAP2, the former of somewhat slower mobility by SDS PAGE (14–16, 20, 23, and see Figs. 2–6). Each of these in turn can be further resolved into at least two distinct bands (termed here MAPs 1.1, 1.2, 2.1, and

2.2) by SDS PAGE. As illustrated in Figs. 2–6, the HMW phosphoprotein consistently co-migrated with MAP1, and at higher resolution, with the more rapidly migrating MAP1.2.

To test the possibility that the HMW phosphoprotein corresponds to a HMW MAP, PC12 cells were treated with or without NGF for 10–16 d, phosphorylated for 2 h, solubilized in 1% SDS in the presence of inhibitors of proteolysis, and equal numbers of TCA-precipitable cpm from each type of culture were subjected to immunoprecipitation with antiserum directed against purified HMW MAPs. When the immunoprecipitates were analyzed by SDS PAGE, a major band was observed that co-migrated with MAP1.2 (for example see Fig. 4 *E–H*). In addition, the relative intensities of these bands from cultures maintained with and without NGF reflected the differences in intensities of these bands in the original cell homogenates. The immunoprecipitations were not blocked by the addition of excess (1 mg/ml) MAP2 protein. If the proteolytic inhibitors were omitted, a second labeled band appeared that had a mobility slightly faster than MAP2.

Similar experiments were also performed with PC12 cultures labeled for 48–72 h with [^{32}S]-methionine. A major band of identical mobility with the phosphorylated band was specifically immunoprecipitated (Fig. 4 *A–D*). Significantly, even though equal numbers of TCA-precipitable cpm were used for immunoprecipitation, the band from the NGF-treated cultures was again more intense. This suggests that NGF causes an increase in the absolute amount of this protein which is detectable either by labeling with [^{35}S]-methionine or [^{32}P]-orthophosphate.

Association of the HMW Protein with Microtubules

Two types of experiments were performed to test whether the NGF-affected protein could associate with microtubules. PC12 cells treated with or without NGF were labeled with [^{35}S]-methionine or [^{35}P]-orthophosphate, homogenized under microtubule-destabilizing conditions and then centrifuged at high speed (see Materials and Methods). The HMW phosphoprotein and a corresponding methionine-labeled band were found to be present predominantly in the supernatant fraction. In one type of experiment, the latter fraction was submitted to several consecutive cycles of assembly-disassembly in the presence of excess purified brain microtubules (see Materials and Methods). Fig. 5 illustrates an experiment with [^{35}S]-methionine-labeled cultures that had been treated with NGF. A high molecular weight band co-migrating with MAP1.2 was highly enriched through two cycles of co-assembly. In contrast, when similar levels of TCA-precipitable radioactivity derived from NGF-untreated cultures were co-cycled, this band was not detected, even after the first cycle. Similar results were achieved with [^{32}P]-orthophosphate-labeled cultures. In addition to the $M_r \gtrsim 300{,}000$ band, as illustrated in Fig. 5, a band of $M_r \simeq 210{,}000$ was also highly enriched upon co-cycling. A second type of experiment involved the use of taxol, an alkaloid that greatly promotes assembly and stabilization of microtubules (36). As recently described by Vallee (23), MAPs associated with taxol-stabilized tubules can be quantitatively solubilized with high salt while leaving the tubules intact. Labelled supernatant fractions prepared as described above were incubated in the presence of purified MAP-free brain tubulin and taxol (see Materials and Methods). The mixture was then centrifuged at high speed to pellet the resulting microtubules. For both the [^{32}P]- and [^{35}S]-labeled cultures, a band co-migrating with MAP1.2 was present in the supernatant prior to taxol addition and, after taxol polymerization, was nearly quantitatively shifted to the pellet fraction. Fig. 6 illustrates an experiment in which the washed taxol-assembled microtubule pellet was exposed to 0.35 M NaCl to solubilize MAPs. Note that the band co-migrating with MAP1 is nearly quantitatively removed from the original supernatant fraction after taxol treatment (lanes *B* and *C*) and then solubilized by the salt treatment (lanes *D* and *E*).

