

Sequential Phosphorylation of Chartin Microtubule-associated Proteins Is Regulated by the Presence of Microtubules

John M. Aletta and Lloyd A. Greene

Department of Pharmacology, New York University Medical Center, New York, 10016

Abstract. Chartins are a unique class of three families of microtubule-associated proteins, each consisting of several isoforms possessing varying degrees of phosphorylation. The most highly phosphorylated chartin isoforms are highly enriched in neuronal cell fractions containing microtubules and there is evidence that their phosphorylation may play a role in promoting neurite outgrowth. The present work describes the relationship between the phosphorylation state of chartins and the presence of intact microtubules in long-term cultures of NGF-treated, neurite-bearing PC12 cells. Cultures were depleted of microtubules by exposure to high concentrations of depolymerizing agents for 2–24 h. Radiolabeling of cellular proteins with [³²P]orthophosphate or [³⁵S]methionine revealed that both the ongoing and steady-state phosphorylation of chartins is markedly altered under these conditions. Two-dimensional isoelectric focusing by SDS-PAGE of whole cell extracts demonstrated that the more acidic, highly phosphorylated isoforms are diminished with a concomitant increase in the more basic, less phos-

phorylated isoforms. These phosphorylation changes were relatively specific for the chartins and were not observed for phosphorylated MAP 1.2, phospho- β -tubulin, or most other phosphoproteins. Thus, the phosphorylation state of chartins, but not of other phosphoproteins, is regulated by the presence of native microtubules. Despite depolymerization of microtubules, neurites remained extended for at least 24 h. Neurite elongation, however, was arrested. Microtubules, therefore, may be required for extension, but not for short-term maintenance of well-established neurites. Taxol, which promotes tubule assembly and stability, does not, conversely, drive phosphorylation of the chartins. Instead, taxol appeared to decrease the turnover of phosphate in microtubule-associated, acidic chartin isoforms. These data suggest several models as to how chartin phosphorylation is regulated in neurite-bearing cells and indicate that phosphorylation of cytoplasmic and microtubule-associated chartins occurs via different mechanisms.

THE structural and functional properties of the microtubular cytoskeleton appear to depend, by essentially unknown mechanisms, upon a diverse set of molecules known as microtubule-associated proteins (MAPs).¹ High molecular mass (36 and tau (9, 49) MAPs promote the polymerization of microtubules from monomeric tubulin *in vitro*, suggesting a role for these proteins in the regulation of the assembly state of cellular microtubules. MAPs have also been thought to mediate cross-bridging between microtubules and other cytoskeletal elements (26, 30, 44). Recently, evidence has accumulated linking increases in these and other MAPs with neuritogenesis (2, 7, 15, 20). Additional functions which may be performed in part by MAPs include maintenance of cell shape (41), stability (24), and intracellular motility (48).

Since most MAPs are known to be substrates for protein kinases (10, 20, 32, 37, 39, 45), modulation of phosphoryla-

tion may be one mechanism for the regulation of MAP functions. This may be particularly true for a class of MAPs first described by Pallas and Solomon (39) and recently designated "chartins" (34). Several lines of biophysical and immunological evidence have demonstrated that chartins are distinct from other classes of MAPs, including tau proteins (2, 4, 34, 40, 49). In their initial study with cultured neuroblastoma and PC12 cells cultured without nerve growth factor (NGF), Pallas and Solomon demonstrated that the most highly phosphorylated variants of chartin MAPs are selectively enriched in cellular fractions containing components of the assembled microtubular cytoskeleton (39). Partly on this basis, these workers hypothesized that enhancement of chartin phosphorylation could be involved in microtubule assembly. Moreover, since microtubules are major components of the neuronal cytoskeleton and since microtubule formation appears to be required for generation of neurites (11, 18, 51), it is possible that phosphorylated chartins may consequently play a role in neurite outgrowth.

In consonance with a possible role of phosphorylated

1. *Abbreviations used in this paper:* 2-D IEF×SDS-PAGE, two dimensional isoelectric focusing by SDS polyacrylamide gel electrophoresis; MAP(s), microtubule-associated protein(s); NGF, nerve growth factor.

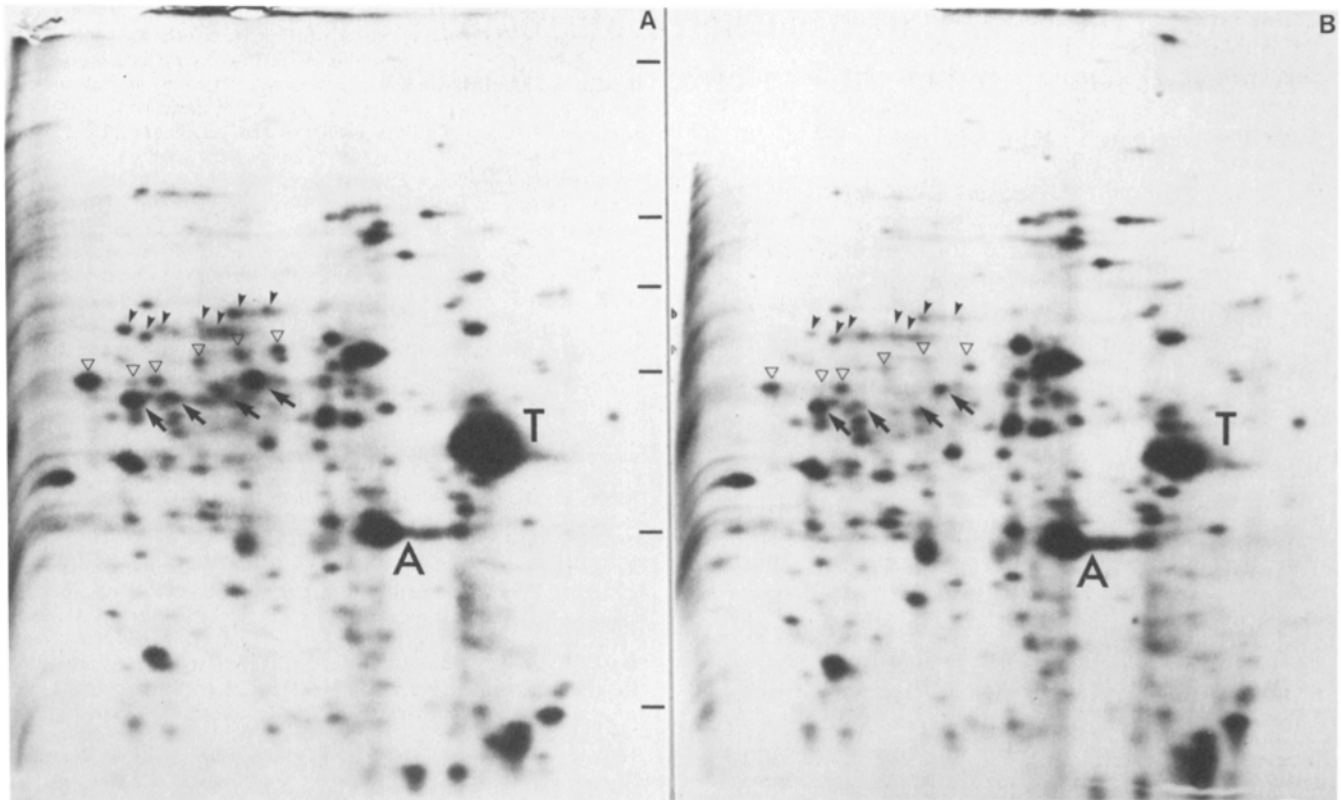


Figure 1. Identification of chartins in a MAP-enriched fraction of long-term (>2 wk) NGF-treated PC12 cells. Cultures were incubated in the presence of 100 $\mu\text{Ci/ml}$ of [^{35}S]methionine for 3 d and processed, as described in Materials and Methods, to isolate proteins of the microtubular cytoskeleton for separation by 2-D IEF \times SDS-PAGE. Equal numbers (90,000) of TCA-precipitable counts of this material were loaded onto IEF gels. Fluorograms of 4-d exposures from the SDS-PAGE are reproduced here. On the left is the pattern typical of long-term NGF-treated PC12 cells. The fluorogram on the right was generated from a culture plated and treated with NGF in the same manner and time as that on the left, the only exception being that 50 μM nocodazole was present in the culture medium for the last 6 h of incubation. All three chartin families, depicted by arrows (60–64 kD), triangles (64–72 kD), and arrowheads (80 kD), were markedly diminished in this fraction due to depolymerization of the microtubular cytoskeleton by nocodazole and their consequent extraction during a prior step. Similar results were obtained in three independent experiments. *T*, tubulin; *A*, actin. The separating gels were 7.5–12% acrylamide with an isoelectric point range between 4.0 and 6.8. The basic end of the gel is to the left. Molecular mass markers are indicated by the black bars: 200, 116, 92, 66, 45, and 31 kD.

chartins in the nervous system, these MAPs are present in the brain (34) as well as in murine neuroblastoma cells (39), cultured sympathetic neurons (4), and in the PC12 clonal line of rat pheochromocytoma cells (2, 7, 39, 52). The latter finding is of particular practical importance since PC12 cells have been widely used as a model to study neuronal differentiation and neuritogenesis (21, 22), and, recently, to detect and assess the functional role of MAPs in the nervous system (2, 7, 15, 20, 39, 52). When exposed to NGF, PC12 cells stop dividing, and, over the course of several days to weeks, elaborate an extensive network of neurites filled with microtubules. After long-term (2–3 wk) exposure to NGF, there occurs an enhancement in the stability of PC12 cell microtubules (3) and substantial increases in the amount (15) and proportion (2) of total cellular tubulin that is assembled. Along with this, the relative abundances of tau MAPs (15) and of a phosphorylated high molecular mass MAP (15, 20) rise substantially. Moreover, under these conditions the composition of chartin MAPs shifts such that the more acidic, phosphorylated forms are markedly elevated as compared with the situation in non-NGF-treated PC12 cells (2). The latter observation is consistent with the hypothesis that char-

tin MAP phosphorylation may be involved in regulation of microtubule properties and in neurite outgrowth. In further support of this, exposure of PC12 cells and sympathetic neurons to lithium ion has been found to selectively inhibit both NGF-promoted chartin MAP phosphorylation and neuritogenesis (7). Recently, forskolin and cholera toxin, activators of adenylate cyclase, were also observed to suppress both chartin phosphorylation and neurite outgrowth in PC12 cells (19).

