PC12 Cells Express Juvenile Microtubule-associated Proteins during Nerve Growth Factor–induced Neurite Outgrowth

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Abstract. Microtubule-associated proteins (MAPs) are believed to play an important role in regulating the growth of neuronal processes. The nerve growth factor–induced differentiation of PC12 pheochromocytoma cells is a widely used tissue culture model for studying this mechanism. We have found that contrary to previous suggestions, the major MAPs of adult brain, MAP1 and MAP2, are minor components of PC12 cells. Instead two novel MAPs characteristic of developing brain, MAP3 and MAP5, are present and increase more than 10-fold after nerve growth factor treatment; the timing of these increases coinciding with the bundling of microtubules and neurite outgrowth. Immunocytochemical staining showed that MAP3 and MAP5 are initially distributed throughout the cytoplasm. Subsequently MAP5 becomes associated with microtubules in both neurites and growth cones but MAP3 distribution remained diffuse. Thus MAP3 and MAP5, which are characteristic of developing neurons in the juvenile brain, are also induced in PC12 cells during neurite outgrowth in culture. In contrast MAP1, which is characteristic of mature neurons, does not increase during PC12 cell differentiation. These results provide evidence that one set of MAPs is expressed during neurite outgrowth and a different set during the maintenance of neuronal form. It also appears that the PC12 system is an appropriate model for studying the active neurite growth phase of neuronal differentiation but not for neuronal maturation.

THE assembly of microtubules is an essential step in the growth of neuronal processes (6, 35, 41), and it is therefore important to identify those components of the neuronal cytoplasm that modulate tubulin polymerization. Much recent work has focused on microtubule-associated proteins (MAPs)[1] because they stimulate tubulin polymerization in vitro and are particularly abundant in the brain (5, 8, 23, 31, 39). Some of these proteins are particularly associated with neuronal processes. For example MAP2 is concentrated in dendrites (30) and tau MAPs are concentrated in axons (2). Several of the MAPs are also implicated in neuronal differentiation because large changes in their content in brain coincide with the completion of axon and dendrite growth (1, 9, 27, 33, 34). The sequential induction of different MAPs might thus be correlated with the different steps in neurite initiation, extension, and stabilization (28, 32).

Many recent studies of neurite outgrowth have used the PC12 rat pheochromocytoma cell line (12), which can be induced to form axon-like processes by treatment with nerve growth factor (NGF; 4, 7, 13, 14, 16, 20, 25). Several lines of evidence suggest that MAPs play an important part in the molecular mechanism of neurite outgrowth in PC12 cells (4, 7, 14). Drubin et al. (7) have shown that changes in tubulin levels in PC12 cells exposed to NGF do not parallel the degree of microtubule polymerization activity in the cell. They proposed instead that it is biochemical changes in MAPs, which are induced by NGF, that are primarily responsible for the formation of microtubules and for microtubule bundling, and that therefore MAPs regulate the formation of neurites. Other studies have shown that microtubules in NGF-treated cells are more stable to the tubulin-depolymerizing drugs and low temperatures than are microtubules in cells grown without NGF (3, 21, 22). It has been suggested that MAPs, synthesized in response to NGF, confer this stability.

The exact identity of the MAP components involved in neurite formation has yet to be settled. It is known that the relative amounts of both a high molecular mass MAP species and the tau MAPs in PC12 cells increase dramatically (10–20-fold) after long-term NGF-treatment (14). However, NGF also induces changes in the phosphorylation state of the chartins (4), a recently discovered group of MAPs similar in size to, but chemically distinct from tau MAPs (26), and it is unclear what the relative importance of tau and the chartins are. The situation with respect to the high molecular mass MAPs is even more uncertain. Using a sensitive immunoassay, Black et al. (4) detected increased MAP2 levels

1. *Abbreviations used in this paper*: MAPs, microtubule-associated proteins; NGF, nerve growth factor.

in differentiated compared to undifferentiated PC12 cells. Drubin et al. (7) tentatively identified the high molecular mass NGF-induced MAP of PC12 cells as MAP1 and Greene et al. (14) termed it MAP1.2. However, there are several high molecular mass MAP components that migrate on SDS-polyacrylamide gels above MAP2, in the region of MAP1, and it is not clear which of these was studied in these experiments. The protein in this size range that we describe in relation to PC12 differentiation in this paper is a novel MAP, (MAP5) that is not closely related to MAP1 and that we have characterized in detail elsewhere (34, 36, 37).

The exact identification of NGF-induced MAPs is an essential prerequisite for determining their role in PC12 cell neurite outgrowth. In this study we have used mAbs against MAP1, MAP2, MAP3, and MAP5, which are MAPs that are well-characterized in brain microtubules (1, 17, 18, 29) and have been found to undergo large changes during postnatal brain development (33, 34). We document changes in the relative amounts of these MAPs during the differentiation of PC12 cells and demonstrate that the one that undergoes the largest change in expression, MAP5, also undergoes a significant change in its cytoplasmic distribution. Our results indicate parallels between MAP synthesis in the NGF-induced outgrowth of PC12 cells and early events during brain morphogenesis in terms of MAP induction.

Materials and Methods

Cell Culture

PC12 cells were grown in DME (Gibco, Renfrewshire, Scotland) containing 5% heat-inactivated FCS and 10% horse serum. Cells exposed to NGF (100 ng/ml) were grown in the same medium supplemented with 1% horse serum. For exposure to NGF, cells were plated at 2×10^4 cells/cm^2 on polystyrene tissue culture dishes coated with 100 μg/ml collagen R (Serva Biochemicals, Heidelberg, Federal Republic of Germany).