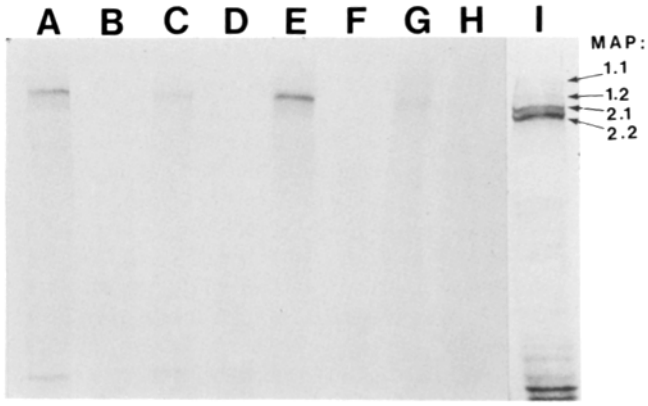

FIGURE 4 Immunoprecipitation with anti-MAPs. Lanes *A* and *C* show the specific immunoprecipitation of [^{35}S]-methionine-labeled material from NGF-treated and untreated cultures respectively, whereas *B* and *D* are controls in which preimmune serum was used. Lanes *E* and *G* are immunoprecipitates from [^{32}P]-orthophosphate-labeled cultures with (*E*) and without (*G*) NGF. *F* and *H* are the corresponding controls with preimmune serum. [^{35}S]-methionine labeling was for 2 d after 11 d of culture with (*A* and *B*) or without (*C* and *D*) NGF. Labeling with [^{32}P]-orthophosphate was for 2 h after 13 d culture with (*E* and *F*) and without (*G* and *H*) NGF. Aliquots containing 500,000 TCA-precipitable cpm were used for immunoprecipitation in each case and the entire immunoprecipitates were dissolved and loaded on the gel (5% acrylamide). Radioautography was for 11 d. Lane *I* shows the positions of stained HMW MAPs in a sample of bovine brain microtubule proteins loaded on the same gel.

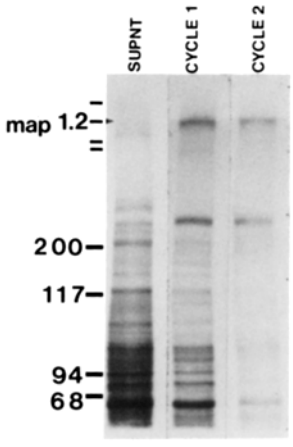

FIGURE 5 Co-cycling of PC12 HMW MAPs with brain microtubules. PC12 cultures were maintained with NGF for 10 d and then labeled in complete medium for 48 h with [^{35}S]-methionine. The cultures were homogenized, a high speed supernatant was prepared (*SUPNT*) and the material was processed through two consecutive cycles of assembly and disassembly with carrier brain microtubule proteins. Left-hand lane, 50,000 TCA-precipitable cpm of high speed supernatant; middle lane 6,800 TCA-precipitable cpm from the disassembled microtubule pellet of *cycle 1*; and right-hand lane, 2,300 TCA-precipitable cpm from the microtubule pellet of *cycle 2*. Resolution was by SDS PAGE on 5% acrylamide gels and visualization by 1 (*SUPNT*) and 10 (cycles *1* and *2*) days of fluorography. Left-hand side indicates positions in the same gel of stained bovine brain microtubule HMW MAPs (1.1, 1.2, 2.1, and 2.2 from top) and of molecular weight standards (mol wt $\times 10^{-3}$).

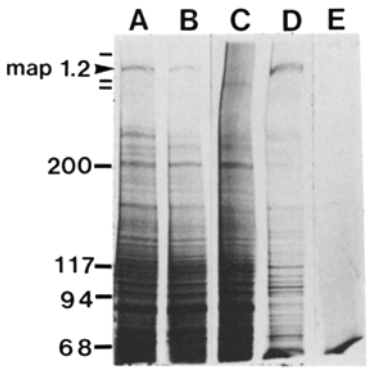

FIGURE 6 Co-assembly of PC12 HMW MAP with tubulin in the presence of taxol and its release by high salt. PC12 cultures were maintained with NGF for 7 d and then labeled with [^{35}S]-methionine in complete medium for 3 d. The cultures were homogenized in the presence of carrier tubulin (*A*), a high-speed supernatant was prepared (*B*) and the latter was treated with taxol as described in Materials and Methods and centrifuged at high speed. An aliquot of the supernatant fraction from the latter centrifugation is shown in lane *C*. The pellet was washed once, suspended in buffer containing 0.35 NaCl and recentrifuged at high speed. Lanes *D* and *E* contain material from the resulting supernatant and pellet, respectively. Approximately 40,000 (*A*–*C*), 6,000 (*D*), and 4,200 (*E*) TCA-precipitable counts were loaded. Resolution was by SDS PAGE on 5% acrylamide gels. Fluorography was for 13 d. Left-hand side indicates positions of stained HMW MAPs (1.1, 1.2, 2.1, 2.2, from the top) and of molecular weight standards (mol wt × 10^{-3}) all loaded on the same gel.

