The Phosphoprotein Stathmin Is Essential for Nerve Growth Factor–stimulated Differentiation

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Abstract. Stathmin is a ubiquitous cytosolic protein which undergoes extensive phosphorylation in response to a variety of external signals. It is highly abundant in developing neurons. The use of antisense oligonucleotides which selectively block stathmin expression has allowed us to study directly its role in rat PC12 cells. We show that stathmin depletion prevents nerve growth factor (NGF)–stimulated differentiation of PC12 cells into sympathetic-like neurons although the expression of several NGF-inducible genes was not affected. Furthermore, we found that stathmin phosphorylation in PC12 cells which is induced by NGF depends on mitogen-activated protein kinase (MAPK) activity. We conclude that stathmin is an essential component of the NGF-induced MAPK signaling pathway and performs a key role during differentiation of developing neurons.

In the developing central nervous system, neurite outgrowth as well as the elimination of neurons are essential processes underlying the formation of precise patterns of synaptic connectivity (O'Leary et al., 1986; Catsicas et al., 1987; for review see Goodman and Shatz, 1993). In an attempt to identify molecules and mechanisms involved in these processes, we have employed a subtractive cloning strategy to isolate cDNAs differentially expressed during specific developmental stages in the chick retina (Lebeau et al., 1991). Subtracted clone D6 which was transiently upregulated during the period of neurite outgrowth and synapse formation encoded the chick homologue of stathmin (Catsicas, S., and G. Grenningloh, unpublished data). Here we describe the functional analysis of this molecule.

Stathmin, also known as p19 (Pasmantier et al., 1986), prosolin (Braverman et al., 1986), p18 (Hanash et al., 1988), 19K (Gullberg et al., 1990), and Op18 (Hailat et al., 1990) is a cytosolic protein whose phosphorylation is correlated with the action of multiple extracellular stimuli regulating proliferation and differentiation. While these observations implicate stathmin in processes underlying signal transduction (for review see Sobel, 1991), a role in the control of cell cycle has also been proposed (Luo et al., 1994; Brattsand et al., 1994; Marklund et al., 1994). Stathmin is expressed in a variety of cell types and tissues, including neurons, where it is highly abundant during development (for review see Sobel, 1991). In rat brain, stathmin expression reaches a peak around birth, although its expression remains significant in specific areas of adult brain (Peschanski et al., 1993; Himi et al., 1994). Previous studies had demonstrated that stathmin is expressed in PC12 cells where its phosphorylation, but not its expression, is stimulated by the addition of NGF (Doye et al., 1990). Despite considerable interest in the expression and phosphorylation of stathmin in neurons, to date, there has been no direct evidence implicating this protein in specific neuronal functions.

As a first step towards a functional analysis of stathmin in neurons, we have used antisense oligonucleotides to decrease stathmin protein levels in rat pheochromocytoma PC12 cells. This cell line is considered an excellent model system to analyze molecular mechanisms underlying NGF-mediated neuronal differentiation. PC12 cells undergo differentiation into sympathetic-like neurons upon chronic NGF treatment, exhibiting characteristic morphological and molecular properties of a neuronal phenotype (Greene and Tischler, 1976).

The intracellular signaling pathways stimulated by NGF are triggered after activation of the high affinity receptor tyrosine kinase TrkA. Of several identified downstream effector targets, activation of p21ras and an associated cascade leading to activation of mitogen-activated protein kinase (MAPK)[1] family members plays an essential role in processes mediating neuronal differentiation (for reviews

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1. *Abbreviations used in this paper*: BrdU, bromodeoxyuridine; MAPK, mitogen-activated protein kinase; PON, phosphorothioate oligonucleotide.

see Keegan and Halegoua, 1993; Kaplan and Stephens, 1994; Heumann, 1994; Pang et al., 1995). MAPKs phosphorylate a diverse range of nuclear, membranous, and cytoplasmic proteins (for review see Davis, 1993) although targets crucial for neurotrophic responsiveness are unknown. Interestingly, recent evidence has demonstrated that stathmin is a substrate for MAPK in vitro (Leighton et al., 1993; Marklund et al., 1993) and may also be a physiological substrate for this kinase system in intact cells (Beretta et al., 1993; Marklund et al., 1993; Beretta et al., 1995).

In this report, we have induced stathmin depletion by chronically exposing PC12 cells to phosphorothioate antisense oligonucleotides (PONs). We show that a selective blockade of stathmin expression prevented the differentiation-promoting actions of NGF. Moreover, inhibition of the MAPK pathway prevented NGF-induced phosphorylation of stathmin in PC12 cells. Taken together, our results suggest that stathmin plays a critical role in the NGF-activated MAPK cascade mediating withdrawal from the cell cycle and differentiation.