Microtubule Preparation

Cells were washed twice with PBS, harvested in 1.5 vol of diluted (1:1,000) microtubule assembly buffer (0.1 M 2-[*N*-morpholino] ethanesulfonic acid, pH 6.4, containing 2 mM EGTA, and 1 mM $MgCl_2$ without GTP) supplemented with phenylmethylsulphonyl fluoride (PMSF), pepstatin A, and antipain, each at 10 μM to inhibit proteolysis during preparation. The cells were disrupted with a teflon pestle tissue grinder. One-tenth volume of 10× microtubule assembly buffer was added to the homogenate and after 10 min at 4°C, the homogenate was centrifuged at 180,000 *g* for 90 min in an Airfuge (Beckman Instruments, Geneva, Switzerland). Microtubule formation was induced by the addition of taxol (National Institute of Cancer, Bethesda, Maryland) to 20 μM, and GTP to 1 mM. The microtubules were harvested by centrifugation for 15 min at 180,000 *g* (40). The pellet was dissolved in SDS-containing sample buffer and the proteins were separated by electrophoresis in a 3–14% polyacrylamide gel (24). Proteins were blotted on nitrocellulose and immunoperoxidase-stained with antibodies as described by Towbin et al. (38). Anti-MAP1 (17), anti-MAP2 (17), anti-MAP3 (18), and anti-MAP5 (34) were used as previously described. Monoclonal anti-β-tubulin was obtained from Dr. L. I. Binder (University of Alabama). All mAbs were used as a 1:20 dilution of the hybridoma supernatant.

Immunodot Assay

Cell homogenate was centrifuged at 180,000 *g* for 90 min at 4°C and the supernatant fractions (3.5 mg/ml) were immobilized as 0.5 μl dots on nitrocellulose. Changes in the relative amount of each MAP during NGF-induced cell differentiation were determined by scanning the anti-MAP immunoperoxidase-stained dots of supernatant proteins and measuring the peak height for each dot using a Shimadzu reflectance densitometer (Burkard Instruments, Zurich, Switzerland). A twofold dilution series of the supernatant proteins was made to establish that the measurements made with each antibody lay in the linear range of the logarithmic relationship between densitometric peak height of the immunoperoxidase-stained sample and the amount of antigenic protein attached to the nitrocellulose (15). Series of dots from each time point were stained with Amido black and scanned to verify that equal amounts of protein were dotted onto the nitrocellulose. None of the MAPs were detectable in the cold pellet, indicating that supernatant fractions are representative of total PC12 MAPs.

Immunofluorescent Labeling of Cultured PC12 Cells

Cells were plated at a density of 2×10^4 cells/cm^2 in 35-mm tissue culture dishes coated with R collagen (100 μg/ml). The cells were rinsed with 100 mM PBS (150 mM NaCl, pH 7.2), fixed with 3% paraformaldehyde in PBS for 30 min at room temperature and then treated with 0.05% Triton X-100 in PBS for 10 min to permeabilize the cell membrane. The fixed cells were treated with PBS containing 5% FCS for 10 min to block nonspecific binding and incubated with mAbs. All mAbs were used as a 1:50 dilution of the hybridoma supernatant. After incubation overnight, the cells were washed with PBS and incubated further with rhodamine-labeled goat anti-mouse immunoglobulin (DAKOPATTS, Copenhagen, Denmark) diluted 1:200 for 2 h.

Results

Changes in MAPs during PC12 Cell Differentiation

To determine the MAP content and changes in the relative amounts of MAPs, equivalent amounts of taxol-precipitated PC12 microtubule protein from undifferentiated and differentiated (10-d NGF-treated) cells were loaded on SDS-gels, blotted onto nitrocellulose, and immunostained for MAP proteins.

Anti-MAP1 detected a minor protein band that comigrated with rat brain MAP1 (350 kD) in microtubules from both undifferentiated and differentiated PC12 cells (Fig. 1). There were no detectable changes in the amount of MAP1 between NGF-treated and control cultures. Anti-MAP2 stained a very faint band with the same M_r as rat brain MAP2 (280 kD). 10 d after NGF-induced differentiation there was approximately a twofold increase in MAP2 (Figs. 1 and 2). Both MAP1 and MAP2 are present at very low levels in PC12 cells; to detect them at all we had to overload the gel by loading 20 μg/mm of protein compared with 5 μg/mm for optimal Coomassie Blue staining of whole PC12 proteins.

In contrast, the anti-MAP3 gave strong staining of a pair of polypeptides whose size, M_r = 180 kD, was identical to that of MAP3 in adult rat brain microtubules. We observed a 10-fold increase in the level of this protein in NGF-treated PC12 cells compared to that in undifferentiated cells (Figs. 1 and 2). However there was an even larger change in MAP5 (320 kD, similar to rat brain) that underwent a 15-fold increase during the 10 d of NGF treatment (Figs. 1 and 2).

To determine the exact timing and the change in the relative amounts of each MAP induced by NGF, PC12 supernatant protein was collected at daily intervals after NGF treatment and quantified by immunodot assay. The levels of MAP1, -2, -3, -5, and tubulin were measured in dilution series of these supernatants using appropriate mAbs (Fig. 2). To be certain that we were measuring the appropriate protein, the supernatant fractions from days 0, 1, 2, 3, 4, and 10 were separated on an SDS-gel, blotted and stained with antibodies specific for MAP3, MAP5, and β-tubulin (Fig. 3).

