Phosphorylation of Stathmin and Other Proteins Related to Nerve Growth Factor-induced Regulation of PC12 Cells*

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We previously identified a set of soluble proteins whose phosphorylation could be originally related to the multihormonal regulations of anterior pituitary cells. Among these proteins, stathmin (proteins 7 and 8) was found to be ubiquitous and mostly abundant in neurons. Interestingly, stathmin and some other phosphoproteins of the same set could be identified also in PC12 cells in culture. Their phosphorylation was stimulated in these cells by nerve growth factor (NGF) in a way associated with its short term actions, probably corresponding to the early steps of its neuronal differentiating activity. In addition, the same proteins had their phosphorylation stimulated in the presence of fibroblast growth factor, known to stimulate PC12 cell differentiation in a way similar to NGF. A pharmacological analysis allowed us to distinguish three characteristic subsets of phosphoproteins, respectively, affected by CAMP-dependent agents, by CAMP-independent ones, or by both types of agents. Moreover, phosphorylation of stathmin and some other proteins was additive in the presence of NGF and of the CAMP-promoting agent forskolin. Altogether, the present results unravel some intracellular mechanisms related to the regulation of PC12 cells by extracellular effectors. They extend to the regulation of cell differentiation in our recent model for stathmin (Sobel, A., Boutterin, M.-C., Beretta, L., Chaeiwehe, H., Doye, V., and Peyro-Saint-Paul, H. (1989) J. Biol. Chem. 264, 3765–3772) as an ubiquitous intracellular relay possibly integrating the actions of diverse second messenger pathways involved in cell regulations.

Many extracellular agents regulate cell differentiation and functions through their binding to specific receptors at the cell surface. In the subsequent intracellular transduction of these signals, phosphorylation-dephosphorylation of proteins is known to be a major regulatory mechanism in all biological systems (1–5).

We previously identified, by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE),1 a set of cytoplasmic proteins designated proteins 1–16, whose phosphorylation was associated with the regulation of the diverse pituitary cell types by various extracellular effectors (6–9). Some of these proteins were also detected in other biological systems, such as muscle cells (10).

One 19-kDa phosphoprotein, corresponding to the proteins that we originally designated as 7 and 8 and that we recently proposed to name “stathmin,” appears actually to be ubiquitous (11) and identical to proteins P19 (12), p17/ or prosolin (13), and p18 (14). We proposed that it might play a general role as an intracellular relay integrating the various second messenger pathways activated by diverse extracellular signals (11). Two distinct isoforms α and β of stathmin have been identified (15), differing by post-translational modifications (16). Their common cDNA was recently cloned from PC12 cell (16) and rat testis (17) cDNA libraries. However, stathmin is most abundant in neurons and displays a peak of expression during ontogenesis of the central nervous system around birth (12, 16, 18, 19).

The rat pheochromocytoma cell line PC12 (20) has proved to be a very good model for studying the mechanisms and regulations of neuronal differentiation. Indeed, when treated for several days with nerve growth factor (NGF), the chromaffin-resembling cells cease proliferating and acquire a sympathetic neuron-like phenotype with the outgrowth of neurites (reviewed in Ref. 21). This long term action of NGF is preceded by a number of short term responses such as membrane ruffling, activation of ornithine decarboxylase, and protein phosphorylation (reviewed in Ref. 21). PC12 cells also respond to other regulatory agents such as fibroblast growth factor (FGF), which has been shown to reproduce most if not all the biological actions of NGF (22). Other agents trigger only some of the actions of NGF such as epidermal growth factor (23, 24), tumor promoters (25), adenosine and analogues (26), or CAMP analogues which induce some neurite outgrowth but with different characteristics than with NGF (27).

The second messengers involved in NGF’s action are not elucidated, although the involvement of cAMP has been proposed (28) but also challenged (29). The possible role of Cα2 (30–33), as well as diacylglycerols (34), has been also considered (reviewed in Ref. 35).

NGF has been shown to activate several protein kinases (31, 36, 37) including a specific NGF-activated protein kinase (38). Stimulation of these kinases results in the phosphorylation of a number of intracellular proteins, some of which have been characterized by their 2D-PAGE migration properties (39–41).