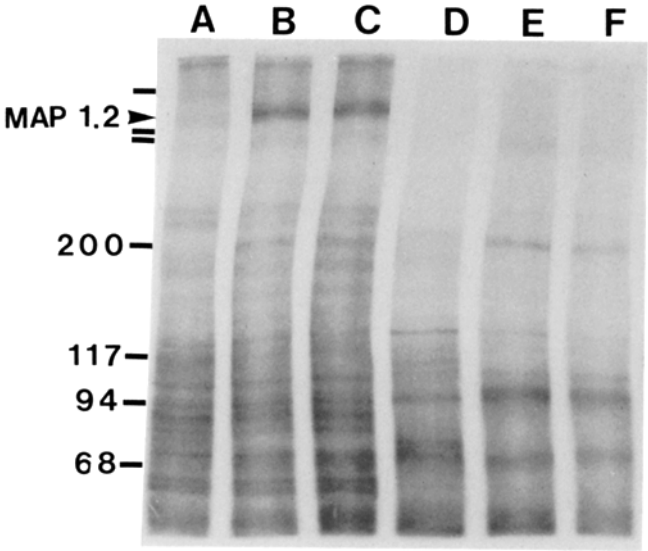

FIGURE 7 Thermal lability of the NGF-responsive MAP. Cell cultures were grown for 10 d in the presence or absence of 50 ng/ml NGF and labeled for 2 h with [^{32}P]-orthophosphate under the same conditions. Cultures were then homogenized, centrifuged at high speed, and the supernatants boiled, cooled, and recentrifuged (see Materials and Methods). Carrier microtubule proteins were added to samples *A*, *B*, *D*, and *E* and omitted from *C* and *F*. Lanes *A* and *D* are from the supernatants of cultures grown without NGF before and after boiling, respectively. Lanes *B* and *C* are from NGF-treated cultures before boiling whereas *E* and *F* are from the same cultures after boiling. ~30,000 TCA-precipitable cpm were loaded per lane and autoradiography was for 4 d. The positions of microtubule associated proteins and molecular weight standards in the same gel are indicated at left (mol wt × 10^{-3}).

Thermal Lability of the HMW Phosphoprotein

The mobility of the NGF-responsive phosphoprotein suggests that it corresponds to MAP1 rather than to MAP2. A distinguishing feature between MAP1 and MAP2 is that the latter, but not the former is stable to boiling (20, 24). NGF-treated and -untreated PC12 cultures were labeled with [^{32}P]-orthophosphate, homogenized in the presence of depolymerized carrier microtubules and soluble supernatants were prepared as described above. These were then boiled for 5 min as described in Materials and Methods. As illustrated in Fig. 7, as a result of this treatment, the HMW phosphoprotein was lost from the supernatant fractions. This material could be recovered however, from the precipitate fraction that formed upon boiling (data not shown). Panels *C* and *F* of Fig. 7 demonstrate that the susceptibility of the HMW phosphoprotein to boiling was not affected by the absence of carrier microtubule proteins. Staining of the gels revealed that as expected, the carrier MAP1, but not MAP2, was lost from the supernatant upon boiling.

Immunofluorescent Staining

The presence of HMW MAPs in PC12 processes was demonstrated by indirect immunofluorescence. Staining with the antiserum against HMW MAPs was evident (in comparison with controls processed with preimmune serum) in all neurites as well as in the somas of NGF-treated cells. Positive, though apparently significantly less intense, staining was also obtained with NGF-untreated cultures. Identical patterns of staining were observed with anti-HMW-MAP antiserum to which an excess (~0.2 mg/ml) of purified MAP2 had been added. Fibrillar staining was not seen in any of the cells. Positive staining of somas and processes was also present with stabilized cytoskeletal preparations of the cultures, both with and without pretreatment with taxol, but again a fibrillar pattern could not be detected. The absence of fibrillar staining is difficult to interpret because the fine caliber of the PC12 processes may have precluded such an observation. In support of the latter possibility, monoclonal anti-α-tubulin presented a strongly positive but nonfibrillar pattern of staining with both whole cell and stabilized cytoskeletal preparations.

DISCUSSION

Rapid Effects of NGF on Protein Phosphorylation

Our results corroborate previous reports (37, 38) that NGF evokes rapid changes in phosphorylation of selected proteins in PC12 cultures. In particular, the $M_r \simeq 60{,}000$ band appears to correspond to the band identified by Halegoua and Patrick (38) as tyrosine hydroxylase. The cause of the fall off of relative labeling of this species after several days is not clear, but could correlate with the drop in tyrosine hydroxylase activity that occurs in PC12 cultures after a similar period of NGF treatment (2). The effect of NGF on the $M_r \simeq 56{,}000$ and 64,000 bands appear to have been previously undescribed. The significance of such changes is not clear, but as suggested previously for NGF (37, 38) as well as for rapid phosphorylations triggered by other peptide hormones, these could play a causal role in subsequent cellular responses.