Materials and Methods

PC12 Cell Culture

PC12 cell lines were routinely cultured as described previously (Greene and Tischler, 1976), maintained in RPMI 1640 containing 10% horse serum and 5% FCS.

Antisense Experiments and Phosphorothioates Oligonucleotides

PC12 cells were plated in 24-well collagen-coated multi-well dishes at a density of 10,000 cells per well in RPMI 1640 supplemented with 1% horse serum. The uptake of PONs by PC12 cells was tested with three different FITC-labeled PONs (two 18-mers and one 20-mer) as previously described (Catsicas et al., 1995). Fluorescence was visible in more than 90% of the cells 24 h after the addition of PONs. To establish the dose-response, 10 μM HPLC-purified antisense or control PONs, respectively, were added on the first day followed by 1 μM, 2 μM, or 4 μM every day (antisense PON AS1: 5′-ctccagctctttcacctg-3′; antisense PON AS2: 5′-tgaatatcagaagatgccat-3′; nonsense PON NS: 5′-cttcccactgctcgctat-3′; unrelated PON UR: 5′-gcatgttcttggtca-3′). The second day and every other day, 50 ng/ml NGF was added and cells were incubated for another 5 d. The cells were photographed using an Axiovert 135 microscope (Zeiss). For Western blot analysis, the cells were removed from the culture dish, pelleted, and resuspended in sample buffer for PAGE.

Gel Electrophoresis, Immunoblotting, and Antibodies

Proteins were separated on 14% or 8–16% SDS-PAGE after electrophoretic transfer to nitrocellulose, immunoblots were immersed in blocking solution consisting of 5% milk powder in PBS/0.15% Tween-20. Blots were subsequently incubated with one of the following antibodies diluted in 2.5% milk powder in PBS: rabbit anti-stathmin (Koppel et al., 1990), mouse anti-actin (Boehringer Mannheim C4), rabbit anti-VGF8a (Possenti et al., 1989; a generous gift from A. Levi, Rome), rabbit anti-peripherin (Djabali et al., 1991; a generous gift from M.-M. Portier, Paris), rabbit anti-Gqα (Edgerton et al., 1994), and rabbit anti-SCG10. The rabbit anti-SCG10 serum was generated against a fusion protein containing the entire coding rat sequence (generous gift from N. Mori, Kyoto, Japan) fused to the bacterial TrpE protein (Spindler et al., 1984). Antibody binding was detected using the ECL Western blotting kit (Amersham International, Buckinghamshire, England). The intensity of the bands on the autoradiograms was quantified by optic densitometry using a 3 CCD video camera linked to a VIDAS (Kontron, Zürich, Switzerland) image analyzer. The protein expression values were normalized to those of the Gqα protein, whose expression is not affected by NGF treatment which can be used as a marker of equal loading. In the experiment dealing with the NGF-induced proteins, the protein expression values were normalized to those of the samples treated with NGF alone, which represented arbitrarily the 100% values.

Cell Counts and Bromodeoxyuridine-Incorporation Assay

For cell proliferation assays, PC12 cells were plated in 24-well collagen-coated multi-well dishes at a density of 10,000 cells per well. Addition of oligos (4 μM every day) and NGF was performed as above. Triplicate samples of cells were counted in a hemacytometer. For the bromodeoxyuridine (BrdU)-incorporation assay, BrdU was added on the fourth day of incubation for 40 h. Cells were transferred to slides using the cytospin technique, fixed, and stained according to the manufacturer's protocol (Boehringer, Mannheim, Germany, BrdU labeling kit). Triplicate fields containing 200–300 cells were counted manually for labeled vs nonlabeled cells on the image analyzer.

Analysis of MAPK and Stathmin Phosphorylation in PC12 Cells

PC12 cells were plated at 2×10^6 cells in six-well collagen-coated multi-well dishes and maintained in RPMI 1640, 0.5% horse serum for 48 h followed by incubation in serum-free medium for 2 h. Then, the PC12 cells

