Regulation of protein kinase activities in PC12 pheochromocytoma cells

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Stimulation of serine protein kinase activity (referred to as S6 kinase) occurs within minutes of addition of nerve growth factor (NGF) to PC12 rat pheochromocytoma cells. This enzyme activity is not related to the cAMP-dependent protein kinase (protein kinase A) or the Ca^{2+} - and phospholipid-dependent protein kinase (protein kinase C), two other protein kinases potentially involved in signal transduction. Two peaks of NGF-stimulated S6 phosphotransferase activity are observed upon ion exchange chromatography; one that co-migrates with the serine kinase previously described in chicken embryo fibroblasts and another with distinct elution properties. Several other factors are also found to regulate S6 phosphotransferase activity in PC12 cells including epidermal growth factor, insulin, and phorbol myristate acetate. Dibutyl cAMP stimulates S6 phosphotransferase activity; however, this activity is strongly inhibited by the protein kinase A heat stable inhibitor. At least two mechanisms exist through which the NGF-stimulated S6 kinase activity can be regulated, one that apparently can use protein kinase C whereas the other(s) does not. The potential roles of these protein kinase activities in signal transduction and regulation of cell growth and differentiation is discussed.

Key words: nerve growth factor/protein kinase A/protein kinase C/S6 kinase/signal transduction

Introduction

A protein kinase that has the potential to transmit signals from the plasma membrane to appropriate targets within the cell has recently been partially characterized. The serine protein kinase activity, which is assayed *in vitro* by its capacity to phosphorylate the 40S ribosomal protein S6, is rapidly stimulated in quiescent cells within minutes after addition of a variety of growth promoting factors (Wettenhall *et al.*, 1983; Novak-Hofer and Thomas, 1984; Blenis and Erikson, 1985; Tabarini *et al.*, 1985), phorbol esters (Blenis and Erikson, 1985; Tabarini *et al.*, 1985) or following expression of the Rous sarcoma virus (RSV) transforming gene product, pp60^{v-src} (Blenis and Erikson, 1985, 1986). S6 kinase activity is also stimulated in *Xenopus* oocytes upon maturation to unfertilized eggs (Erikson and Maller, 1985; Stefanovic *et al.*, 1986). In chicken embryo fibroblasts (CEF), regulation of S6 kinase activity by serum growth factors, phorbol ester or pp60^{v-src} correlates with complete phosphorylation of S6 (Blenis *et al.*, 1984; Blenis and Erikson, 1985) supporting the likelihood that this kinase is responsible for the increased S6 phosphorylation observed in the stimulated cell.

Several other protein kinases have also been proposed to be involved in signal transduction including protein kinase C, cAMP-

dependent protein kinase (protein kinase A) and Ca^{2+} /calmodulin-independent protein kinases. Partially purified S6 kinase is distinct from these kinases based on several criteria including: distinct substrate specificity, different chromatographic properties and inability of Ca^{2+} , Ca^{2+} /phospholipid, Ca^{2+} /calmodulin, or the heat stable inhibitor of protein kinase A to alter activity (Blenis and Erikson, 1985 and unpublished observations). The ability to stimulate S6 kinase activity and S6 phosphorylation following treatment with phorbol ester, which is known to bind to and activate protein kinase C (Nishizuka, 1984), suggests that this phospholipid- and Ca^{2+} -dependent protein kinase plays a role in regulating the S6 kinase. However, the regulation of S6 kinase activity by serum growth factors or pp60^{v-src} is mediated by protein kinase C-independent as well as protein kinase C-dependent pathways (Blenis and Erikson, 1986). Protein kinase C-dependent and -independent pathways have also been proposed in the transduction of signals regulating specific gene expression (Coughlin *et al.*, 1985; Kaibuchi *et al.*, 1986). It is worth noting that a strong correlation exists between agents that stimulate S6 kinase activity and those that modulate mRNA levels of several genes, including the proto-oncogenes *c-myc* and *c-fos*.