In the present studies we sought to further define the relationship between chartin MAP phosphorylation and the polymerization state of cellular tubulin in a neuronal model. That is, if phosphorylation of chartin MAPs may play a role in microtubule properties, does the converse occur—does formation or maintenance of highly phosphorylated chartins require the presence of microtubules? To approach this issue, we exposed long-term cultures of NGF-treated PC12 cells to agents known to either stabilize or destabilize microtubules in intact cells. We find that phosphorylation of chartins, but not of several other MAPs, is tightly linked to the presence of native, intact tubules. Drug-induced depolymerization of microtubules leads to a marked diminution in the formation

and maintenance of acidic chartin isoforms and to a reciprocal increase in the more basic isoforms. However, in contrast, promotion of cellular tubulin assembly and stabilization by exposure to taxol does not enhance chartin phosphorylation but, in fact, appears to decrease the turnover of phosphate in the chartins. We also find that the neurites in long-term NGF-treated cultures can remain extended, but do not elongate, during at least 24 h of exposure to drug treatments that lead to a substantial reduction in the number of cellular microtubules. These and additional observations are used here to refine hypotheses regarding the role of chartins and their phosphorylation in neurite outgrowth.

Materials and Methods

Reagents

Nocodazole (Aldrich Chemical Co., Milwaukee, WI) and podophyllotoxin (Sigma Chemical Co., St. Louis, MO) were dissolved in dimethyl sulfoxide (Fisher Scientific Co., Pittsburgh, PA) at concentrations of 50 mM and used at a final dilution of 1:1000 in culture medium. An equal volume of the dimethyl sulfoxide carrier was added to control cultures. Taxol, stored frozen as a 20-mM stock solution in dimethyl sulfoxide, was diluted to a final concentration of 2 μ M during exposure to the cultured cells. NGF was prepared according to the method of Mobley et al. (35). The chemicals used for gel electrophoresis were from Bio-Rad Laboratories (Richmond, CA) and those for sample preparation were from Fisher Scientific Co. and Sigma Chemical Co. Ampholytes (5-7 and 3.5-10) were purchased from LKB Instruments, Inc. (Gaithersburg, MD). Protein A-Sepharose beads were from Pharmacia Fine Chemicals (Piscataway, NJ).

Cell Culture

PC12 cells were grown as previously described (21) on collagen-coated 35-mm tissue culture dishes in RPMI 1640 medium supplemented with 10% horse serum and 5% fetal calf serum. When non-NGF-treated cultures were required, PC12 cells were typically plated at densities of 5×10^5 to 1×10^6 cells per 35-mm dish and harvested for analysis after 1-4 d.

Cultures for NGF treatment were initially seeded with 5×10^5 cells per 35-mm dish in RPMI 1640 medium initially containing 1% horse serum and 50 ng/ml NGF. Decreasing the serum concentration minimizes cell clumping and thus maximizes the extensiveness of the neuritic network (3). Once these cultures had elaborated a robust outgrowth of neurites (10 d-2 wk), the medium was replaced with complete medium plus 50 ng/ml NGF. Clumping at this time after NGF treatment was not a problem. These cultures were always grown in the presence of NGF for 2 wk or longer before use in experiments. A previous study has established that the serum concentration in the culture does not affect the NGF-promoted regulation of chartin phosphorylation (2).

To examine the growth of individual neurites, more sparsely plated cultures were required. Approximately 60,000-125,000 cells were seeded onto 35-mm culture dishes in complete medium containing 50 ng/ml NGF for 2 wk, at which time nocodazole was added to some of the cultures at a final concentration of 50 μ M. Individual neurites were photographed just before drug treatment and then 2-14 h afterwards.

Radiolabeling

Metabolic radiolabeling of PC12 cell proteins was carried out as previously described (2, 20) in the presence of either carrier-free [32 P]orthophosphate (50-500 μ Ci/ml) or 50-100 μ Ci/ml [35 S]methionine (1,100 Ci/mmol), both from New England Nuclear (Boston, MA). For NGF-treated cultures, the factor was also present during the exposure to isotope. In some experiments, as noted in the text, nocodazole, podophyllotoxin, or taxol were also present during part of the labeling period.

Sample Preparation

Whole Cell Lysates. Total cell proteins of cultures washed with PBS (16) were solubilized immediately in SDS sample buffer (62.5 mM Tris-HCl,

2% SDS, 2.5 mM EGTA, 2.5 mM EDTA, 10% glycerol, 0.001% bromophenol blue, pH 6.8) and placed in a boiling water bath for 5 min before analysis by SDS-PAGE. For two-dimensional isoelectric focusing by SDS-PAGE (2-D IEF \times SDS-PAGE), cells were solubilized in a harvest solution appropriate for loading onto the IEF gels (8.5 M urea, 4.5% NP-40, 5% beta-mercaptoethanol, 0.2% SDS, 250 mM NaCl, 2.4 mM EDTA, 2.4 mM EGTA, 3% ampholytes 5-7, and 0.8% ampholytes 3.5-10).

Extraction Procedures. Preparations enriched in MAPs were generated from cells attached to culture dishes as described by Burstein et al. (7) using modifications of the procedures of Solomon et al. (46). The solubilized proteins in the Triton/glycerol (stabilized) and calcium (destabilized) extracts were precipitated with 9 vol of ice-cold methanol and centrifuged at 40,000 g. The pellets were solubilized either in the harvest solution used for IEF or in PBS containing 1% SDS for immunoprecipitations. The cellular residues remaining on the dish after these extractions were, in some cases, scraped up and solubilized in the IEF harvest solution.

Gel Electrophoresis

Discontinuous slab gel electrophoresis was carried out by the method of Laemmli (27) on 32-cm, 6-12% gradients of polyacrylamide. Molecular mass standards (Bio-Rad Laboratories or Sigma Chemical Co.) were run alongside the samples in each experiment. The gels containing proteins labeled with [32 P]orthophosphate were fixed, (50% ethanol, 10% acetic acid), stained (0.2% Coomassie Brilliant Blue in 50% ethanol, 10% acetic acid), and destained (10% ethanol, 10% acetic acid), after which they were dried and placed in contact with Kodak XAR-5 x-ray film to produce an autoradiographic image.