In the present work, we show that proteins comigrating on two-dimensional gels with stathmin and other proteins of the set 1–16, previously identified in pituitary cells, are also present in PC12 cells. Their phosphorylation is stimulated in

1 The abbreviations used are: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; FGF, fibroblast growth factor; NGF, nerve growth factor; SCG, superior cervical ganglion; TPA, 12-O-tetradecanoyl-phorbol-13-acetate.

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response to NGF and other pharmacological effectors of PC12 cell neuronal differentiation. More generally, the pharmacological analysis of their phosphorylation gives further clues on the intracellular mechanisms involved in PC12 cell regulations. It also documents the proposed ubiquitous role of stathmin and some of the other proteins 1–16 as intracellular relays for the actions of extracellular agents including the regulation of cell differentiation.

**EXPERIMENTAL PROCEDURES**

**Materials**

Culture media and fetal calf serum were from Gibco Laboratories; horse sera were either from KC Biologicals (for PC12 cells) or from Gibco Laboratories (for GH4C1 cells). Phosphate-free Eagle’s minimum essential medium was from Flow Laboratoires, Inc. Culture dishes were from Falcon Labware or Nunc. The PC12 and GH4C1 cell lines were kindly provided, respectively, by Drs. J. Mallet, Centre National de la Recherche Scientifique (CNRS, Gif-sur-Yvette, France), L. Greene (Columbia University, New York), and Dr. Arnaud Tashjian (Harvard University, Boston). 7 S NGF purified according to Burton et al. (42) and primary cultures of newborn rat superior cervical ganglion (SCO) neurons (45) were kindly provided by Dr. M. Verdier (Institut National de la Santé et de la Recherche Médicale, Paris, France). Basic FGF, the antisem to tyrosine hydroxylase, and the reagents for homogenization buffer were, respectively, gifts from Drs. Y. Umesono, P. Chavakis, and J. Fellous. Other chemicals and their sources were as follows: 2.5 S NGF ( Collaborative Research); adenosine, forskolin, 12-O-tetradecanoylphorbol-13-acetate (TPA), apropin, pepstatin, leupetin, dithiotreitol (Sigma); DNaSe, RNase, and trypsin (Worthington); reagents for polyacrylamide gels (Serva and Bio-Rad); sodium dodecyl sulfate (Fluka); amphiocaines (Pharmacia LKB Biotechnology, Inc.); silver nitrate (Prolabo, France); 32PO4- (Commissariat à l’Energie Atomique, France).

**Methods**

**Cell Culture—GH4C1** cells were cultivated as described previously (6) in Ham’s F-10 medium supplemented with 15% horse serum and 2.5% fetal calf serum. PC12 cells were grown at 37°C with 7.5% CO2 in RPMI 1640 medium supplemented with 10% horse serum and 5% fetal calf serum. The medium was changed after 3 days in culture, and the cells were used for labeling and pharmacological experiments 4 days after plating. For nonradioactive experiments, PC12 cells were plated at a density of 106 cells/100-mm dish.

For 32PO4- labeling of GH4C1 and undifferentiated PC12 cells, cells were pretreated for a density of 5–10 x 106 cells/16-mm well. For differentiated PC12 cell labeling, cells were plated at a density of 2 x 106 cells/35-mm dish in medium supplemented with 7 S NGF (200 ng/ml).

**Radioactive Labeling and Pharmacological Treatments—32PO4-** labeling was performed by preincubating the cells for 4 h with 0.3 mCi of 32PO4- in 250 µl of phosphate-free culture medium/10-mm well or 750 µl/35-mm dish. Test agents were added directly to the radioactive medium, usually for the last 15 or 30 min as indicated on the figures. The labeling was stopped as described previously (6) except that the addition of micrococal nuclease solution was suppressed, and the samples were prepared for electrophoresis according to Garrels (44) for most modifications.

For NGF treatments, the most purified 2.5 S form was used for most short term experiments, the more economical 7 S form being used essentially for long term repetitive treatments.