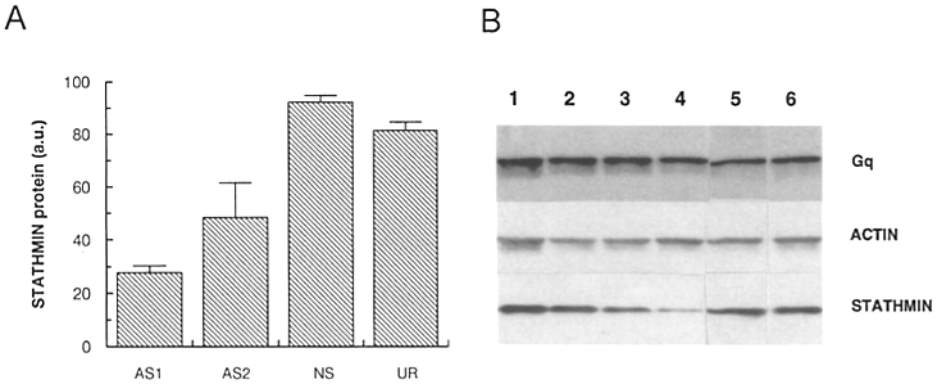

Figure 1. Blockade of stathmin expression with antisense PONs. PC12 cells were maintained in RPMI 1640 supplemented with 1% horse serum and treated in the following way: on the first day, 10 μM antisense or control PONs, respectively, were added, followed by 1 μM, 2 μM, or 4 μM every day in parallel samples. The second day and every other day, 50 ng/ml NGF was added and the cells were incubated for another 5 d. *A* shows the effect of 4 μM antisense PONs, AS1 and AS2, and 4 μM control PONs (NS, nonsense and UR, unrelated) on stathmin protein levels in PC12 cells. Normalized mean gray values of the signals in Western blots (from three independent experiments) are shown in arbitrary units. Values denote means ± SEM. AS1 differs statistically from NS and UR ($P < 0.01$); AS2 differs from NS ($P < 0.01$); and from UR ($P < 0.05$). *B* shows the Western blot of protein extracts from cultures incubated in the absence of PON (lane *1*), in the presence of antisense PON AS1, 1 μM (lane 2), 2 μM (lane *3*), or 4 μM (lane *4*), and nonsense PON, 1 μM (lane *5*) and 4 μM (lane *6*). Blots were incubated with antibodies specific for stathmin, actin, and Gqα. Inhibition of stathmin expression following antisense treatment is specific, dose-dependent, and reaches ~75% of control values.

were incubated for various times (0, 5, 10, 30, 60, and 180 min) with NGF (50 ng/ml) in RPMI 1640, 1% horse serum. To inhibit MAPK kinase activity, 20 μM PD98059 was added 30 min before the addition of NGF (Pang et al., 1995). For the analysis of MAP kinase phosphorylation, the cells were subsequently washed with cold PBS and lysed by adding 100 μl of SDS sample buffer. After sonication and heat denaturation, 20 μl of each sample was loaded on an 8–16% SDS-PAGE. As controls, 25 ng of phosphorylated and nonphosphorylated MAP kinase protein (New England