The dot immunoassay (Fig. 2) and the supernatant immunoblots (Fig. 3) show clearly that both MAP3 and MAP5 protein induction occurs after a lag phase of 2 d after NGF

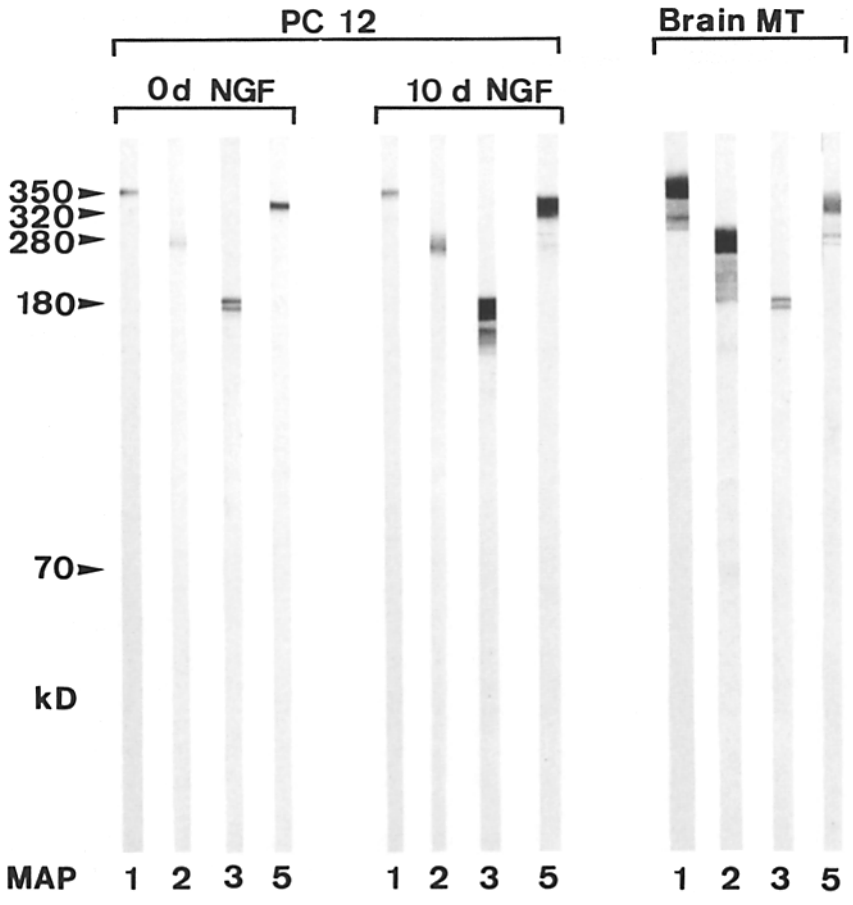

Figure 1. Changes in PC12-MAP content after 10 d NGF (100 ng/ml) treatment. Taxol-precipitated PC12 microtubule proteins and rat brain microtubule proteins were loaded onto an SDS gel (20 μg/mm slot) and transferred to nitrocellulose. The nitrocellulose was cut into strips and incubated with mAbs against MAP1, MAP2, MAP3, and MAP5.

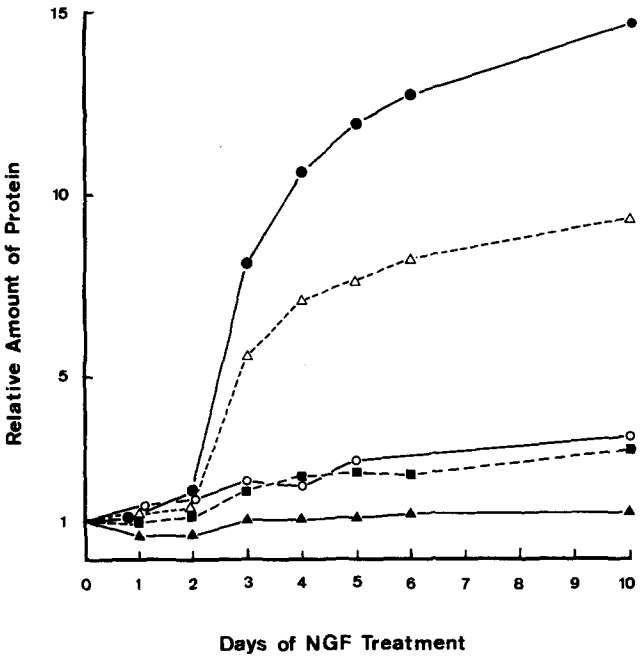

Figure 2. Changes of the relative amount of MAPs and tubulin during PC12 differentiation. Equal amounts of PC12 supernatant fraction protein after different periods of exposure to NGF were dotted in a dilution series (twofold) on nitrocellulose and stained with mAbs. Changes in the relative amount of each MAP and tubulin were determined by scanning the immunoperoxidase-stained dots using a reflectance densitometer. ●, MAP5; △, MAP3; ■, MAP2; ▲, MAP1; and ○, tubulin.

treatment, after which there was a steady accumulation of both MAPs. In contrast to MAP3 and -5, tubulin levels changed by only twofold over the 10-d period of NGF treatment and there was no lag phase. MAP1 levels did not change detectably and MAP2 showed about the same twofold increase as tubulin (Fig. 2).