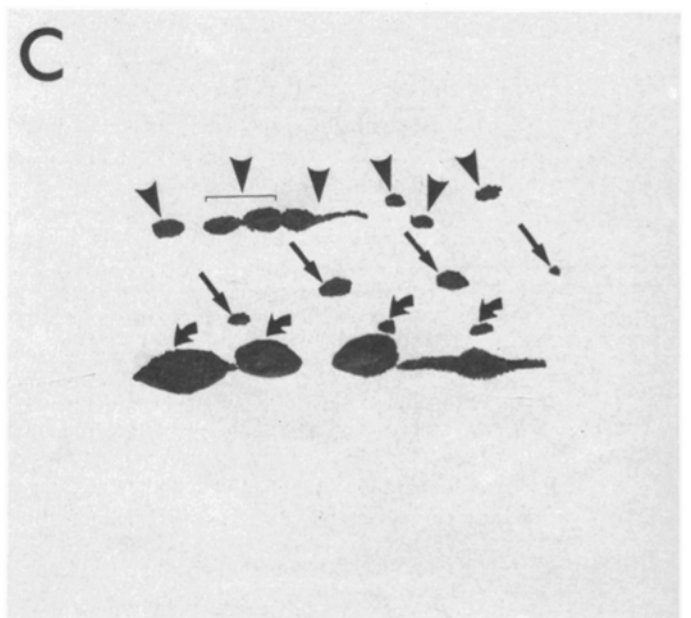
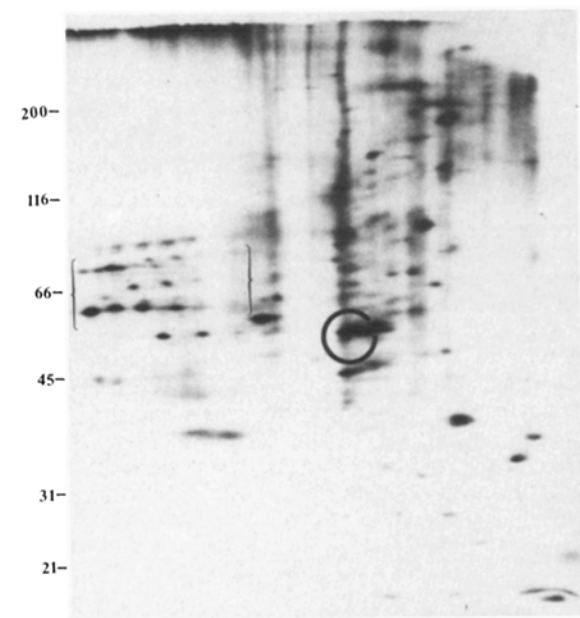
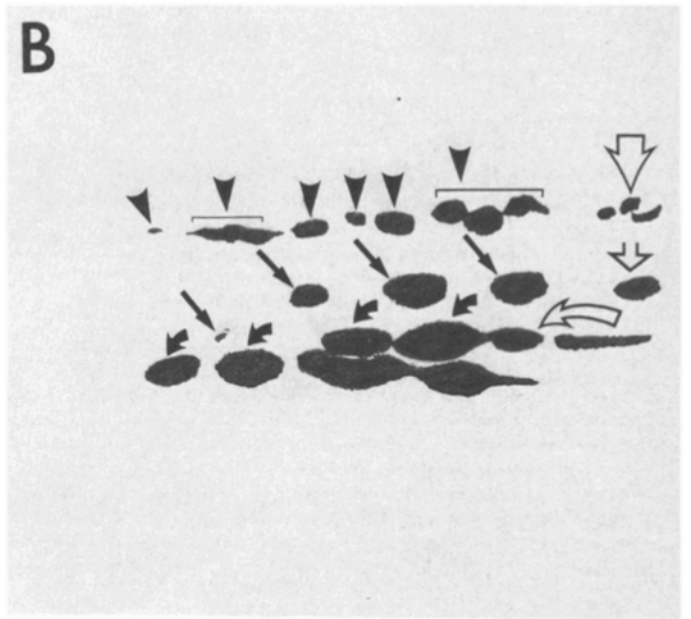
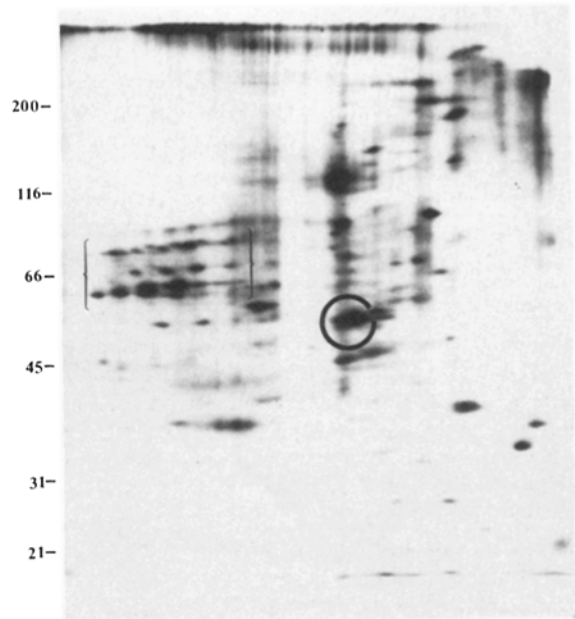
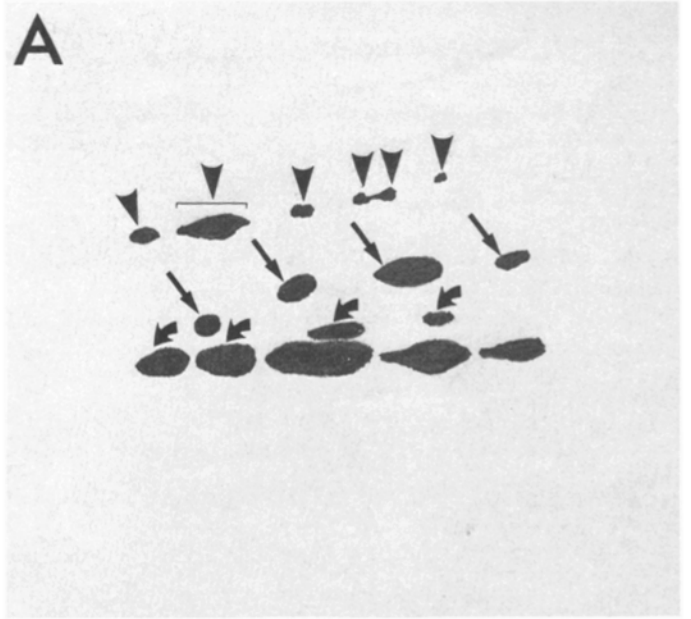
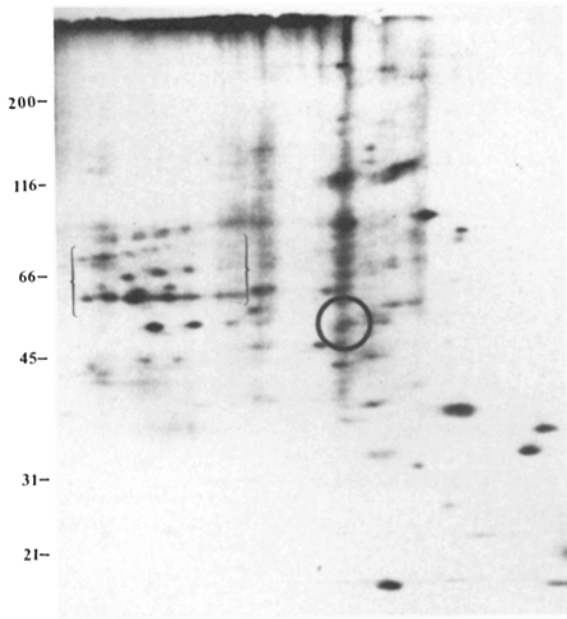
Two-dimensional electrophoresis of protein samples was performed according to the method of O'Farrell (38), with the following modifications. IEF gels for the first dimension (10 M urea, 4.1% acrylamide, 2.2% NP-40, 3.0% ampholytes 5-7, 0.8% ampholytes 3.5-10) were pre-run at constant voltage for 600 Vh (15 min at 200 V, 30 min at 300 V, and 60 min at 400 V) after which samples were loaded and subjected to a constant voltage of 500 V for 17 h. Separation of proteins in the second dimension was on 16-cm slab gels containing 7.5-12% gradients of polyacrylamide. Molecular mass standards, similarly embedded in 5-mm sections of tube gels, were run along with each experimental IEF gel sample. Gels were fixed, stained, and destained as described above, and those labeled with 32 P were dried and placed in contact with Kodak XAR-5 x-ray film and a Cronex Lightning Plus enhancing screen (DuPont Co., Wilmington, DE). Exposures of 5 d-3 wk were used to produce autoradiograms. Gels containing 35 S-labeled proteins were immersed in 1 M salicylic acid after removing fixative by three 20-min washes in deionized water (8). After drying, the gels they were exposed to preflashed XAR-5 x-ray film (28) and kept at -70°C (6) for 1 d-3 wk.

Immunoprecipitations

Whole cell cultures or MAP fractions prepared as above from 32 P-phosphate labeled PC12 cells were solubilized in sample buffer containing 1% SDS. These were incubated at 4° overnight with a chartin antiserum (1:50) (34) provided by Dr. Frank Solomon (Dept. of Biology, Massachusetts Institute of Technology, Cambridge, MA), a MAP 1 antiserum (1:200) provided by Dr. Nicholas Cowan (Dept. of Biochemistry, New York University Medical Center, New York), or an antibody to β -tubulin (1:300, Amersham Corp., Arlington Heights, IL). The immunoreaction was carried out in Tris-buffered (pH 8.0) saline containing 0.1% SDS, 1% Triton X-100, 25 mM NaF, 5 mM EGTA, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 100 kallikrein inhibition units/ml aprotinin followed by precipitation of antigen-antibody complexes with protein A-Sepharose beads. Proteins were then solubilized in sample buffer and separated either on a one-dimensional slab gel or by 2-D IEF \times SDS-PAGE as described above.

Electron Microscopy

Cultures were rinsed three times with warm PBS followed by fixation (2.5% glutaraldehyde in PBS, pH 7.4, for 1 h), osmication (1% osmium for 20 min), and dehydration. After infiltration and embedding with fresh Epon 812, silver-light gold sections were cut on an ultramicrotome and stained with lead citrate and uranyl acetate for observation with a JEOL S100 transmission electron microscope at 80 kV. In most cases, each experimental treatment was replicated in a second independent trial.



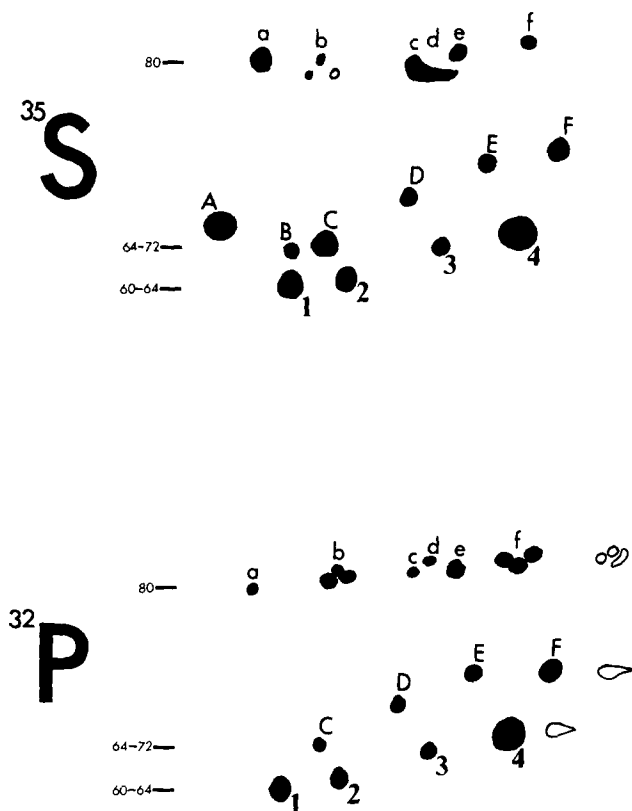


Figure 3. Metabolic radiolabeling of microtubule-associated chartins from NGF-treated PC12 cells using [³⁵S]methionine (72 h incubation) or [³²P]orthophosphate (1.5–2 h incubation) reveal similar, but not identical patterns. Microtubule fractions were prepared from the labeled cells and resolved by 2-D IEF×SDS-PAGE. Tracings of the chartin MAPs present in the resulting fluorograms and autoradiograms are enlarged here for easier comparison. The members of the 60–64-, 64–72-, and 80-kD chartin families are denoted by numbers, upper case letters, and lower case letters, respectively. The patterns are very similar except for isoforms A and B, which are not detectably phosphorylated. Isoform B is present at very low levels and does not appear in photographic reproduction. The open spots in the ³²P-labeled material were not detectable in non-NGF-treated PC12 cells nor in the ³⁵S-labeled material. There is also one open spot within the 80-kD string of the ³⁵S pattern that does not appear to be a MAP since it is still present in abundance after depolymerizing treatments.

Results

Detection and Identification of the Chartin MAPs

Detection and identification of the three chartin families in NGF-treated PC12 cells and of their characteristic associa-

tion with microtubules was achieved using the principles described by Solomon et al. (46) for preparing Ca⁺⁺-extracts enriched in MAPs (see Materials and Methods for details). 2-D IEF×SDS-PAGE separation of the proteins in such extracts of long-term NGF-treated cultures labeled for 72 h with [³⁵S]methionine is shown in Fig. 1. The chartin MAPs are present as three characteristic strings of protein spots in the molecular mass ranges of 60–64, 64–72, and 80 kD and in the pI range between 6.3 and 6.8 (Fig. 1; see also references 2, 4, 39, 52). Peptide mapping studies have previously demonstrated that each of these series of protein spots constitutes a distinct family (2, 39). As expected of proteins associated with polymerized microtubules, all three series of proteins are substantially diminished or absent from MAP fractions of cells treated with microtubule-depolymerizing agents before extraction (Fig. 1 B). As noted below, further verification of the identity of the 60–64-kD proteins as chartins was obtained on the basis of immunological cross-reactivity.