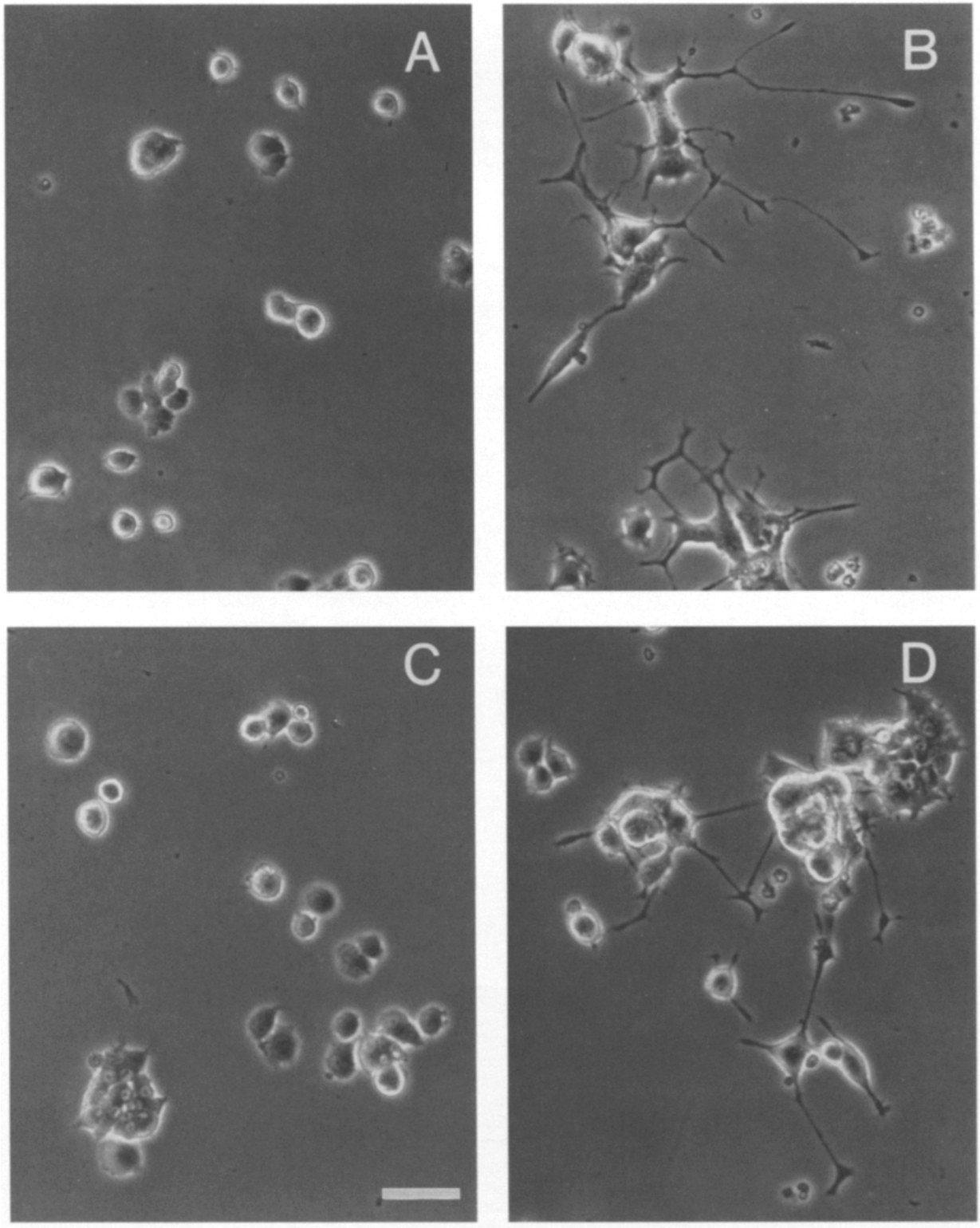

Figure 2. Effect of stathmin antisense PON AS1 on NGF-induced neurite outgrowth in PC12 cells. Phase contrast micrographs of PC12 cells maintained in RPMI 1640 supplemented with 1% horse serum for 6 d without any addition (*A*), with NGF (*B*), with NGF and 4 μM antisense PON AS1 (*C*), or with NGF and 4 μM nonsense PON (*D*). Neurite outgrowth in response to NGF is seen only in *B* and *D*. Antisense-treated cells (*C*) are similar to cells not induced with NGF (*A*). The same results were obtained in replicate experiments. Bar, 25 μm.

Biolabs, Schwalbach, Germany) was also loaded. After electrophoretic transfer to Immobilon membrane, phosphorylated MAP kinase was detected with the Phospho Plus antibody (New England Biolabs) which specifically recognizes phosphorylated p44 and p42 MAP kinase. For analysis of stathmin phosphorylation, we used a native PAGE gel system that separates stathmin according to the charge differences introduced by phosphorylation (Marklund et al., 1993). 20 μg of total protein were loaded on a 10% nondenaturating gel. Western blotting was performed as described above.

Results

Inhibition of Stathmin Expression in PC12 Cells

To block stathmin expression, we chronically added to the culture medium phosphorothioate-modified antisense oligonucleotides (PONs) complementary to position 1 to 20 (AS2) and 19 to 36 (AS1) relative to the translation initiation site of the rat stathmin coding region (Doye et al., 1989; Schubart et al., 1989). Controls included a nonsense (NS) and an unrelated (UR) PON. Western blot experiments and subsequent measurements of protein levels by optic densitometry of the autoradiograms allowed us to monitor stathmin expression after the addition of PONs at different concentrations. The two antisense PONs were able to decrease significantly stathmin protein levels between 50 (AS2) and 75% (AS1) in a specific and dose-dependent manner (Fig. 1 and data not shown). Fig. 1, *A* and *B* show that the strong decrease in expression of stathmin after one week of antisense treatment was not observed in cells treated with control PONs, and that the expression of internal control proteins was not affected by the antisense PONs.

Stathmin Antisense Treatment Blocks PC12 Cell Differentiation

Visual inspection of cultures treated with the antisense PONs revealed an apparent inability of the PC12 cells to respond to NGF. The most dramatic effects were observed with PON AS1, which correlated with its efficiency in inhibiting stathmin expression by 75% (Fig. 2). Whereas in control PON-treated cultures, cells extended processes after 5 d of NGF exposure, no neurite outgrowth was detectable in cultures treated with antisense PON AS1 and the cells maintained the characteristic round shape, phase-bright feature of proliferating PC12 cells (Fig. 2). Furthermore, we found a consistent and significant increase in the number of cells in the antisense-treated cultures in the presence of NGF. When cultures were treated with antisense PON AS1, the number of cells at the end of the treatment was increased by a factor of 3.5 to 4, whereas in the presence of NGF and control PONs, we found only a twofold increase in cell number.