Intracellular Distribution of MAPs in PC12 Cells

Undifferentiated PC12 cells have a round or polygonal cell-shape with short, thin cytoplasmic extensions (Figs. 4 *a* and 5 *a*). These cells contain a dense microtubule network that spreads out to the border of the thin cytoplasmic extensions (Figs. 4 *d* and 5 *b*). The microtubules at this stage are not bundled and are characteristically "curly". Undifferentiated cells stained with MAP5 (Fig. 4 *g*) and MAP3 mAbs (Fig. 4 *k*) show a strong but diffuse cytoplasmic staining. The staining of the thin cytoplasmic extensions is not as strong as in the nuclear region, even allowing for the lesser cytoplasmic volume in the cell periphery.

After treatment with NGF for 1 h, PC12 cells spread on the substratum and grow short, flat processes with small growth cones (Figs. 4 *b*, and 5, *c* and *d*). The majority of the cells remain at this state of differentiation until day 3. At this stage the microtubules are still not bundled (Figs. 4 *e* and 5 *c*). MAP3 and MAP5 are still evenly distributed throughout the cytoplasm of the cell body as well as in the short processes and growth cones, but do not present a fibrillar staining pattern (Fig. 4, *h* and *i*). This can be compared to the distribution of tubulin, which is arranged in filaments (Fig. 4 *e*).

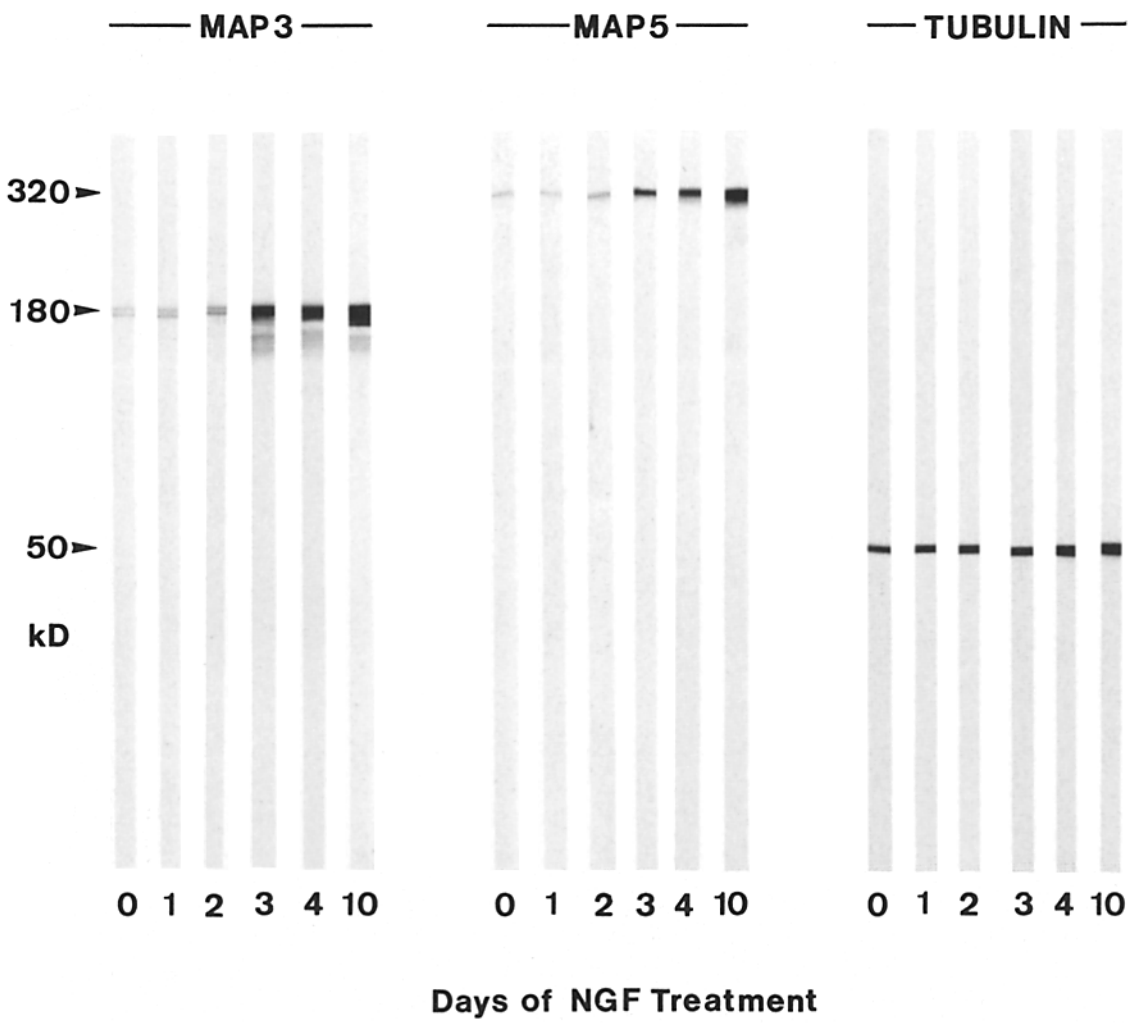

Figure 3. Induction of MAP3 and MAP5 in PC12 cells. Supernatant fraction protein from days 0, 1, 2, 3, 4, and 10 were loaded on an SDS gel (20 μg/mm slot), transferred to nitrocellulose, and immunostained. MAP3 and MAP5 induction occurs 2 d after NGF exposure in contrast to tubulin, which shows a linear increase from the beginning of NGF treatment.