**Preparation of Stathmin-enriched Samples—PC12 cells** were harvested in cold phosphate-buffered saline and centrifuged, and the pellet was resuspended in homogenization buffer with protease inhibitors (10 mm Tris-HCl, pH 7.4, 0.02% NaN3, 10 µg/ml leupeptin, 25 µg/ml apropin, 10 µg/ml pepstatin, 1 mm EDTA sodium salt). Soluble cell extracts were obtained by sonication and centrifugation at 100,000 rpm for 6 min in a Beckman TL-100 ultracentrifuge. They were adjusted to 100 mm NaCl, treated at 100°C for 2 min, and centrifuged again anew (11). In these conditions, stathmin remained in the resulting “Ss” supernatant which was further prepared for electrophoresis as described above.

**Two-dimensional Polyacrylamide Gel Electrophoresis 2D-PAGE—** 2D-PAGE was performed according to Garrels (44) and as previously described (6). The isoelectric focusing gels contained 5% total ampholines, pH 5–7 and 3–10, in the proportions 4:1. The second dimension was run on 12% acrylamide gels. The fixed gels were either first stained or dried directly and exposed for autoradiography (3–15 days) with Kodak XAR-5 films. Silver staining was performed according to Morrissey (45). In each group of samples, the same amount of either trichloroacetic acid-precipitable radioactive (5–8 x 106 cpm) in the case of radioactive samples or of protein (2–5 µg assayed by the method of Bradford (46)) in the case of unlabeled cells were electrophoresed, allowing direct comparison of autoradiograms or silver-stained gels within a given series.

**RESULTS and DISCUSSION**

Cells were labeled with 32PO4- and treated with the appropriate pharmacological agents, and the phosphorylated proteins were analyzed by 2D-PAGE autoradiography. This approach led previously to the characterization, in anterior pituitary cells, of the set of cytoplasmic phosphoproteins designated as proteins 1–16 (6, 8, 9). More recently, it has been shown that the radioactive spots numbered 7 and 8 (M, ∼19,000) correspond to the phosphorylated forms P1 and P2 of an ubiquitous protein that we proposed to name stathmin (11). Two isoforms, α and β, of this protein were identified (15), whose respective nonphosphorylated (α0, β0) and phosphorylated forms (α1–α3, β1–β3) yield corresponding spots of decreasing pI (6.2–5.5) on 2D-PAGE gels: N1 (α0), N2 (β0), P1 (α1), P2 (β1, β2), and P3 (β2, α3, β3).

**Compared 2D-PAGE Protein Phosphorylation Patterns in GH4C1, PC12, and SCG Cells**

The comparison of 32P-labeled 2D-PAGE patterns allowed us to detect, in PC12 cells, phosphoproteins migrating at the same positions as proteins 7–10 and 16 of the rat anterior pituitary GH4C1 cell line (Fig. 1A) (proteins A and B in Fig. 6 are also tentatively identified with the pituitary protein 11). Equal amounts of trichloroacetic acid-precipitable radioactive material extracted from GH4C1 and PC12 cells were mixed and coelectrophoresed. A single spot was detected at the 2D-PAGE location of each of the phosphoproteins 7–10 and 16 as illustrated on Fig. 1B for the P1 and P2 spots of stathmin (proteins 7 and 8) and for protein 16. Their homology with the corresponding GH4C1 proteins is further stressed by a comparable regulation of their phosphorylation by various extracellular effectors (see below). In addition, the PC12 cell proteins which migrated at the positions of the unphosphorylated (N1 and N2) and phosphorylated (P1, P2, and P3) spots of stathmin shared the characteristic heat resistance property (11) of this protein (see Fig. 4) and were all recognized by an antibody against brain stathmin (not shown). We also recently cloned a cDNA for stathmin from PC12 cells, coding for amino acid sequences identical to sequences determined for rat brain stathmin (16). Together, these observations indicate that the comigrating proteins are at least homologous, if not identical. We therefore use here the same designations (stathmin and proteins 9–10 and 16) for the proteins in PC12 cells as for the corresponding phosphoproteins in other biological systems.