Since these experiments were performed in differentiation medium containing only 1% horse serum where the proliferation rate of PC12 cells is reduced, we then measured PC12 cell proliferation using the bromodeoxyuridine (BrdU)-incorporation method in order to evaluate more precisely whether the cells responded to NGF after antisense treatment. PC12 cells were treated for 6 d with 4 μM PON and incubated in the presence of BrdU for the last 40 h of PON treatment. In control cultures, we found that ~50% of the cells incorporated BrdU after 5 d of NGF treatment, whereas ~90% were positive in the absence of NGF (Fig. 3). After blockade of stathmin expression with antisense PON AS1, the rate of BrdU-incorporation was highly increased ($P < 0.001$) as compared to NGF-treated controls in the presence or in the absence of nonsense PON (Fig. 3). Also, PON AS2 had a highly significant effect on NGF-stimulated cessation of proliferation, although less prominent than PON AS1, which is consistent with its lower efficiency in stathmin depletion (Fig. 3). In control experiments, nonsense PON (NS) was ineffective on BrdU-incorporation of NGF-treated cultures (Fig. 3). Interestingly, both antisense PONs had a significant effect ($P < 0.05$) on BrdU-incorporation in the absence of NGF, showing reduced proliferation (Fig. 3). This observation indicates that stathmin depletion also interferes with cell proliferation as previously proposed (Marklund et al., 1994; Luo et al., 1994).

Depletion of Stathmin Does Not Affect Expression of NGF-induced Proteins

In addition to withdrawal from the cell cycle and morphological changes, NGF also stimulates the expression of specific proteins in PC12 cells, some of which are the products of the so-called "late-induced genes" (for review see Levi, 1991). To determine whether stathmin is involved in the signaling events promoted by NGF resulting in specific gene expression, we examined the effect of blockade of stathmin expression on the expression of three late-induced proteins: SCG10, which is a neuronal protein related to stathmin (Stein et al., 1988*a*,*b*), VGF8a, a secreted protein of unknown function (Possenti et al., 1989), and peripherin, an intermediate filament protein sharing important structural features with neurofilaments (Aletta et al., 1989). After the PC12 cells had been exposed for 5 d to NGF, samples in triplicate were collected and used for Western

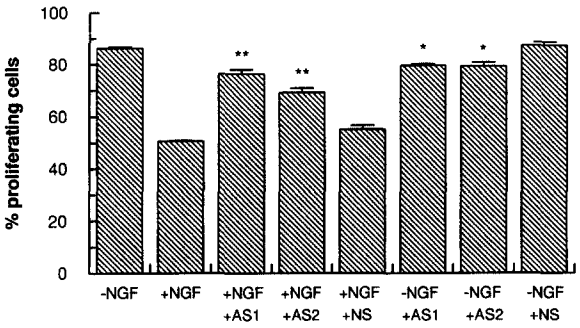

Figure 3. Effect of stathmin antisense PONs on NGF-induced cessation of proliferation. PC12 cell proliferation was measured by BrdU-incorporation. The asterisks indicate statistically significant differences (**, $P < 0.001$; *, $P < 0.05$). Values denote means ± SEM ($n = 3$); − NGF, culture without NGF; + NGF, NGF-stimulated control; + NGF + AS1/AS2/NS, cultures with NGF and antisense PON AS1, AS2, and nonsense PON, respectively; − NGF + AS1/AS2/NS, cultures without NGF with antisense PON AS1, AS2, and nonsense PON, respectively. After blockade of stathmin expression with antisense PONs, AS1 and AS2, NGF-treated cells incorporate BrdU at a much higher rate as NGF-treated control, whereas nonsense PON is ineffective on NGF-treated cultures. In the absence of NGF, both antisense PONs caused a significant decrease of BrdU-incorporation.