In addition to rapid changes in the expression of several proto-oncogenes upon addition of growth promoting factors to quiescent cell cultures, it now appears that similar genes are regulated in several cell lineages induced to differentiate (for review, see Müller, 1986). Much of this work has been completed with the rat pheochromocytoma cell line PC12 (Green and Tischler, 1976). PC12 cells differentiate from cells exhibiting a chromaffin-like phenotype to cells with sympathetic-neuron-like characteristics following addition of nerve growth factor (NGF) to the culture medium (for review, see Greene and Tischler, 1982). These cells exhibit a variety of short- and long-term NGF-induced responses that duplicate responses of normal neurons to NGF and thus provide a valid *in vitro* cell culture system for studying neuronal differentiation. In addition to the rapid NGF-induced expression of the proto-oncogenes *c-fos* and *c-myc* (Greenberg *et al.*, 1985; Curran and Morgan, 1985), the oncogene products pp60^{v-src} and pp21^{v-ras} have also been implicated in neuronal differentiation, as expression of the appropriate retroviruses or microinjection of the *ras* oncogene product into PC12 cells results in NGF-independent neuronal differentiation (Alema *et al.*, 1985; Noda *et al.*, 1985; Bar-Sagi and Feramisco, 1985). These results may indicate that the molecular events which regulate cell proliferation overlap with some of the events involved in signalling the induction of cell differentiation.

We report here on the regulation of S6 protein kinase and protein kinase A activities in PC12 cells by a variety of agents. In addition, we demonstrate the existence of protein kinase C-independent and -dependent mechanisms for regulation of the S6 kinase activity by NGF. These experiments were designed in an initial attempt to understand further the early steps involved in the NGF-stimulated mechanism of neuronal cell differentiation and to identify biochemical pathways involved in controlling cell proliferation and cell differentiation.

Fig. 1. Effect of NGF on the extent of S6 phosphorylation in PC12 cells. Growing PC12 cells were labeled with 5 μ Ci/ml [35 S]methionine for 24 h in methionine-free DME medium containing non-dialyzed fetal bovine serum (10%, v/v) and horse serum (5%, v/v). The extent of S6 phosphorylation was analyzed by two-dimensional polyacrylamide gel electrophoresis and fluorography (Materials and methods). A indicates the position of unphosphorylated S6. Positions a–e represent S6 phosphorylated with 1–5 mol phosphate per mol S6, respectively. (A) Untreated PC12 cells. (B) PC12 cells incubated for 2 h with 100 ng/ml NGF.

Fig. 2. Effect of increasing NGF on S6 kinase activity in PC12 cells. Cell-free lysates were prepared from PC12 cells 1 h after the addition of increasing amounts of NGF. Equal amounts of lysate protein were assayed for S6 phosphotransferase activity. S6 phosphorylation was monitored by SDS–polyacrylamide gel electrophoresis and autoradiography. Lane 1, 0.1 ng/ml; lane 2, 1 ng/ml NGF; lane 3, 10 ng/ml NGF; lane 4, 100 ng/ml NGF.

Results

NGF-stimulated S6 phosphorylation

The extent of S6 phosphorylation in PC12 cells incubated with NGF was examined by two-dimensional alkaline- and acid-urea gel electrophoresis (Figure 1). This gel electrophoretic system separates unphosphorylated 40S ribosomal protein S6 and its five phosphorylated derivatives (a–e) and provides information concerning the stoichiometry of S6 phosphorylation (Thomas *et al.*, 1982). As shown in Figure 1B, incubation of [35 S]methionine-labeled PC12 cells cultured on a collagen matrix with 100 ng/ml NGF for 2 h resulted in phosphorylation of up to 4–5 mol phosphate/mol S6 (forms d and e). Little S6 phosphorylation was observed in untreated PC12 cells (Figure 1A).

NGF-stimulated S6 kinase activity

To determine whether the increase in S6 phosphorylation in NGF-treated PC12 cells correlated with the activation of a protein kinase, cell lysates were prepared as described previously (Blenis

and Erikson, 1985) and assayed for 40S ribosomal protein S6 phosphotransferase activity. PC12 cells were incubated with 0.1, 1, 10 and 100 ng/ml NGF for 1 h and lysates assayed for S6 kinase activity. Stimulation of S6 kinase activity in PC12 by NGF was observed at 10 ng/ml and reached a plateau at 50–100 ng/ml (Figure 2). Extracts of NGF-treated cells yielded activities 2- to 10-fold greater than those found in extracts of untreated cells during the course of these experiments. Analysis of the kinetics of stimulation of S6 kinase demonstrated that maximal activity was obtained within 15 min of NGF (100 ng/ml) treatment and this level of activity was maintained for up to 10 h following NGF addition (Figure 3A). Examination of the early kinetics of S6 kinase activation revealed stimulation by 2 min following addition of NGF, with half maximal stimulated activity obtained by 7.5 min (Figure 3B).