Phosphoproteins characteristic of PC12 cells were also identified on the 2D-PAGE autoradiograms (Fig. 1A). The different forms of tyrosine hydroxylase, first detected by reference to Nose et al. (39), were further identified on immunoblots with a specific antisemur (not shown). Chlartin microtubule-associated proteins were also identified by analogy with the 2D-PAGE patterns presented by Black et al. (40) and by the observation of some of their previously described properties (47) such as long-term increased phosphorylation induced by NGF and inhibition of this effect by lithium (not shown).
Since PC12 cells can be converted by NGF to sympathetic neuron-like cells (21), we compared their phosphorylation pattern with that of SCG neurons in primary culture (Fig. 1A). As in PC12 cells, phosphoproteins of the set 7–16, including stathmin, were present in these peripheral neurons from nontumoral origin (some of these proteins, which appear faintly on the figure, were clearly detectable on the original autoradiograms). Since PC12 cells can be converted by NGF to sympathetic neuron-like cells (21), we compared their phosphorylation pattern with that of SCG neurons in primary culture (Fig. 1A). As in PC12 cells, phosphoproteins of the set 7–16, including stathmin, were present in these peripheral neurons from nontumoral origin (some of these proteins, which appear faintly on the figure, were clearly detectable on the original autoradiograms).

Regulation of PC12 Cell Protein Phosphorylation by NGF

2D-PAGE Phosphoprotein Pattern Changes Induced by NGF—NGF has been shown to induce the phosphorylation of various well identified intracellular substrates like tyrosine hydroxylase (28), ribosomal protein S6 (37), vinculin (48), synapsin (49), histones (28), and other nuclear proteins (60, 51). When PC12 cells were prelabeled with [32P]orthophosphate and treated with NGF (200 ng/ml) for 30 min, the phosphorylation of several proteins detected on 2D-PAGE autoradiograms, including tyrosine hydroxylase, was stimulated. This latter effect was thus used in all experiments as an internal control for effective NGF stimulation. Phosphorylation of stathmin, protein 16 (Figs. 2–6), and of the additional protein 17 (Fig. 6) was clearly and consistently stimulated by NGF. In parallel, a decrease in the intensity of spot 9 was accompanied by a concomitant increase of the slightly more acidic spot 10 (Figs. 3 and 5), appearing, like in pituitary cells (6, 8, 9), as a possible conversion of phospho-

protein 9 to 10, due to phosphorylation or to another molecular modification.

Stimulation of PC12 cells by NGF simultaneously induced a conversion of both α and β isoforms of stathmin from their unphosphorylated to their phosphorylated forms, as reflected on silver-stained gels by a shift from spots N1 (α1) and N2 (β0) toward P1 (α1) and P2 (β1, α2). Spot P3 (β2, α3, β3) was not clearly detected by silver staining in our study (Fig. 4). This result indicates that the NGF-stimulated 32P incorporation reflects not only an activated phosphate turnover but an actual increase in the degree of phosphorylation of stathmin. Together with the observation that forskolin and adenine induced the phosphorylation of the β1 and α2 forms of stathmin migrating as P2 (Figs. 5 and 6), it also demonstrates that, as in GH cell regulations (9), both the α and β isoforms

![Fig. 1. 2D-PAGE identification of common phosphoproteins in GH4C1, PC12, and SCG cells in culture. A, the figure shows 2D-PAGE autoradiograms of 32PO4−-labeled GH4C1, PC12, and SCG cells. GH4C1 and PC12 cells were treated for 30 min, respectively, with thyrotropin-releasing hormone (100 nm) and 7 S NGF (200 ng/ml) to enhance the detection of proteins of the set 7–16; SCG neurons were cultured and labeled in the continuous presence of 7 S NGF (1 μg/ml) as required for their survival in culture. Arrowheads indicate the locations of proteins previously identified in pituitary cells (6, 8) and the corresponding spots detected on the PC12 and SCG phosphorylation patterns. Numbers 7 and 8 refer to the previous nomenclature of the stathmin spots P1 and P2. Spots 9–10 and 16 were more clearly detected on the original autoradiograms. The locations of tyrosine hydroxylase (7X!) and chartin (9–10 and 16) were more clearly detected on the original autoradiograms, indicating that the corresponding PC12 proteins exactly comigrate with their respective GH4C1 homologs.](image)
Protein Phosphorylations Related to PC12 Cell Regulations