To facilitate resolution of the three families in whole cell extracts (cf. Figs. 2, 6, and 9), our gel system has been somewhat modified from those previously used to detect the chartins (2, 39). This has allowed us to study the relative abundance of chartin family member spots in whole cells after experimental manipulations and to minimize the uncertainties associated with possible translocations among different cellular compartments and/or generation of artifactual polypeptides during extraction procedures.

Past studies have revealed that the chartin MAPs are phosphorylated (2, 7, 39, 52). After [³²P]orthophosphate labeling of PC12 cells, most of the chartin MAP isoforms in whole cell extracts can be detected in autoradiograms generated from 2-D IEF×SDS-PAGE. Long-term treatment of the cells with NGF results in an increased intensity as well as an increased number of polypeptide spots in the acidic region of each family of chartin MAPs (Fig. 2; see also reference 2). These NGF-inducible changes appear to be generated post-translationally, at least in part, by progressive phosphorylation reactions that convert the substrate proteins to more negatively charged isoforms (2, 39). Several of these acidic forms were not detectable in the non-NGF-treated cells (open arrows in Fig. 2 B).

Fig. 3 compares the 2-D IEF×SDS-PAGE profiles of chartins present in the MAP fraction of NGF-treated PC12 cells labeled either with [³²P]orthophosphate (2 h) or [³⁵S]methionine (3 d) (see also reference 2). For the most part, the patterns are similar, with the notable exceptions being the proteins designated A and B of the 64–72-kD family which are not detected by phosphate labeling. The absence of phosphate label in these and several other chartin proteins has

Figure 2. Effects of nocodazole on the incorporation of [³²P]orthophosphate into chartins as detected by 2-D IEF×SDS-PAGE of whole PC12 cell extracts. Non-NGF treated PC12 cells (A), NGF-treated (>2 wk) PC12 cells (B), and NGF-treated (>2 wk) PC12 cells incubated with 50 μM nocodazole for 13 h (C) were labeled with [³²P]orthophosphate for 2 h. In the latter case, nocodazole was also present during the time of labeling. Whole cell extracts were prepared and equal numbers of TCA-precipitable cpm (250,000) were loaded onto the gels. The autoradiograms shown (left) were obtained after a 5-d exposure in the presence of an enhancing screen. The chartin proteins are found within the area demarcated by brackets. The spots within these areas were traced and are shown enlarged approximately four and a half times (right). The curved arrows mark the members of the 60–64-kD chartin family; the straight arrows, the 64–72-kD family; and the arrowheads, the 80-kD family. Open symbols point out phosphorylated chartins not detectable in non-NGF-treated and drug-treated cells. Phosphorylated β-tubulin, identified by its comigration with the Coomassie-stained tubulin visible on the dried gel, is circled in the autoradiograms. Molecular mass markers (kD) appear along the left side. The basic end of each gel is to the left.

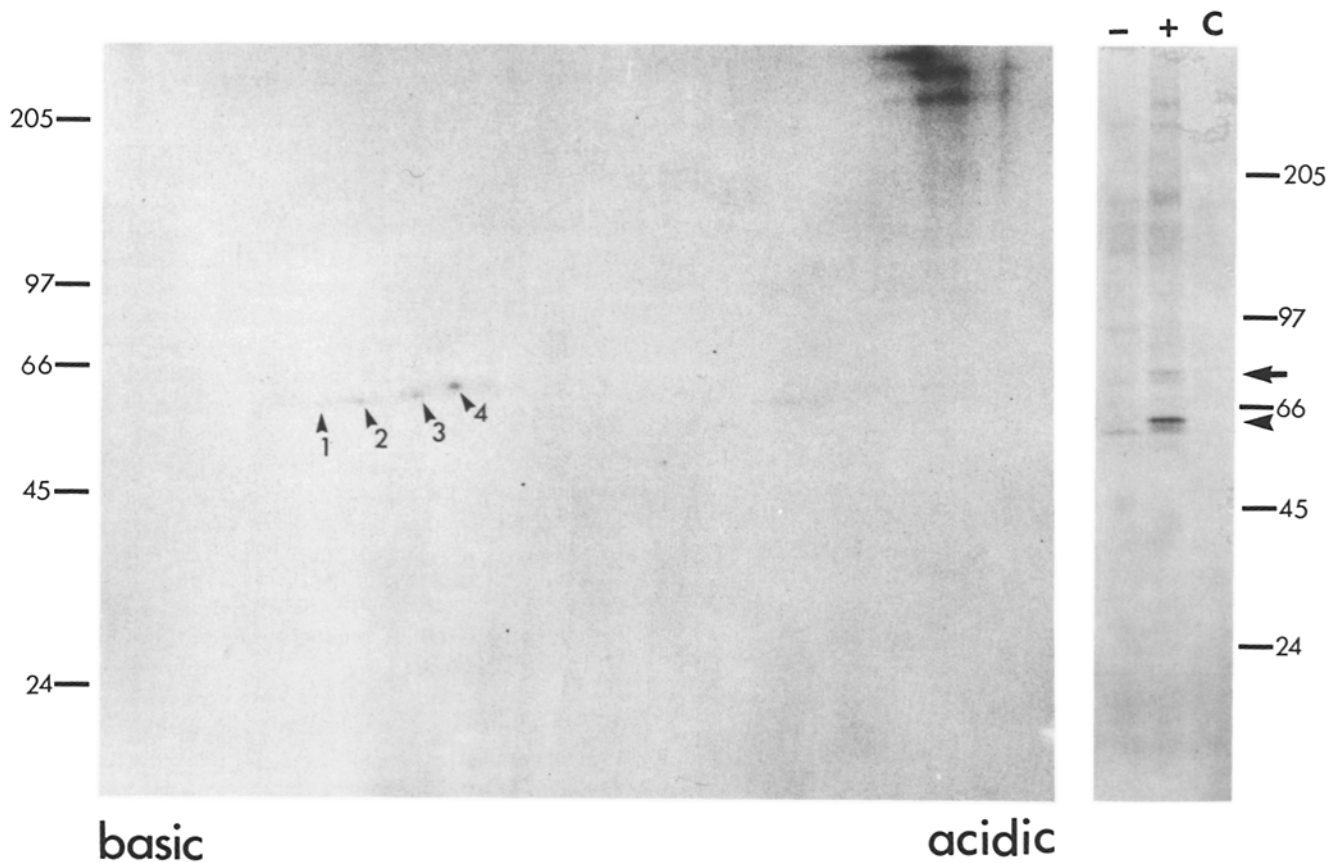


Figure 4. Immunoprecipitation of phosphorylated chartins. A MAP-fraction from long-term NGF-treated PC12 cells, metabolically labeled with [32 P]orthophosphate, was incubated with an anti-chartin antiserum provided by Dr. Frank Solomon (34). After immunoprecipitation (see Materials and Methods) from 385,000 cpm (two-dimensional) or 275,000 cpm (1-D), antigen-antibody complexes were solubilized in sample buffer and subjected to either 2-D IEF \times SDS-PAGE or 1-D SDS-PAGE. The resultant autoradiograms shown here were produced by exposing the dried gels to film either for 3 wk (2-D) or 2 wk (1-D) with enhancing screens. By overlaying with a two-dimensional autoradiogram generated from the same MAP fraction before immunoprecipitation, the four spots (arrowheads) were found to line up precisely with spots 1-4 of the 60-64-kD chartin family (cf. Fig. 3). By immunoprecipitation followed with 1-D analysis, the 64-kD (arrowhead) and 80-kD (arrow) families were present in the MAP fraction from NGF-treated (+), but not from untreated (-) PC12 cells. Lane C is a control incubation of the (+) material without the chartin antiserum.

been noted before (2, 39). The patterns in the MAP fractions are also similar to those seen in whole cell extracts (cf. Figs. 2 B and 3, bottom, and 6 B and 3, top; see also reference 2).

To further confirm the identity of the proteins in our study as chartins, cells were metabolically radiolabeled, MAP fractions thereof were subjected to immunoprecipitation with affinity-purified antibodies (from antiserum 4-81) prepared against brain chartins (34), and the starting and immunoprecipitated proteins were analyzed by 2-D IEF \times SDS-PAGE and autoradiography or fluorography. Superimposition of the autoradiograms or fluorograms revealed that each of the members of the 60-64-kD family (corresponding to Nos. 1-4 in Fig. 3) labeled with either [32 P]orthophosphate (Fig. 4) or [35 S]methionine (not shown) was specifically recognized by the antiserum. Several acidic members of the 80-kD family were also detectable after long-term exposure of fluorograms of 35 S-labeled material (data not shown), but these proteins were more efficiently detected as a single band after 32 P labeling and separation on a one-dimensional gel (Fig. 4). Attempts to immunoprecipitate detectable amounts of chartins from whole cell lysates were not successful.

Nocodazole Treatment Markedly Alters the Phosphorylation Pattern of the Chartin MAPs

Since it has been reported that there is a selective association of the more highly phosphorylated chartins with microtubules (39), we were interested in establishing the relationship between the phosphorylation state of these proteins and the assembly state of the microtubular cytoskeleton. As a means toward this end, we assessed the effects of drug-induced depolymerization of microtubules on the phosphorylation of chartins in whole PC12 cells.