After day 3 of NGF treatment the PC12 cells undergo a transition to a neuron-like morphology. The cell body rounds up and the short processes generate growth cones (Fig. 5, *g* and *h*). The neurites start to grow very rapidly, and in cells maintained for 7 d with NGF, the microtubules are bundled in neurites and the fibrillar staining pattern seen earlier with tubulin antibody is only detectable in the cell body, the neurite branching region, and in the growth cones (Fig. 4 *f*).

Throughout this period of rapid neurite growth MAP3 continues to be evenly distributed throughout the cytoplasm (Fig. 4, *k*, *l*, and *m*). However, we noticed a decrease in MAP3 staining in growth cones (Fig. 4 *m*, *arrow*, and Fig. 6, *b* and *d*) and a noticeable change from a diffuse cytosolic to a somewhat punctate distribution.

MAP5, on the other hand, shows a distinct change in its relationship to microtubules. After being distributed throughout the cytoplasm on day 3, it becomes associated with microtubules by day 7 (Fig. 7, *b* and *d*). The tubulin and the MAP5 antibodies show the same staining pattern in neurites, neurite branching regions, and in the growth cones (Fig. 4, *f* and *i*).

Anti-MAP1 stains the cells at all stages of differentiation very weakly and seems to be limited to the cell body. In contrast, the MAP2 antibody staining is distributed over the entire differentiated cell, but this staining is also very faint and is not fibrillar. There is no staining with rhodamine-labeled second antibody alone (results not shown).

Discussion

The majority of the components that have been identified in brain microtubules (MAP1, MAP2, MAP3, MAP5, tau proteins, and chartins; 1, 2, 4, 7, 13, 18, 34) can also be detected in the PC12 cell line. This, and the fact that undifferentiated PC12 cells can be induced by NGF to differentiate into cells with typical neuronal morphology, makes this cell line an ideal model system to study the putative role of MAPs in neuronal differentiation.

Expression of MAP3 and MAP5 Seems To Be a General Feature of Early Neuronal Differentiation

Our results suggest that there are significant parallels between NGF-induced neurite outgrowth in PC12 cells and