FIG. 4. Time course of NGF action on the phosphorylation-dephosphorylation of stathmin. PC12 cells were treated for increasing times with 7 S NGF (200 ng/ml). Approximately 5 μg of protein of stathmin-enriched samples (see under “Methods”) were submitted to 2D-PAGE followed by silver staining. This method allows the detection of spots corresponding both to the unphosphorylated (N1 and N2) and phosphorylated (P1 and P2) forms of stathmin. Spot P3 was too weakly detected by silver staining and is not shown. Note the NGF-induced conversion of stathmin from its unphosphorylated to its more acidic and phosphorylated forms and the subsequent reverse conversion occurring in the continuous presence of NGF.

of stathmin are involved in the biological regulations of PC12 cells.

Dose Response and Kinetics of NGF Effects—Phosphorylation of tyrosine hydroxylase, stathmin, and proteins 16 (Fig. 2) and 17 and the apparent conversion of protein 9 to 10 (not shown) were stimulated in the concentration range where NGF elicits its biological actions, with a maximum effect at 20 ng/ml, stable up to 200 ng/ml.

In the presence of 200 ng/ml 2.5 S NGF, stimulation of the phosphorylation of all the above described proteins occurred rapidly (Fig. 3). The apparent conversion of protein 9 to protein 10 was the earliest signal detected (1 min), followed by stimulation of stathmin (P1-P3) and proteins 16 and 17 (2 min). Maximal effects were reached at 5 min for proteins 9 and 10 and at 1 h for the other proteins. In addition, the time course of the NGF-stimulated conversion of stathmin isoforms from N1 and N2 to P1 and P2 (Fig. 4) paralleled that of 32P incorporation into P1-P3.

After its maximal conversion at 1–2 h, stathmin was dephosphorylated, the distribution of its various forms returning to its basal state at 8 h (Fig. 4) and remaining in that state for up to 24 h in the continuous presence of NGF (not shown). In agreement with these data, the basal levels of 32P incorporation into stathmin and proteins 9–10, 16 (Fig. 5), and 17 (not shown) were similar in PC12 cells treated with NGF (200 ng/ml 7 S NGF) for 4 days and in untreated cells, and addition of fresh NGF induced no further stimulation (not shown). This relatively rapid dephosphorylation and subsequent “insensitivity” of stathmin thus reflects a “desensitization” of the stathmin phosphorylation pathway for the action of NGF, the cellular and molecular levels at which it takes place remaining to be determined.

Altogether, the relatively rapid stimulation and subsequent reversion of the phosphorylation of stathmin and proteins 9–10, 16, and 17 is thus most likely related to the acute biological actions of NGF, which themselves are likely to be the first steps of its long term neuronal differentiating activity. In addition to previous correlations with the regulations of cell proliferation and functions in diverse biological systems, this is the first example of a correlation between the regulation of cell differentiation and the phosphorylation of stathmin and proteins 9–10, 16, and 17.

Phosphorylation Patterns Induced by Other Pharmacological Agents

The same approach as with NGF was used to examine the effects of other pharmacological effectors whose biological, morphological, and/or biochemical actions on PC12 cells have been described. Since in NGF-pretreated cells phosphorylation of the above described proteins was no longer stimulated by NGF, we also determined if it could still be stimulated by other pharmacological effectors.

Fibroblast Growth Factor FGF—Among various effectors regulating PC12 cell functions or differentiation, FGF induces the neuronal differentiation of PC12 cells in a way similar to NGF, whose wide spectrum of biological effects it most closely reproduces (22), including phosphorylation of several proteins like tyrosine hydroxylase (52) or a nuclear protein and Nsp100 (22).

Basic FGF (5 ng/ml) stimulated phosphorylation of stathmin and protein 16, as well as the apparent conversion of
and they also induced phosphorylations not detected with NGF, such as proteins A and B (Fig. 6). These observations extend previous ones by Halegoua and Patrick (28) of a partial overlap of the spectrum of proteins phosphorylated by NGF and cAMP.