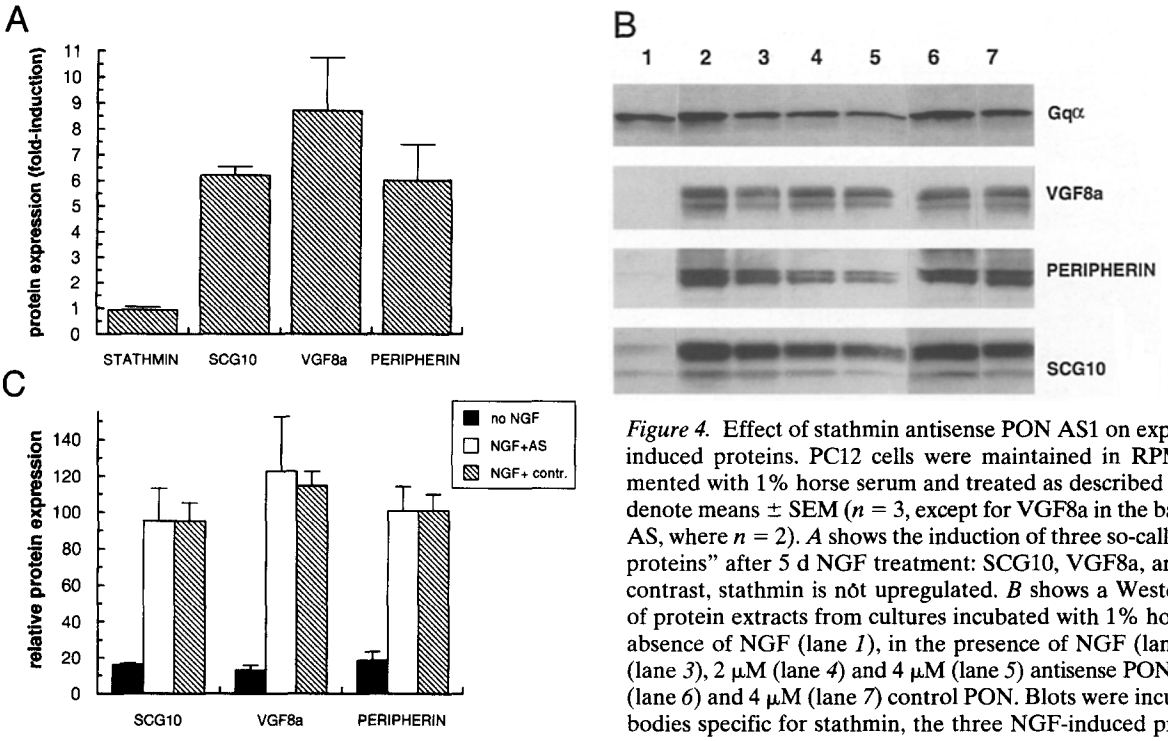

Figure 4. Effect of stathmin antisense PON AS1 on expression of NGF-induced proteins. PC12 cells were maintained in RPMI 1640 supplemented with 1% horse serum and treated as described in Fig. 1. Values denote means ± SEM ($n = 3$, except for VGF8a in the bar height NGF + AS, where $n = 2$). *A* shows the induction of three so-called "late-induced proteins" after 5 d NGF treatment: SCG10, VGF8a, and peripherin; in contrast, stathmin is not upregulated. *B* shows a Western blot analysis of protein extracts from cultures incubated with 1% horse serum in the absence of NGF (lane *1*), in the presence of NGF (lane *2*), with 1 μM (lane *3*), 2 μM (lane *4*) and 4 μM (lane *5*) antisense PON AS1, with 1 μM (lane *6*) and 4 μM (lane *7*) control PON. Blots were incubated with antibodies specific for stathmin, the three NGF-induced proteins and Gqα protein, as equal loading marker. *C* shows protein expression levels of NGF-induced proteins in the presence or absence of 4 μM antisense AS1 and controls PONs. The values were obtained from Western blot signals of three different experiments and normalized to the equal loading marker, Gqα. The values are expressed as percentages of the samples treated with NGF alone (100%). The graph indicates that, in the absence of NGF, relative expression levels of NGF-induced proteins are very low, as shown in Fig. 4 *B* (lane *1*). NGF + contr. represents the induction levels in the presence of NGF and nonsense or unrelated control PONs. The expression of the three NGF-inducible proteins is significantly induced ($P < 0.001$, for SCG10 and peripherin) after antisense treatment (NGF + AS), although stathmin levels are downregulated by ~75% in these experiments (Fig. 1).

blot analysis as described in the experimental procedures. Quantitation of the three different markers of differentiation in control cultures allowed us to confirm that a significant upregulation (6–9-fold) of these proteins, but not of stathmin, was promoted by NGF (Fig. 4 *A*). In parallel experiments, antisense, nonsense, and unrelated PONs were chronically added to the cultures at different concentrations (Fig. 4, *B* and *C*). We found that in antisense-treated cultures the expression of the three specific markers of PC12 cell differentiation was induced to similar levels as in control cultures (Fig. 4, *B* and *C*). Thus, despite antisense-dependent block of NGF-induced proliferation arrest and neurite outgrowth, stathmin-depleted PC12 cells were still capable of responding to neurotrophin stimulation with induction of a range of specific genes.