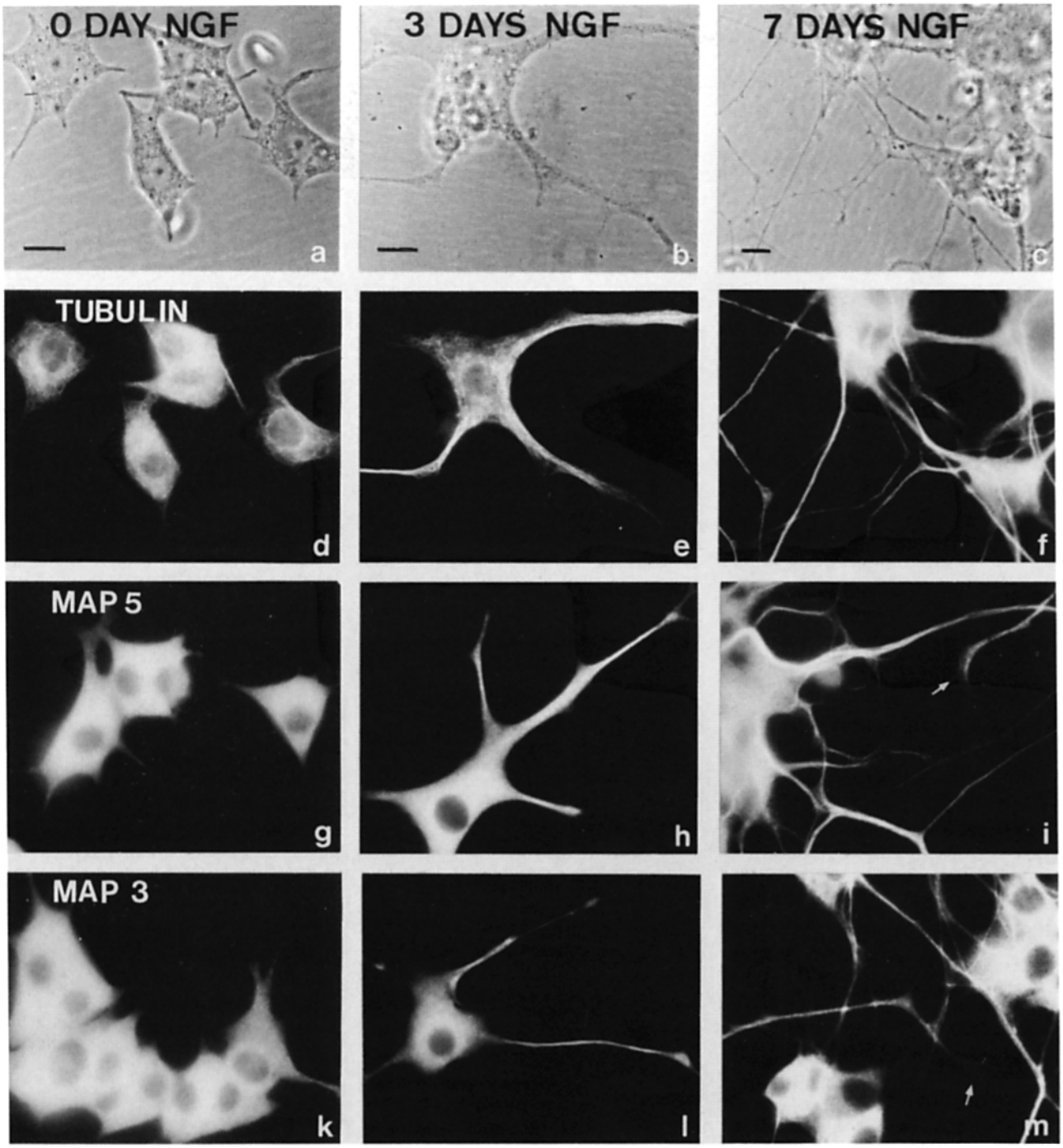

Figure 4. Microtubule arrangement and distribution of MAP5 and MAP3 in differentiating PC12 cells. In undifferentiated PC12 cells (*a*, *d*, *g*, and *k*) polymerized tubulin is distributed throughout the cell extending to the tip of the thin cytoplasmic extensions (*d*). MAP5 (*g*) and MAP3 (*k*) show a diffuse distribution. Cells treated for 3 d with NGF (*b*, *e*, *h*, and *l*) generate short processes. At this stage microtubules are still not bundled (*e*), and MAP5 (*h*) and MAP3 (*l*) remain cytosolic. After 7 d NGF treatment (*c*, *f*, *i*, and *m*), the cells produce long neurites with well-developed growth cones. MAP5 (*i*) is colocalized with microtubules in growth cones (*arrow*) and neurites (see also Fig. 6, *c* and *d*). MAP3 staining (*m*) changes from a diffuse to a more punctate staining pattern and decreases in growth cones (*arrow*). Bar, 10 μm.

early events during the morphogenesis of brain neurons. MAP3 and MAP5 are abundant in PC12 cells as they are in developing rat brain where neurons are not fully differentiated (18, 34). Even in undifferentiated PC12 cells, MAP3 and MAP5 are readily detectable on immunoblots; immunocytochemical staining for MAP3 and MAP5 is very bright and diffuse throughout the cytoplasm. MAP5 in brain appears to be distributed throughout axons, dendrites, and cell bodies of neurons that have just started to differentiate (34), and anti-MAP3 stains cell bodies and the outgrowing processes in juvenile brain (28).

During brain development MAP3 and MAP5 are "early"

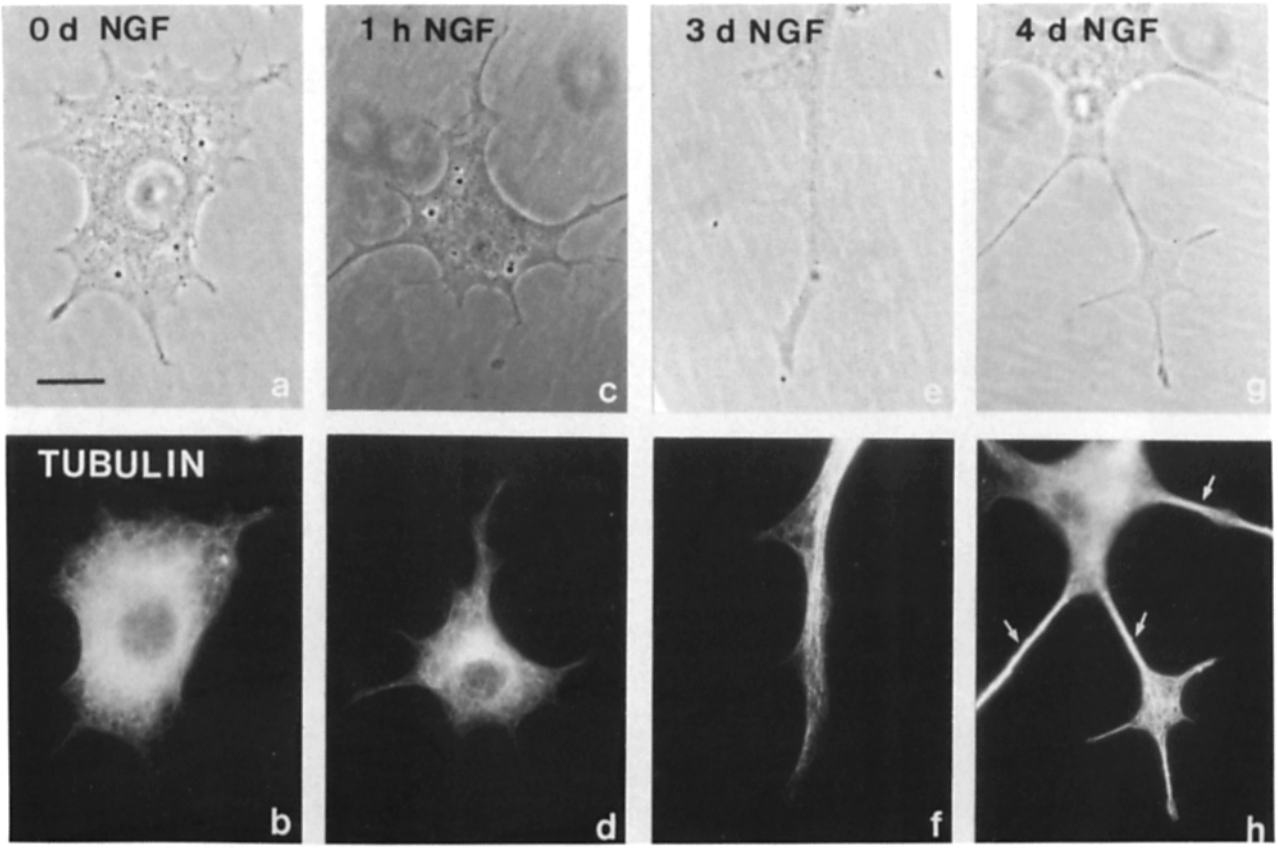

Figure 5. Bundling of microtubules during PC12 differentiation. Cells were fixed and stained with β-tubulin antibody after different NGF exposure times (*a* and *b*, no NGF; *c* and *d*, 1 h; *e* and *f*, 3 d; *g* and *h*, 4 d). Until day 3, microtubules curl and twist as individual filaments, then they start to bundle and become visible as thick cords in neurites (*h*, *arrows*). Bar, 10 μm.