The lack of stimulation of proteins 16 and 17 (Fig. 6) by agents acting through cAMP indicated, however, that the spectrum of protein phosphorylation stimulated by NGF or FGF was partially distinct from that induced by pharmacological effectors enhancing intracellular CAMP. We thus completed this approach by studying the additivity of phosphorylation patterns induced by saturating concentrations of 2.5 S NGF (200 ng/ml) and forskolin (10 μM) (Fig. 6). Although they are not quantitative, our data indicate that the effects of NGF and forskolin were at least partially additive; phosphorylation of stathmin was higher than with either agent alone, as attested by the strong intensity of its most phosphorylated spot, P3. In addition, forskolin and NGF mutually potentiated or inhibited some of their respective phosphorylation effects (Fig. 6).

These results, together with the fact that long term NGF pretreatment did not affect forskolin (Fig. 5) or adenosine (not shown) responses, are also in agreement with the fact that NGF and cAMP trigger distinct regulatory pathways, which only partially overlap in their downstream effects.

TPA—Ca\(^{2+}\) (30–33, 36) and diacylglycerols (34) were also proposed to be involved in the biological actions of NGF. Since both agents are known to regulate the Ca\(^{2+}\)/phospholipid-dependent protein kinase (protein kinase C), we also examined the effects on protein phosphorylation of the tumor promoter TPA, a direct activator of this enzyme which mimics some of the actions of NGF (26). TPA (100 ng/ml) had only a small effect on undifferentiated PC12 cells, significantly enhanced after the 4-day differentiating treatment by NGF. It induced the apparent conversion from spot 9 to 10 and the phosphorylation of spots P1, P2, 16 (Fig. 5), and 17 (not shown).

Interestingly, phosphorylation of stathmin (spots P1 and P2) and proteins 16 and 17 was shown in NGF-pretreated cells to be stimulated also by K\(^+\) depolarization (39), most likely through the resulting increase of the intracellular Ca\(^{2+}\) concentration. Phosphorylation of these proteins is thus related to the activation of diverse second messenger pathways, the Ca\(^{2+}\) and/or phospholipid one(s) being more effective in differentiated than in untreated PC12 cells for reasons still unclear at the present stage.

**Characteristic Features of Phosphoproteins Related to NGF- and cAMP-triggered Intracellular Regulatory Pathways**

**Proteins 9–10**—The apparent conversion of phosphoprotein 9 to 10, although possibly occurring through a modification other than phosphorylation, is a general and ubiquitous phenomenon observed in diverse biological systems (6, 8, 9). It was related in each case to activations of cell responses by various extracellular agents and, as for stathmin, in a way not specific to any particular second messenger pathway.

**Proteins A and B**—On the other hand, proteins labeled A and B on Fig. 6 were stimulated only by cAMP-related effectors. Interestingly, they most likely correspond to protein 11 previously identified in pituitary cells as a specific cAMP-responsive protein (6, 8, 9). Unlike stathmin, they appear thus more generally as ubiquitous markers of the activation of a distinct and specific second messenger pathway.

**Proteins 16 and 17**—Conversely, phosphorylation of proteins 16 and 17 was stimulated only in the presence of cAMP-independent effectors. This result with PC12 cells generalizes...
previous observations with putitary (8), muscle (10), or neuronal (18) cells, where protein 16 was also affected only by growth factors and other CAMP-independent agents. Proteins 16 and 17 thus appear also as ubiquitous markers of the activation of distinct and specific second messenger pathways.

However, the decrease of the intensities of spots 16 and 17, after the simultaneous addition of both forskolin and NGF, together with the increase of slightly more acidic spots at the same M, level (Fig. 6) suggests that these latter spots derive, respectively, from spots 16 and 17, possibly by further phosphorylation. If this model is correct, the actions of the two phosphoproteins related to the biological actions of various second messenger pathways activated by extracellular regulators are preliminary observations indicate that proteins 16 and 17 might actually be very closely related to stathmin at the molecular level.

Stathmin—Among proteins 7–11, 16, and 17, stathmin is the best characterized on both the biological and biochemical levels. It was detected in many different biological systems, such as brain neurons in culture, indicating that they might be, like stathmin, ubiquitous phosphoproteins related to the biological actions of various second messenger pathways activated by extracellular regulators. Preliminary observations indicate that proteins 16 and 17 might actually be very closely related to stathmin at the molecular level.

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