Induction of MAPs

The present studies reveal that NGF significantly induces the level of a high molecular weight phosphoprotein in PC12 pheochromocytoma cells. Evidence for the identification of this protein as a MAP include its (*a*) cross-reactivity with brain HMW MAPs, (*b*) co-association with tubules through several cycles of assembly and disassembly, and (*c*) quantitative assembly with tubulin in the presence of taxol and its quantitative

removal from taxol-stabilized tubules by 0.35 M NaCl. Furthermore the induced species appears to correspond to MAP1 rather than MAP2, as indicated by its co-migration with brain MAP1 in SDS PAGE gels, its lability to boiling, and the failure of excess MAPs to block its immunoprecipitation. High-resolution SDS PAGE in 5% acrylamide gels further indicated co-migration with MAP1.2. Because the molecular properties of HMW MAPs are largely unknown, one cannot yet ascertain whether the induced PC12 MAP is identical to previously described brain HMW MAPs.

HMW MAPs are found in relative abundance in brain (14–16, 20, 24). MAP2 appears to be significantly enriched in neuronal dendrites (39), whereas MAP1 may have a more widespread neural distribution (23). A variety of different types of experiments (16, 20, 24) suggest that HMW MAPs may promote the formation and stabilization of microtubules as well as cross-link these structures with other elements of the cytoskeleton (40). There is also abundant evidence that microtubules are required for the production and maintenance of neurites by neurons (41, 42) as well as by NGF-treated PC12 cells (9). Hence, it is reasonable to speculate that one step in the mechanism of action of NGF is the induction of MAP1 protein, which in turn enhances the formation and/or stabilization of microtubules. This could play a major role in promotion and maintenance of neurite outgrowth. The presence of MAP1 in PC12 neurites, as revealed by immunofluorescent staining, is consistent with this hypothesis.

Of particular relevance to the above possibility is the recent finding that long-term treatment of PC12 cells with NGF appears to alter the properties of their microtubules (27). That is, microtubules in neurite-bearing PC12 cells exposed to NGF for 3 wk are considerably more resistant to the depolymerizing effects of colchicine than are microtubules in PC12 cultures grown either without NGF or with NGF for 24 h. Also, MAPs are reported to decrease the susceptibility of microtubules to calcium-induced depolymerization in vitro (43).

MAP Induction and Priming

Prior experiments have provided evidence for a "priming" mechanism whereby NGF promotes via a slow-onset, transcription-dependent mechanism, the synthesis and accumulation of material that is required for neurite outgrowth and elongation (5, 9). As for priming and neurite outgrowth, induction of phosphorylated MAP1 by NGF was found here to follow a lag of 1–2 d, to increase progressively thereafter and to require *de novo* synthesis of RNA. Also, as for priming, induction of MAP1 occurred in suspension and was hence independent of neurite outgrowth and substrate attachment. Finally, treatments with a variety of agents that elicit responses from PC12 cells but that do not cause priming, did not induce phosphorylated MAP1. Hence, these findings are consistent with the possibility that induction of MAP1 by NGF could be one of the molecular correlates of priming.

PC12 Cell MAPs

Aside from MAP1, our data suggest that PC12 cells possess at least several other HMW MAPs. Very long overexposure of radioautographs of material immunoprecipitated from labeled cultures, as well as of microtubules produced by cycling and taxol-treatment of such cultures, indicates the presence of a relatively small amount of material that co-migrates with MAP2. The relative labeling of this material appears to be unaffected by NGF treatment and it appears to be stable to boiling (see Fig. 6 for example). In addition, analysis of microtubules formed by co-cycling and taxol treatment of labeled cultures indicates the presence of a phosphoprotein MAP of apparent $M_r \simeq 200{,}000$–$225{,}000$ in NGF-treated cultures (see Figs. 5 and 6). It has not yet been possible to detect this material in corresponding experiments with NGF-untreated cultures. The "boiling" experiment shown in Fig. 7 further indicates that a heat stable phosphoprotein of this size is considerably enhanced in NGF-treated cultures. It is of interest that a MAP of about this size has been reported to be induced in "differentiated" murine neuroblastoma cultures (44) and to be present in certain nonneural cells (45, 46). However, further experiments will be necessary to further substantiate the identity of these and other putative MAPs in PC12 cells.

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