MAPs, that is MAPs that are present in neurons from the earliest phase of differentiation but whose levels decrease when neurons reach their mature form (33). Soon after NGF induction PC12 cells generate short processes with small growth cones and the majority of the cells remain at this differentiation stage until day 3. During this time the MAP3 and MAP5 pools remain at the same level as in uninduced PC12 cells. After day 3 the neurites begin to generate large growth cones and the rate of neurite extension increases rapidly. This morphological change occurs simultaneously with a large increase in MAP3 and MAP5 levels in PC12 cells between days 2 and 3. The MAP3 and MAP5 levels subsequently continue to increase, along with neurite length. By 10 d after NGF induction, we could measure a 10-fold increase in MAP3 and a 15-fold increase in MAP5 compared with undifferentiated PC12 cells. Thus the presence of MAP3 and MAP5 in differentiating neurons seems to be a general feature of brain development, and PC12 cells represent a culture system where these same proteins are induced during neurite outgrowth.

In rat brain, MAP3 and MAP5 levels drop dramatically after brain maturation (33, 34). We did not observe a drop in MAP3 and MAP5 levels in PC12, even after long-term NGF treatment. Tucker and Matus (36) have recently observed a decrease of MAP5 staining in developing avian retinal ganglion cells after the axons reached their target cells in the tectum. It thus seems possible that PC12 cells do not show a similar decrease in MAP3 and MAP5 because the neurites remain in an outgrowth phase, never reaching a final differentiation stage because they cannot make synapses with target cells.

MAP1 and MAP2 are Minor Proteins in PC12 Cells

Immunohistochemical studies in rat brain with the same mAbs as were used here have shown that MAP1 is associated with neurons and is more abundant in dendrites than in axons (1). With respect to brain development, MAP1 is a "late" MAP, its levels increasing when dendrite growth is already well-advanced (28). In PC12 cells MAP1 could be detected as a very minor component in taxol-precipitated PC12 microtubules by immunoassay, but the results show clearly that MAP1 is not induced during PC12 differentiation. Other workers have reported a large increase in levels of a high molecular mass MAP species, referred to as either MAP1 (7) or MAP1.2, (14) after NGF-induced PC12 cells. Judging by our results using antibodies that distinguish between MAP1 and the similarly sized MAP5, it seems probable that the high molecular mass MAP whose increase was observed in these earlier studies was MAP5 and not MAP1.

MAP2 in brain is represented by two high molecular mass components, MAP2a and MAP2b, and a recently described 70-kD component, MAP2c (10, 11). High molecular mass MAP2 is present in PC12 cells and shows an approximately

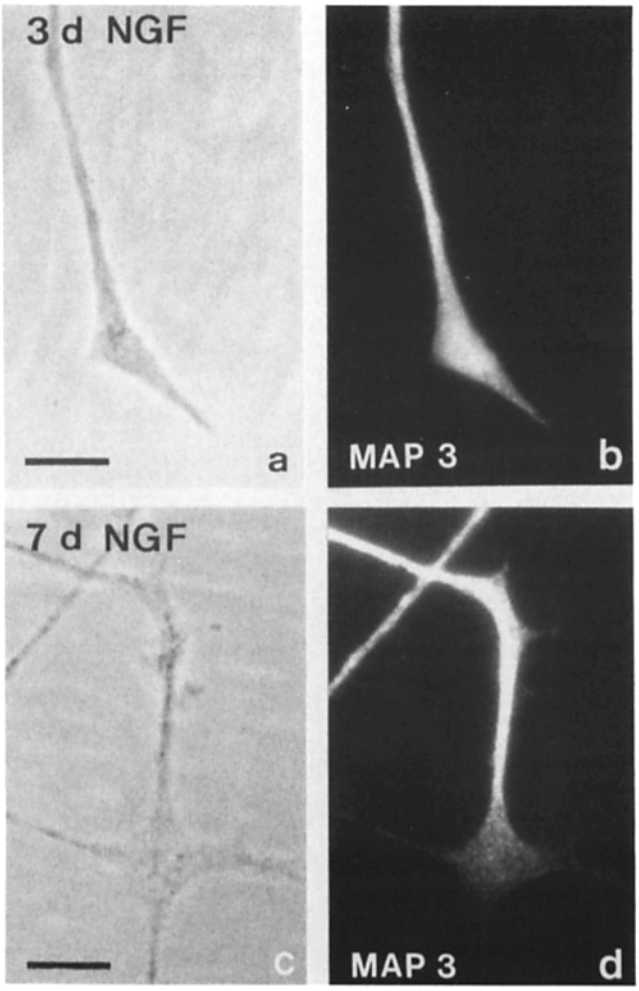

Figure 6. Distribution of MAP3 in growth cones during PC12 differentiation. MAP3 staining decreases in growth cones during differentiation (*a* and *b*, 3 d NGF; *c* and *d*, 7 d NGF) and never shows a fibrillar staining pattern as is seen with anti-tubulin (compare with Fig. 5 *h*). Bar, 5 μm.