NGF-induced Stathmin Phosphorylation Depends on MAPK Activation

To determine whether stathmin is a component of the MAPK signaling cascade initiated by NGF, we used a pharmacological approach. PD 098059 has recently been reported to selectively inhibit the activation of the MAPK-activating enzyme (MAPK kinase or MEK), and, consequently, to prevent the activation of MAPK (Dudley et al., 1995; Alessi et al., 1995). In PC12 cells, PD 098059 blocks NGF-induced differentiation (Pang et al., 1995). We treated PC12 cells for various times with NGF and followed MAPK activation and stathmin phosphorylation by Western blot analysis. Fig. 5, *A* and *B* show that both MAPK- and stathmin phosphorylation are strongly induced after 5 min of NGF exposure. After blockade of MAPK activation with 20 μM PD 098059 (not shown), stathmin phosphorylation was prevented (Fig. 5 *C*). These results suggest that stathmin is a downstream target for MAPK within the NGF signaling pathway regulating neuronal differentiation.

Discussion

To gain insight into the molecular mechanisms underlying the development of the nervous system, we isolated candidate regulatory genes using a subtractive cloning approach based on differential gene expression during neurite outgrowth and synapse formation in the chick embryonic retina (Lebeau et al., 1991). One of the cDNAs isolated in our studies encoded the phosphoprotein stathmin. In this report, we describe the characterization of stathmin function in antisense-treated PC12 cells. Our results indicate that the protein is absolutely required for NGF-promoted differentiation in PC12 cells, although it is not necessary for the expression of several NGF-inducible genes.

PC12 cell differentiation induced by NGF leads to the acquisition of neuronal characteristics including cessation of proliferation, neurite outgrowth and expression of proteins associated with neuronal function. We found that chronic blockade of stathmin expression in PC12 cells inhibited NGF-induced morphological changes such as cell

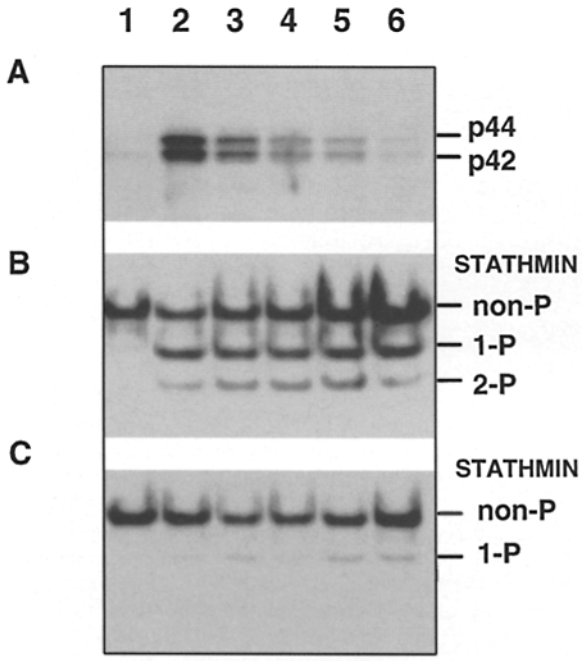

Figure 5. Stathmin phosphorylation in response to NGF is dependent on MAPK activity. PC12 cells treated with NGF for 0 min (lane *1*), 5 min (lane *2*), 10 min (lane *3*), 30 min (lane *4*), 60 min (lane *5*), and 180 min (lane *6*) were analyzed by Western blotting. (*A*) MAPK activation was detected from SDS-lysates of total PC12 cell extracts using phospho-MAPK antibody. (*B*) Stathmin phosphorylation was detected under native gel electrophoresis conditions and paralleled MAPK activation in response to NGF. (*C*) In the presence of 20 μM synthetic inhibitor of the MAPK cascade, PD 098059, NGF-induced stathmin phosphorylation was blocked.

body flattening and extension of processes. To evaluate further the loss of responsiveness to NGF after antisense treatment, we assessed DNA synthesis using BrdU-incorporation. We found that stathmin depletion of NGF-treated PC12 cells resulted in a significantly higher rate of BrdU-incorporation suggesting that these cells continued to proliferate despite the presence of NGF. Increased BrdU-incorporation may also result from an abnormal blockade in S phase. This phenotype was observed by Marklund et al. (1994) in an erythroleukemial cell line overexpressing both antisense and mutant constructs of stathmin, where the target sites for proline-directed kinases were deleted. However, our experiments showed a significant increase in PC12 cell number following antisense treatment suggesting that increased BrdU incorporation indeed reflects continued proliferation. It is also important to note that stathmin depletion caused a significant decrease of proliferation in the absence of NGF, which may reflect a second role of stathmin in proliferating cells. This observation is consistent with recent reports showing that appropriate expression levels and phosphorylation of stathmin are essential for normal cell division in nonneuronal cell lines (Marklund et al., 1994; Luo et al., 1994). Further studies should address whether different states of stathmin phosphorylation account for its dual role in proliferation and differentiation.