Fig. 2 compares the phosphoprotein patterns in extracts of whole PC12 cells, either without NGF-treatment, with NGF treatment for several weeks, or with NGF treatment for several weeks and exposure to 50 μ M nocodazole for 15 h. Labeling with 32 P was carried out during the 2 h immediately before generation of whole cell lysates. Under the latter conditions of nocodazole treatment there is a striking loss of microtubules from the cells (see Fig. 8) and a concomitant substantial reduction of tubulin and chartins in the microtubule fraction (cf. Figs. 1, 2, and reference 7). As shown in Fig. 2, nocodazole pretreatment significantly affects the pat-

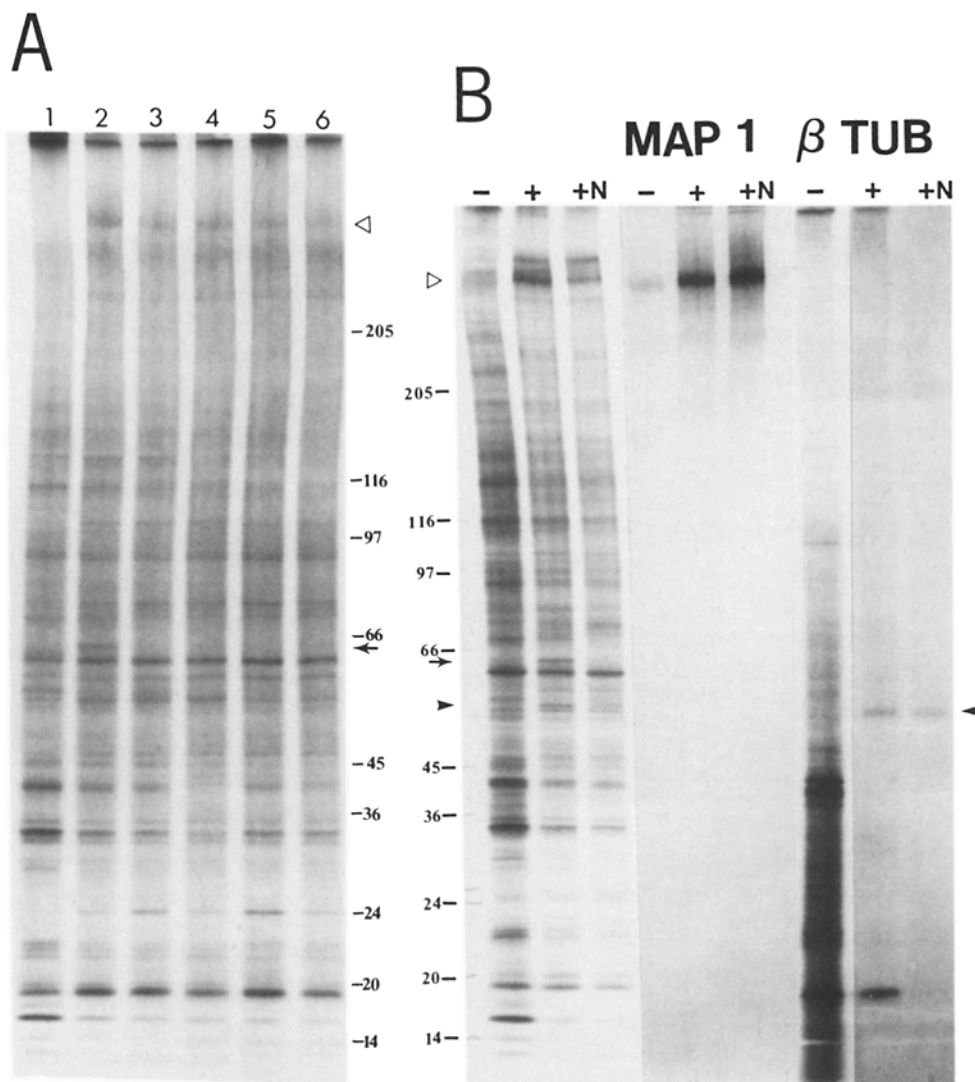


Figure 5. Comparison of the effects of nocodazole and podophyllotoxin on phosphorylation of MAP 1.2 and the 64-kD chartin MAP. (A) PC12 cells were labeled for 2 h with [32 P]orthophosphate, solubilized in sample buffer and subjected to SDS-PAGE (6–12% acrylamide gradient). Equal amounts (100,000 cpm) of TCA-precipitable radioactivity were loaded into each lane. The autoradiograph was obtained by overnight exposure of the dried gel to x-ray film. The lanes contain material from: (lane 1) PC12 cells never exposed to NGF; (lane 2) NGF-treated (>2 wk NGF) cells; (lane 3) NGF-treated cells + 50 μ M nocodazole for 20 h; (lane 4) NGF-treated cells + 50 μ M nocodazole for 5 h; (lane 5) NGF-treated cells + 50 μ M podophyllotoxin for 20 h; (lane 6) NGF-treated cells + 50 μ M podophyllotoxin for 5 h. For cultures treated with nocodazole or podophyllotoxin, the drug was present during exposure to labeled phosphate. The arrow points out the migration distance of the 64-kD MAP and the triangle, that of MAP 1.2. The positions of molecular mass standards are indicated at the right (as $M_r \times 10^{-3}$). (B) Immunoprecipitations (as described in Materials and Methods) of 32 P-labeled MAP 1 or β -tubulin from whole cell lysates of untreated (–), long-term NGF-treated (+), or long-term NGF-treated and nocodazole-treated

(+N) PC12 cells. The first three lanes show the total phosphoprotein patterns from which the subsequent immunoprecipitations were performed (50,000 cpm/lane; nocodazole, when present, for 24 h). The next three lanes are from MAP 1 immunoprecipitations from equal amounts of radioactive material (600,000 cpm; nocodazole, 24 h), and the last three lanes from β -tubulin immunoprecipitations (1.5×10^6 cpm; nocodazole, 2 h).

tern of phosphate incorporation into the chartins. First, the intensity of labeling and the number of acidic variants in each chartin family are reduced. Second, the more basic members of each string of proteins are increased in labeling intensity (Fig. 2 C). Similar qualitative changes are obtained with 2–24 h of nocodazole treatment. Thus, the phosphorylation of the more acidic isoforms within each chartin family is dependent upon the presence of native, polymerized tubulin in the cells.

The amount of β -tubulin which is phosphorylated in long-term NGF-treated PC12 cells is also greatly increased in comparison with that found in PC12 cells cultured without NGF (Figs. 2 and 5 B; see also references 2 and 7). Identification of phosphorylated β -tubulin in our system is based on (a) its electrophoretic co-migration with authentic β -tubulin, (b) selective enrichment in microtubule fractions, (c) loss from these fractions after exposure of cells to depolymerizing agents, and (d) recognition by anti- β -tubulin anti-

body (Fig. 5 B). In comparison with the observed effects on the chartins, however, treatment with nocodazole does not interfere with β -tubulin phosphorylation to the same extent (cf. Figs. 2 and 5 B). In several different experiments, phosphotubulin was somewhat diminished, but still easily detectable under the same conditions that drastically altered chartin phosphorylation.

Comparison with Effects of Depolymerizing Agents on MAP 1 Phosphorylation

We have previously resolved one of the phosphorylated chartin MAPs (apparent M_r 64,000) present in whole cell lysates on one-dimensional SDS polyacrylamide gradient gels (2, 7) along with another phosphorylated MAP of high molecular mass, designated MAP 1.2 (20). The identity of the M_r 64,000 species as a chartin was confirmed by its immunoprecipitation (Fig. 4) with an anti-chartin antiserum (34).

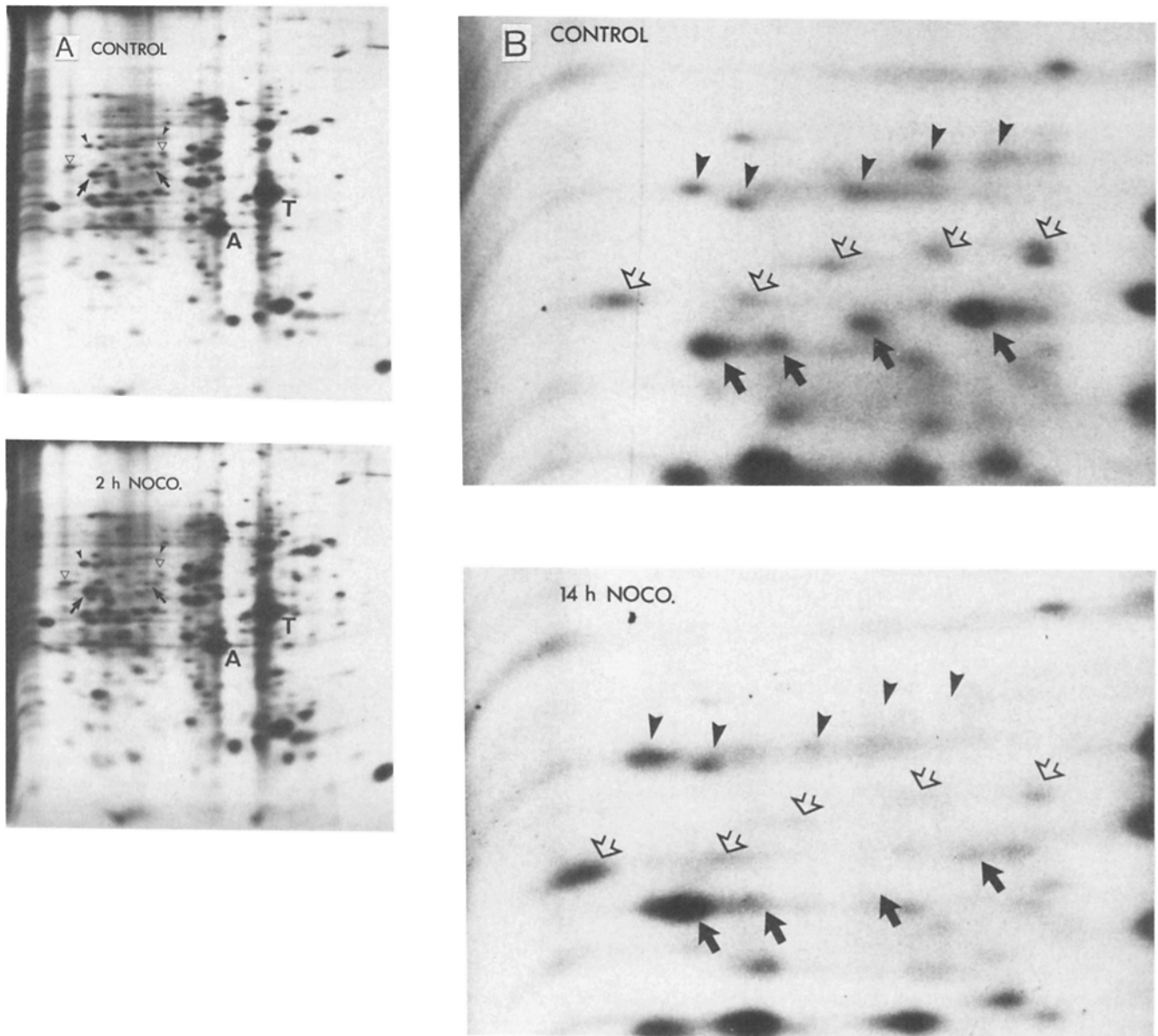


Figure 6. Microtubule depolymerization produced by exposure of NGF-treated PC12 cells to 50 μM nocodazole for 2 h (A) or 14 h (B) causes a decrease in steady-state levels of total cellular acidic chartins and a reciprocal increase in the respective basic isoforms. Cells were solubilized in IEF harvest solution after 72 h of radiolabeling in medium containing 100 $\mu\text{Ci/ml}$ of [^{35}S]methionine. Nocodazole was added for the times indicated before solubilization. Proteins were separated on 7.5–12% acrylamide gradient gels. Arrowheads demarcate the 80-kD family of chartins from the most basic to the most acidic isoforms (left to right). Open symbols signify the 72-kD family, and arrows, the 64-kD family. A displays the entire fluorogram from the shorter time course experiment, which was replicated in a second independent experiment. The results in B, depicting only the area of the gel containing the chartins, were also reproduced in three similar experiments with nocodazole present for 15, 20, or 24 h. A, actin; T, tubulin.