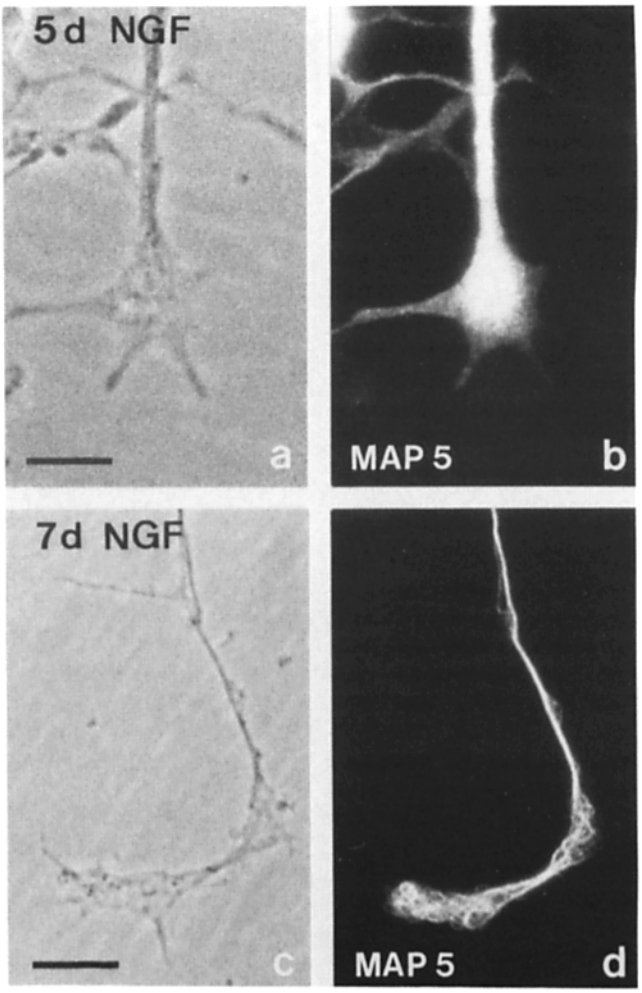

Figure 7. Distribution of MAP5 in growth cones during PC12 differentiation. After being distributed throughout the cytoplasm (*a* and *b*, 5 d NGF), MAP5 starts to be associated with microtubules between day 6 and 7 (*c* and *d*, 7 d NGF). It then shows the same fibrillar distribution as tubulin (compare with Fig. 5 *h*). Bar, 5 μm.

twofold increase in taxol-precipitated PC12 microtubules after 10 d exposure of PC12 cells to NGF. These findings are in agreement with those of Black et al. (4) who also detected low amounts of MAP2 in differentiated PC12 cells using an immunoprecipitation assay. We could not detect MAP2c in PC12 even though this protein is characteristically expressed by neurons in the developing brain (29).

Generally we can say that MAP1 and MAP2 are minor proteins in PC12 cells whose levels do not change greatly after long-term treatment with NGF. Since high levels of MAP1 are characteristic of mature neurons in the rat nervous system (28) this suggests further that NGF-treated PC12 cells remain in a state equivalent to early neuronal differentiation under these culture conditions.

Association of MAP5 with Microtubules Occurs Only after Exposure to NGF

Tubulin appears as filaments at all stages of PC12 cell differentiation. Until day 3, single microtubules are visible even in the short flat processes that the cell generates. The microtubules are not bundled at this stage, but rather curl and twist as individual filaments. Simultaneously with the onset of fast neurite outgrowth and the increase in levels of MAP3 and MAP5, the microtubules become visible as thick cords in the neurites, which are known to correspond to microtubule bundles (20). Single, nonbundled microtubules can still be seen in growth cones and in the cell body. Anti-MAP3 staining did not show a filamentous staining pattern at any stage of PC12 cell development, despite its strong binding to tubulin polymers in vitro (18) and its ability to promote tubulin assembly (19). It is thus unlikely to play a role in the reorganization of microtubules that occurs in the cytoplasm of PC12 cells during neurite induction. MAP5 is also distributed throughout the cytoplasm for the first 5 d after NGF treatment. However, after day 6 anti-MAP5 gives filamentous staining in growth cones and neurites whose appearance is identical to that of anti-tubulin–stained filaments. Although we were unable to obtain a double-label immunofluorescence demonstration of identity between the anti-tubulin

stained filaments and those stained by anti-MAP5, their similarity, together with the fact that MAP5 in vitro binds strongly to microtubules but not to neurofilaments (34), strongly suggest that after day 6 of NGF treatment MAP5 becomes bound to microtubules. Thus the relationship of MAP5 to microtubules changes during the neurite elongation phase. Although there is no obvious morphological change coinciding with this event, Jacobs and Stevens (21) have shown that after 7 d of NGF treatment PC12 neurites become more resistant to microtubule depolymerization by nocodazole. In vitro, both MAP3 and MAP5 promote tubulin polymerization and coassemble with microtubules (18, 34). However, these results make it evident that inside living PC12 cells the distribution and putative function of these molecules is determined by factors in addition to their affinity for tubulin. As the case of MAP5 shows, the balance between these factors may be modulated during differentiation so that the relationship of the molecule to the cytoskeleton is changed.

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