Since chronic inhibition of stathmin expression in undifferentiated PC12 cells led to an apparent loss of responsiveness to NGF, we determined whether the induction of specific markers of PC12 cell neuronal differentiation was also inhibited after antisense treatment. We tested three different proteins which were shown to be highly upregulated in PC12 cells after NGF induction: SCG10, VGF8a, and peripherin. After NGF treatment of stathmin-depleted PC12 cells, which continued to proliferate, the levels of these three proteins increased to control levels. This indicates that stathmin is not required for the expression of the three NGF-inducible genes. This important observation may reflect a dissociation between morphological or cell cycle events and expression of specific markers of neuronal differentiation. A similar finding has been described in another neuronal cell line, following blockade of microtubule component MAP2 expression (Dinsmore and Solomon, 1991). Our data are consistent with a model of neurotrophin action where different cellular responses characteristic of neuronal differentiation are regulated through distinct upstream signaling events (for review see Kaplan and Stephens, 1994).

The intracellular pathways stimulated by NGF have been extensively studied over the past few years. After stimulation of the high affinity NGF receptor TrkA, one major signaling pathway involves p21ras and an associated kinase cascade leading to the activation of MAPK (for reviews see Keegan and Halegoua, 1993; Kaplan and Stephens, 1994; Heumann, 1994). Experiments with dominant negative and constitutively active mutant forms of MAPK kinase have shown that activation of MAPK by MAPK kinase is necessary and sufficient for differentiation of PC12 cells (Cowley et al., 1994). In addition, inhibition of MAPK kinase activity with a small molecular weight inhibitor, PD 098059, provided further evidence for the requirement of the MAPK pathway in PC12 cell differentiation (Pang et al., 1995). Despite the importance of MAPK, it is not clear which of the many substrates of MAPKs are responsible for specific events associated with proliferation arrest and morphological changes.

Recently, several correlative data suggested that stathmin is a physiological substrate for MAPK. First, stathmin, which contains two consensus sites for serine/threonine proline-directed kinase (Ser25 and 38) has been shown to be an in vitro substrate for MAPK (Leighton et al., 1993; Marklund et al., 1993). Second, NGF induction leads to a rapid and strong phosphorylation of stathmin in PC12 cells (Doye et al., 1990), mostly on serine 25 which is the preferential site for MAPK (Beretta et al., 1993). Furthermore, the major stathmin kinases detectable in NGF-induced PC12 cell extracts were the two isoforms of MAPK, p42 and p44 (Leighton et al., 1993). To address the specific position of stathmin in the NGF signal transduction cascade(s), we analyzed stathmin phosphorylation in PC12 cells which have been treated with a selective inhibitor of the MAPK cascade, PD 098059. This inhibitor which blocks activation of MAPK by NGF in PC12 cells prevented NGF-induced stathmin phosphorylation. These data provided pharmacological evidence that stathmin is a physiological substrate for MAPK in the NGF signal transduction pathway leading to neuronal differentiation. Our finding that stathmin is not required for induction of the late-induced genes indicates that the branching point for expression of these genes lies upstream of the MAPK-stathmin interaction. This is consistent with the fact that transcription of

SCG10 and VGF8a can be activated by molecules upstream of MAPK (D'Arcangelo and Halegoua, 1993). Finally, the phenotype we observed in stathmin depleted PC12 cells is similar to the one of PC12 cells in which nitric oxide synthase is inhibited (Peunova and Enikolopov, 1995). Therefore, it may be interesting for the future to see whether induction of nitric oxide synthase is a downstream event of the MAPK pathway and whether stathmin is required for the induction or activity of this gene.

We conclude that stathmin is a major regulatory component of the NGF-signaling pathway in PC12 cells mediating specific aspects of neuronal differentiation. In addition, our subtractive cloning of the chick gene which is differentially expressed during the period of neurite outgrowth and synapse formation in the chick retina (Lebeau et al., 1991) strongly indicates a key role in developing neurons in vivo. The identification of proteins that interact with stathmin will lead to a better understanding of the molecular interactions underlying neuronal differentiation.

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Note Added in Proof. After submission of this manuscript, stathmin was identified as a protein that interacts with tubulin dimers and increases the catastrophe rate of microtubules in vitro (Belmont, L.D., and T.J. Mitchison. 1996. Indentification of a protein that interacts with tubulin dimers and increases the catastrophe rate of microtubules. *Cell.* 84:623–631). With regard to these data, stathmin may be a link between the NGF signal transduction pathway and the modulation of microtubule dynamics.

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