Likewise, a previous study (20) has demonstrated that the high molecular mass species is recognized by anti-MAP 1 antiserum. In the present study, we have used an antibody generated against the fusion protein from a MAP 1 clone (31), kindly provided by Dr. Nicholas Cowan, to corroborate this finding (Fig. 5 B). Long-term NGF treatment results in a large increase in the levels of both of these species (2, 7, 20). Using one-dimensional gels, we asked whether drug-induced depolymerization of microtubules would affect the phosphorylation of both classes of MAPs equally and whether it would affect that of other cellular proteins. ^{32}P -labeled protein extracts of whole cells pretreated with

50 μM podophyllotoxin or 50 μM nocodazole for 2–20 h before labeling were resolved on SDS polyacrylamide gradient gels. After both short (2 h) and longer (20 h) drug treatments, phosphorylation of the 64-kD chartin MAP was suppressed, while phosphorylation of MAP 1.2 was unaltered or only slightly affected. Fig. 5 A is typical of the results obtained in five independent experiments of this type. Overnight drug treatment also often gave rise to increased phosphorylation of a 24-kD protein, the significance of which is unclear.

Despite the effects of podophyllotoxin and nocodazole on chartin MAP phosphorylation, these drugs do not produce

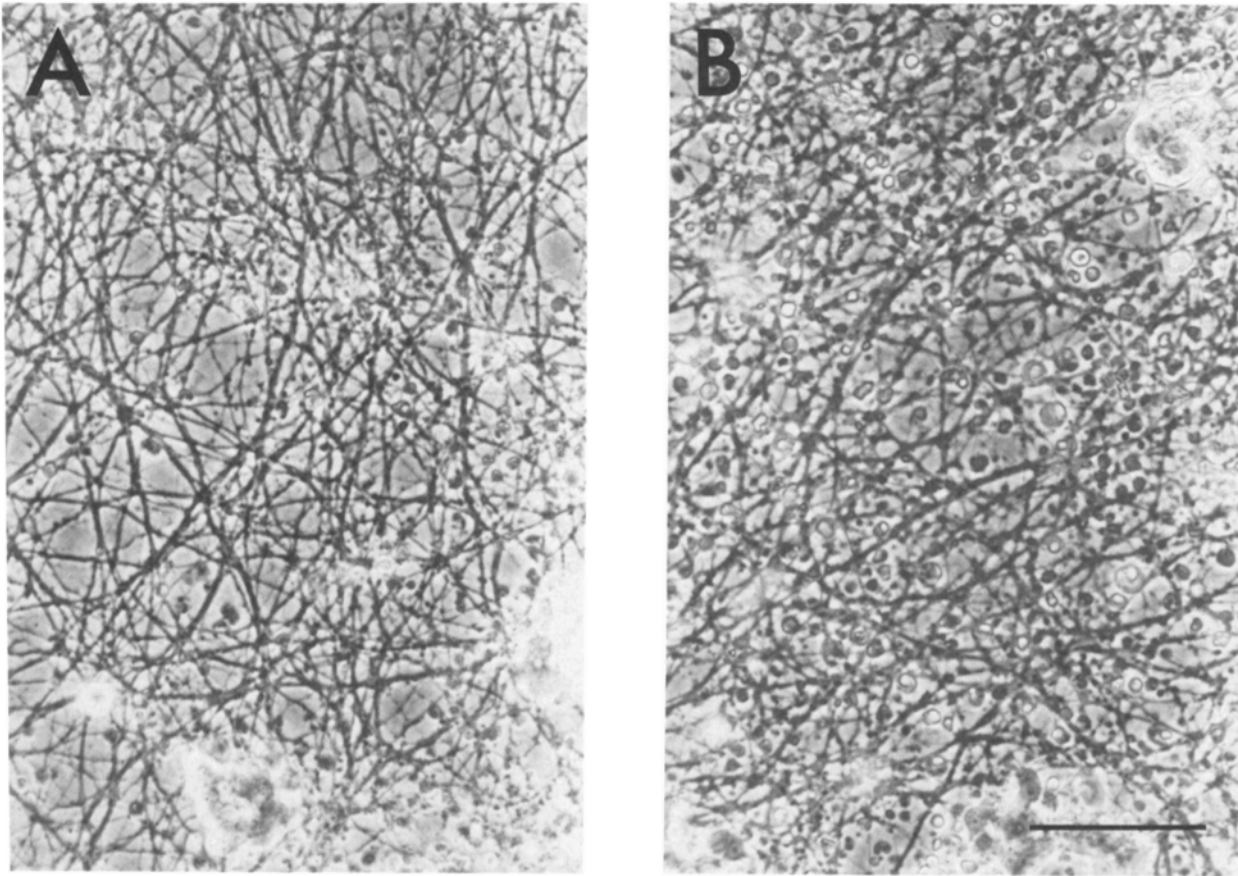


Figure 7. PC12 cell neurites grown in the presence of NGF for 4 wk. No drug treatment (A) or 50 μ M nocodazole for the final 20 h (B). Bar, 100 μ M.

generalized effects on other cellular phosphoproteins. Cultures equally plated with cells and treated with or without drugs exhibited similar incorporation of [32 P]orthophosphate into TCA-precipitable protein as determined by liquid scintillation spectrometry (<1% difference, $n = 5$). Furthermore, with the exceptions of the chartins and occasionally the 24-kD band, as shown in Figs. 2 and 5, nocodazole and podophyllotoxin have little apparent effect on phosphate incorporation into most cellular phosphoproteins.

Steady-State Levels of Chartin MAPs Are Altered by Treatment with Microtubule-depolymerizing Agents

To determine the effect of microtubule depolymerization upon the steady-state levels of the chartin MAPs, we labeled NGF-treated cultures with [35 S]methionine for 3 d. In some cultures, microtubule depolymerizing drugs were added during the last 2–20 h of incubation. The characteristic pattern of chartins could be observed in 2-D IEF \times SDS-PAGE gels of whole cell extracts prepared from control, untreated cultures (Fig. 6, A and B, top). As with [32 P]orthophosphate labeling (Fig. 2), there was a clear shift in the distribution of chartin isoforms in response to microtubule depolymerization. Fig. 6 A demonstrates that even after only 2 h of nocodazole treatment, the quantities of the acidic members of the three families of chartin proteins are reduced, with concomitant increases in levels of the more basic family members. Fig. 6 B shows a more pronounced effect after 14 h

of drug treatment. Comparable findings were obtained with podophyllotoxin. These observations appear to reflect the effects of depolymerization on chartin phosphorylation and show that this response occurs for the entire cellular content of chartin proteins.

Neurites Can Remain Extended Even after Drug-induced Depolymerization of Most Microtubules

To assess the effects of anti-microtubule treatments at the morphological level and to determine the effects of microtubule loss on neurite maintenance and growth, drug-treated cultures were examined by both light and electron microscopy. In long-term NGF-treated cultures (>2 wk) the presence of depolymerizing drugs for up to 24 h did not produce widespread retraction or dissolution of neurites (Fig. 7 B). Beyond this time the drugs appeared to elicit toxic responses, as evidenced by progressive deterioration of both cell bodies and neurites.

Ultrastructural analysis of the neurites by transmission electron microscopy indicated that after 2 h of drug treatment, microtubules were no longer oriented in parallel bundles, but instead appeared as disjointed, wavy tubular segments (data not shown). Overnight drug treatment resulted in further losses of microtubules so that only a few of the aberrant type of tubules remained (Fig. 8). In addition to the loss of microtubules, we noticed the presence of filamentous structures not typically observed in control material (arrow-