To determine whether the measured activity consisted of the regulation of one or more S6 protein kinases, cell lysates from PC12 cells incubated with and without NGF (100 ng/ml – 1 h) were fractionated by cation-exchange (Pharmacia Mono S) and anion-exchange (Pharmacia Mono Q) chromatography (Figure 4). Two peaks of stimulated S6 kinase activity were observed upon Mono S chromatography from NGF-treated PC12 cells compared to untreated cells (Figure 4A). These results suggest the stimulation of two kinases capable of phosphorylating S6 *in vitro* or the presence of modified forms of a single kinase. Neither activity was affected by protein kinase A inhibitor or the absence of Ca^{2+} and both showed elution properties distinct from those of protein kinase C (data not shown). As shown, peak I eluted at ~225 mM NaCl and peak II eluted at 335 mM NaCl. Peak I NGF-stimulated PC12 S6 kinase activity co-eluted on the Mono S column with the single S6 kinase activity stimulated in CEF by a variety of agents (Blenis and Erikson, 1985, 1986). We observed S6 phosphotransferase activity which did not bind under these conditions to the Mono S column. This activity, however, was not regulated by the factors studied here and therefore was not further analyzed. Analysis of the activities detected after Mono S chromatography on a Mono Q column showed the PC12 peak I activity co-eluted with the CEF activity (300 mM NaCl) whereas the PC12 peak II eluted at 260 mM NaCl (Figure 4B). The relationship of the Mono S peak II from NGF-treated PC12 cells to peak I and the S6 kinase stimulated in CEF is under further investigation.

Regulation of S6 kinase activity by other agents

In addition to regulation of S6 kinase activity in PC12 cells by NGF, a variety of other agents that do not induce differentiation in these cells was observed to stimulate this activity. As shown in Figure 5, incubation of growing PC12 cells with EGF (100 ng/ml, lane 3), insulin (100 μ g/ml, lane 4), dibutyryl cAMP (dbcAMP) and isobutylmethylxanthine (IBMX) (1 mM each, lane 5), phorbol myristate acetate (PMA) (100 ng/ml, lane 6) and cycloheximide (100 μ M, lane 8) all increased the S6 kinase specific activity. Since these agents do not induce differentiation of PC12 cells, these results suggest that the S6 kinase activity in these cells is not sufficient for the differentiation process. It should be noted that sodium vanadate at concentrations (1 mM) observed to give maximal stimulation of S6 kinase activity in CEF (Blenis and Erikson, 1986) and Swiss 3T3 cells (Novak-Hofer and Thomas, 1985), had little effect in PC12 cells (lane 7). Furthermore, addition of fresh fetal calf serum and horse serum to a final concentration of 10% and 5%, respectively, had little effect on the S6 kinase activity (data not shown). The level of induction of S6 kinase activity by NGF was found to be greater

Fig. 3. Time-dependent regulation of S6 kinase activity in NGF-treated PC12 cells. (A) Cell-free lysates were prepared from PC12 cells at various times following addition of 100 ng/ml NGF. Equal amounts of lysate protein were analyzed for S6 kinase activity and *in vitro* S6 phosphorylation monitored as described (Materials and methods). 1, untreated; 2, 15 min; 3, 30 min; 4, 1 h; 5, 2 h; 6, 5 h; 7, 10 h. (B) NGF (100 ng/ml) was added to cultured PC12 cells and cell-free lysates prepared 0–15 min later. S6 phosphotransferase activity was measured as described (Materials and methods). 1, untreated; 2, 2 min; 3, 5 min; 4, 10 min; 5, 15 min. The S6 band was excised and associated radioactivity determined. Data are plotted as % maximal S6 kinase activity stimulated during this time course using basal level activity as 0% and activity at 15 min as 100% as was demonstrated in Figure 3A.

than the levels observed stimulated by the various other factors under these culture conditions (see Figures 5 and 6). However, under very dense culture conditions, NGF had less, and EGF more of an effect on S6 kinase activity than shown here (data not shown). This observation is consistent with those of Green and Tischler (for review, 1982) demonstrating the inhibitory affects of high cell density on some NGF-directed responses in PC12 cells. In addition, the dose dependency for EGF-stimulated S6 kinase was linear from 1 to 100 ng/ml (data not shown) in contrast to other EGF-directed responses in PC12 cells (Chandler and Herschman, 1980; Huff *et al.*, 1981; Greenberg *et al.*, 1985).