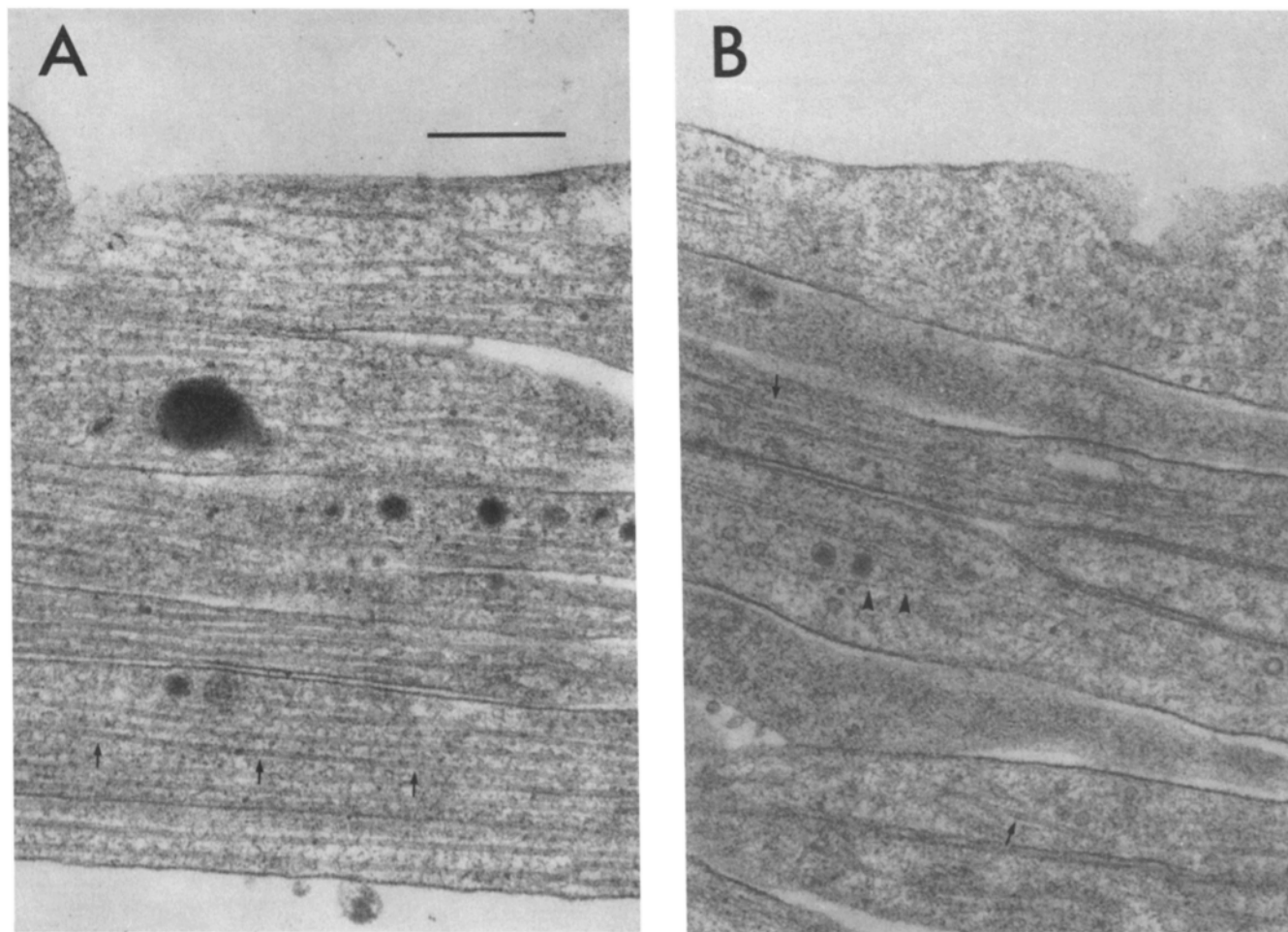


Figure 8. Electron micrographs of PC12 cell neurite bundles generated by 4-wk exposure to NGF and treatment for 18 h with either 50 μ M nocodazole (*B*) or drug carrier alone (*A*). Microtubules (*arrows*) run for long distances in the longitudinally sectioned control material and fill much of the cytoplasm, but they are greatly diminished in number and extent in the drug-treated neurites. Filamentous material (*arrowheads*) was never observed in the control neurites (see also references 29 and 33) but was often encountered in favorably sectioned material from the drug-treated neurites. Bar, 500 nm.

heads in Fig. 8 *B*). These findings confirm that nocodazole causes depolymerization of PC12 microtubules and indicate that long-term NGF-treated cultures can maintain neurites for at least 1 d in the absence of almost all microtubules.

We also examined the effect of nocodazole on the elongation of neurites. This was carried out in low density cultures so that single, nonfasciculated processes could be observed. Measurements from photographs made of the same individual neurites immediately before and 14 h after adding 50 μ M nocodazole to cultures demonstrate that elongation does not occur in the presence of the drug (Table I). Thus, loss of microtubules rapidly affects the elongation, but not maintenance, of neurites.

Taxol Does Not Drive Phosphorylation of Chartin MAPs

Since microtubule depolymerization results in chartin dephosphorylation, we wished to know, conversely, whether conditions favoring microtubule assembly or stability would enhance chartin phosphorylation. Taxol is a plant alkaloid that has been used experimentally to promote microtubule

assembly *in vitro* as well as to protect assembled microtubules from depolymerizing treatments (14, 42, 43). Recently taxol was used to demonstrate that phosphorylation of β -tubulin is polymerization dependent in murine neuroblastoma cells (17). We therefore tested whether this drug would also drive increased phosphorylation of the chartin MAPs.

Table I. Effect of Nocodazole on the Elongation of PC12 Neurites

Treatment	Length change (\pm SEM)	<i>n</i>
	μ m	
Control	+60 \pm 9	14
Nocodazole (50 μ M)	-2 \pm 5	8

Sparsely plated cultures of NGF-treated cells were prepared as described in Materials and Methods. Selected areas containing single, unfasciculated neurites were photographed through phase-contrast optics immediately before addition of nocodazole (50 μ M final concentration) or of an equal volume of the dimethyl sulfoxide solvent (control). These areas were marked on the bottoms of the culture dishes with different colors of ink and a second set of photographs was taken of the same neurites 14 h later. The lengths of neurites before and after treatment were determined from photographic prints of each field.

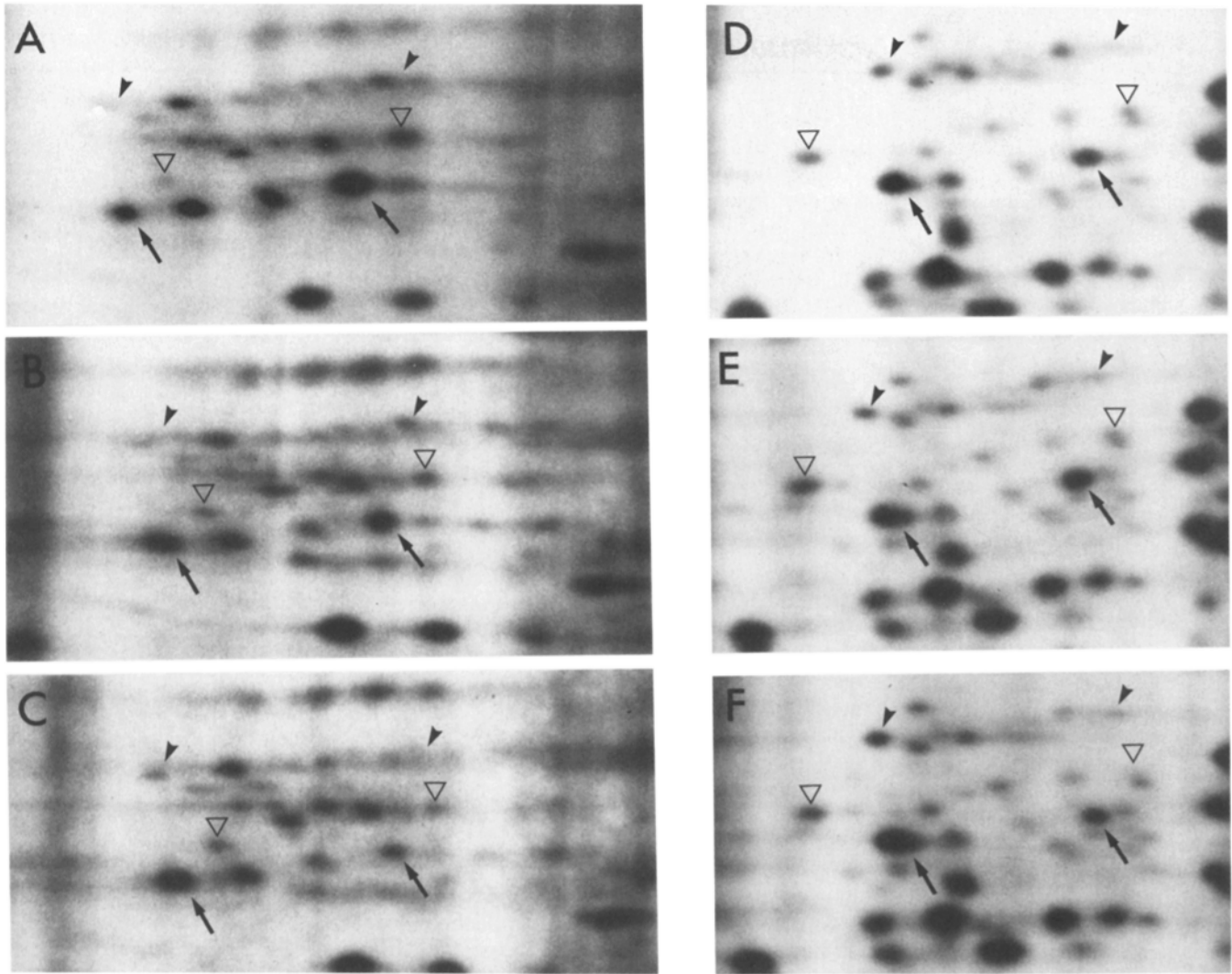


Figure 9 Effects of taxol on the phosphorylation and steady-state levels of the cellular content of chartins. PC12 cells were grown in the presence of NGF for >3 wk along with [³²P]orthophosphate for the final 2 h (A, B, and C) or [³⁵S]methionine for the final 72 h (D, E, and F). The cultures were also either untreated (A and D) or treated with 2 μM taxol for either the final 2.5 (B and E), 24 (C), or 21.5 (F) h of culture. Total cell proteins were then solubilized and resolved by 2-D IEF x SDS-PAGE. The area on the gel containing the chartin MAPs is enlarged. Basic to acidic pIs run from left to right. Each chartin MAP family lies between their respective symbol pairs: (arrowheads) 80 kD; (triangles) 64–72 kD; (arrows) 60–64 kD. Note that the most basic member of the ³⁵S-labeled 72-kD family is not phosphorylated (see Fig. 5). Each radiolabeling experiment submitted to 2-D analysis was replicated in an independent trial and the effect on the ³²P-labeled 64-kD family was verified in two additional experiments with SDS-PAGE.

Long-term NGF-treated (>3 wk) PC12 cells were exposed to taxol for 0.5 h–1 d and then to [³²P]orthophosphate for an additional 2 h in the presence of taxol. Total cell lysates were analyzed for phosphorylated chartins by 2-D IEF x SDS-PAGE. As illustrated in Fig. 9, A–C, it was clear that phosphorylation of the chartin isoforms had not been driven further by taxol. In fact, there was an apparent inhibition of the labeling of several acidic chartin MAPs and an apparent increase in the levels of the more basally charged phosphorylated chartins (Fig. 9, A–C).

Parallel experiments (Fig. 9, D–F) were carried out to assess the effects of taxol on the total cellular composition of chartins. Cultures (>3 wk of NGF treatment) were exposed to [³⁵S]methionine for 3 d. Taxol was added either during the last day or last 2 h of labeling. In consonance with the results achieved with phosphate labeling, taxol did not in-

crease the overall cellular levels of the more acidic chartin isoforms. However, while taxol led to a rise in the relative levels of the more basic isoforms, the taxol-dependent decrease in relative levels of the acidic isoforms shown by phosphate labeling was much less evident with long-term methionine labeling. These observations suggest that taxol may not drive either the phosphorylation or dephosphorylation of the acidic chartin isoforms, but rather that it may interfere with the turnover of phosphate in these MAPs.

To further probe the effects of taxol on the chartin MAPs, [³⁵S]methionine-labeled cultures (with >3 wk of NGF exposure) were treated with this agent for 2 h and then sequentially extracted with a microtubular stabilizing buffer containing 0.1% Triton X-100 and 2 M glycerol, and then with a destabilizing buffer containing 3 mM Ca⁺⁺. The taxol-stabilized material left bound to the culture dish was then

solubilized and analyzed by 2-D IEF×SDS-PAGE. Such experiments revealed that the most acidic forms of the chartin MAPs were highly enriched in the taxol-stabilized, Ca⁺⁺-resistant cytoskeletal fraction and that only trace amounts of these appeared in the Triton and Ca⁺⁺ extractions (data not shown). These observations suggest that the acidic, but not more basic, chartin isoforms can strongly associate with taxol-stabilized tubules. This is consistent with the findings of Pallas and Solomon (39), who reported an enrichment of the more highly phosphorylated chartin isoforms in the microtubule fraction of cultured murine neuroblastoma cells and PC12 cells never exposed to NGF.

Discussion

Dependence of Chartin Phosphorylation on the Presence of Tubules

This study has demonstrated that the state of phosphorylation of three MAP families in process-bearing PC12 cells is dependent on the presence of microtubules. We have distinguished these MAPs from tau proteins and have identified them as chartins based upon (a) their characteristic migration pattern on 2-D IEF×SDS-PAGE (2, 4, 39, 52) and (b) their precipitation upon boiling. In addition, the 64- and 80-kD forms were specifically immunoprecipitated with an anti-chartin antiserum (34). Drug-induced depolymerization of microtubules led to a decrease in incorporation of phosphate into chartins, to depletion of the cellular complement of acidic highly phosphorylated chartin isoforms, and to an increase in the cellular content of more basic, less phosphorylated chartin isoforms.

Dependence of the state of cellular chartin phosphorylation on microtubules could arise from one or more alternative mechanisms. One is that phosphorylation takes place on intact microtubules. In this case, chartins associated with tubules may be exposed to a tubule- or cytoskeletal-associated kinase or, alternatively, could be better substrates for a soluble kinase. A second possibility is that chartin phosphorylation occurs entirely in the soluble phase and is effected by a tubule-activated or -dependent kinase. A third possibility is that the cellular state of chartin phosphorylation is regulated by phosphoprotein phosphatases. For instance, chartins could be protected from such enzymes while associated with tubules, and, upon tubule depolymerization, become vulnerable to dephosphorylation. The present experiments do not permit us to distinguish among such alternatives.

Drug-induced depolymerization of microtubules in living cells was used to assess phosphorylation of chartins present in whole cell lysates. One possible outcome was that each isoform within the three families would simply be released from assembled tubules with merely a change in intracellular location and with no change in either the number of isoforms or in the extent of each one's phosphorylation. Alternatively, de novo phosphorylation of only those isoforms enriched in assembled tubules (39) might have been particularly affected and the cytoplasmic isoforms left unaffected. In fact, we found that both the ongoing phosphorylation and steady-state levels of all of the isoforms were altered. After depolymerization, not only were the more acidic chartin isoforms diminished, but the more basic isoforms in each family were

proportionally increased. This situation after depolymerization more closely resembles the phosphorylation pattern obtained in non-NGF-treated PC12 cells and suggests that chartin isoforms are derived by sequential phosphorylation or dephosphorylation of other family members.

While the main means of modification of chartins appears to be phosphorylation, the observation that two of the isoforms (A and B in Fig. 3) within the more basic region of the 64–72-kD family are not appreciably labeled with [³²P]-orthophosphate invites the possibility that additional types of modification may occur. Also, although microtubule depolymerization caused a diminution in levels of the more highly phosphorylated chartin MAP isoforms, incorporation of phosphate into the more basic isoforms was not blocked. This suggests that separate mechanisms (i.e., different kinases or phosphatases) may be responsible for regulating the phosphorylation of the more basic, soluble isoforms versus the more acidic, tubule-associated isoforms.

In contrast to their effects on chartins, depolymerizing agents had little effect on the incorporation of phosphate into most cellular phosphoproteins. Thus, there appears to be a highly specific mechanism involved in regulation of chartin phosphorylation by tubules. Of particular interest in this regard is MAP 1.2. NGF treatment causes an increase in the phosphorylation of this microtubule-associated species as well as the chartin MAPs (2, 7, 20). However, depolymerizing agents had only a minor influence on the phosphorylation of MAP 1.2. This indicates that different mechanisms underlie the regulation of NGF-promoted increases in chartin phosphorylations as compared with that of other MAPs.

Effects of Taxol on Phosphate Turnover in Chartins

Since dissolution of microtubules caused loss of chartin phosphorylation, we used the microtubule polymerizing agent taxol to test whether enhancement of microtubule assembly and stability would drive chartin phosphorylation. We have shown here that taxol does not enhance phosphorylation of chartins in long-term NGF-treated PC12 cells. Moreover, there was an inhibition of the metabolic labeling of several of the acidic isoforms with [³²P]orthophosphate. This contrasts with the observation that there was much less effect of taxol on the total cellular pattern of chartins as revealed by a 72-h label with methionine. One model consistent with these data is that acidic chartin isoforms are differentially associated with tubules (39) and that when these isoforms are associated with taxol-stabilized microtubules, they undergo a decreased level of phosphate turnover. An additional observation was that taxol does not inhibit phosphate incorporation into the more basic chartins. This further supports the possibility that different mechanisms are involved in regulating phosphorylation of the basic versus acidic chartin isoforms. Finally, we found that when the taxol-stabilized cytoskeleton was analyzed, this fraction was highly enriched in acidic chartin isoforms. This indicates that the more basic chartin isoforms do not associate with taxol-stabilized tubules and that they cannot be driven to do so in the presence of taxol.

Our observations regarding the effects of taxol on chartins are complemented by recent studies in which sympathetic neuron cultures were labeled with [³⁵S]methionine in the continuous presence of taxol (5). Under these conditions,

chartins were synthesized at the same rate as in control cultures, but did not associate with the microtubular cytoskeleton. In our experiments, in which chartins were, in contrast, pre-labeled with [³⁵S]methionine before addition of taxol, there was only a moderate loss of labeled chartins from the microtubular cytoskeleton even after 24 h of drug treatment. Therefore, if, as implied by the sympathetic neuron experiments (5), taxol interferes with association of cytoplasmic chartins with microtubules, then the present observations indicate that introduction of taxol also blocks the capacity of bound chartins to dissociate from these structures. Additional 2D IEF×SDS-PAGE analyses of sympathetic neuron material (1a) have further revealed that the chartins synthesized in the continuous presence of taxol are not converted to the highly phosphorylated isoforms. Our data, on the other hand, suggested that taxol selectively decreases phosphate turnover in tubule-associated chartins. Thus, for cytoplasmic chartins, taxol appears to impede both interaction with microtubules and tubule-dependent phosphorylation, while for tubule-bound chartin isoforms, this drug blocks both dissociation and phosphate turnover. There are several possible mechanisms that could account for such findings. For instance, by stabilizing the association between tubules and bound chartins, taxol could limit exchange of the latter with cytoplasmic chartins. Alternatively, taxol could affect phosphoprotein phosphatase and/or protein kinase activities, and, thereby, chartin phosphorylation and microtubule interaction.

Neurite Stability

The well-established PC12 neurites in long-term NGF-treated cultures used in this study could be maintained under conditions in which most cellular microtubules were lost. The stability of axons in situ and of neurites in culture under conditions favoring disassembly of microtubules has been reviewed by Daniels (13). A number of factors may contribute to maintaining neuritic processes in the absence of the subcellular support afforded by microtubule bundles. At least in cell culture, increased stability is correlated with the formation of long, mature neurites (23). Joshi et al. (25) observed that relatively low concentrations of depolymerizing drugs caused rapid retraction and disappearance of neurites in PC12 cultures that had been exposed to NGF for only a short period of time and in which the processes, consequently, were not well developed. In contrast, the neurites in the presently described, long-term NGF-treated cultures remained extended even in the presence of 10-fold higher drug concentrations that eliminated nearly all microtubules. One possible source of this change in dependence on microtubules is the appearance of intermediate filaments such as neurofilaments (12, 50). Previous studies (47) have revealed that substantial numbers of intermediate filaments can be detected in PC12 cells by electron microscopy only after several weeks of exposure to NGF. While it is presently unclear whether the filamentous features observed in PC12 neurites after exposure to nocodazole were drug induced or were previously present and undetectable due to the densely packed microtubular array, it is possible that these structures could provide a subcellular support within neurites even after depletion of microtubules.

Microtubules and Neurite Elongation

Although we found that well-developed neurites may be maintained for at least 1 d after depolymerization of their tubules, they do not continue to elongate under such circumstances. The mechanism through which intact microtubules serve to promote growth of neurites is unknown. The microtubule-based regulation of chartin MAP phosphorylation described in the present study, however, provides a basis upon which a model can be proposed. Microtubules supply the requisite environment for the maximal sequential phosphorylation of chartins, which in turn may lead to growth either by promoting the stable addition of new tubulin subunits to the distal tips of tubule bundles or by facilitating interactions of microtubules with other cytoskeletal or membranous elements necessary for generating the formation of stable lengths of neurite behind the advancing growth cone. In support of this hypothesis, lithium ion (7) and activators of adenylate cyclase (19) inhibit chartin phosphorylation and also interfere with neurite growth without disrupting microtubules. Both microtubules and phosphorylated chartins, therefore, appear to be required for elongation. In addition, the NGF-promoted increase in levels of the most highly phosphorylated chartins is temporally correlated with neurite outgrowth (2). Moreover, the acidic chartin isoforms are also highly enriched, relative to other phosphoproteins, in neurites versus cell bodies (1). The presently hypothesized roles of microtubules in regulating chartin phosphorylation and of phosphorylated chartins in promoting microtubule elongation represent testable models for future experimentation concerning the molecular events underlying the NGF-triggered growth of neurites.

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