Combinations of various factors were added to PC12 cells to determine whether stimulation of S6 kinase activity was mediated by different mechanisms or whether the factors were activating distinct kinases. In the latter case an additive response might be observed. PC12 cells were treated with NGF, EGF, dbcAMP/IBMX and PMA (Figure 5B, lanes 2–5) and with NGF + EGF, NGF + dbcAMP/IBMX and NGF + PMA (lanes 6–8) for 1 h prior to preparation of cell lysates. The combination of NGF with EGF, dbcAMP/IBMX or PMA did not result in a significant increase of S6 kinase activity over the activity measured in lysates obtained from PC12 cells following addition of NGF alone. These data suggest that if other S6 kinase activities are regulated by these other factors, their contribution to the total S6 kinase activity is small.

Role of protein kinase A

A role for protein kinase A (cyclic AMP-dependent protein kinase) in NGF-mediated phosphorylations in PC12 cells has been

suggested (Halegoua and Patrick, 1980; McTigue *et al.*, 1985). To determine whether protein kinase A was the kinase activated in these cell lysates, reactions were carried out in the presence (+) or absence (–) of the specific heat stable inhibitor of this kinase (PKI, Walsh *et al.*, 1971). As shown in Figure 6, PKI, at a concentration completely inhibiting the ability of purified catalytic subunit of cAMP-dependent protein kinase to phosphorylate S6 (lane 7, – and +, under conditions utilizing approximately equal S6 phosphotransferase activity as that measured in the cell-free lysates), had little effect on the S6 kinase activity stimulated by NGF (lane 2, – and +), EGF (lane 3, – and +), insulin (lane 4, – and +) or PMA (lane 6, – and +). In fact, inclusion of PKI in S6 kinase assays using cell lysates from PC12 cells or CEFs occasionally results in a modest increase in the S6 phosphotransferase activity of the lysate (data not shown). However, in PC12 cells with elevated levels of cAMP (as a result of incubation with IBMX and dbcAMP), the stimulated S6 kinase activity was 50–70% inhibited by PKI (Figure 6, lane 5, – and +). The PKI-resistant S6 kinase activity (lane 5+) was still 2-fold greater than in untreated controls (lane 1, – and +) suggesting the potential activation of at least two distinct kinases in the dbcAMP/IBMX-treated cells. It should be noted, however, that the addition of NGF plus dbcAMP/IBMX appears to have less than an additive effect on S6 kinase activity (Figure 5B, lane 7). Thus, the interpretation of these data must await more experimental information. Interestingly, *in vitro* phosphorylation of a 57-kd protein was observed in all cell-free lysates (greatest stimulation of phosphorylation observed in lane 5–) and the level of this

Fig. 4. Analysis of S6 kinase activities by ion exchange chromatography. Cell-free lysates from 2×150 -mm tissue culture plates of serum-deprived chicken embryo fibroblasts (CEF) incubated with 10% calf serum (v/v) for 1 h or from growing PC12 cells left untreated or incubated with 100 ng/ml NGF for 1 h were fractionated sequentially by cation- and anion-exchange chromatography using the Pharmacia f.p.l.c. system with Mono S and Mono Q columns, respectively. Both columns were eluted with a 0–600 mM linear gradient of NaCl and 1 ml fractions were collected. Aliquots (3 μ l) of alternate fractions were assayed as described (Materials and methods). (A, panel 1) Mono S elution profile of serum-stimulated S6 kinase activity in CEF. (A, panel 2) Mono S elution profile of S6 kinase activity from untreated PC12 cells ($\circ$ — $\circ$) and cells treated with 100 ng/ml NGF ($\bullet$ — $\bullet$). (B, panel 1) Mono Q elution profile of the serum-stimulated CEF S6 kinase activity. (B, panel 2) Mono Q elution profile of NGF-stimulated PC12 S6 kinase activity, peak I. (B, panel 3) Mono Q elution profile of NGF-stimulated PC12 S6 kinase activity, peak II.

phosphorylation was similarly reduced in all reactions assayed in the presence of PKI. These results suggest that protein kinase A can be activated in PC12 cells by raising cAMP levels but that little change in protein kinase A activity toward S6 is detected in these cells after treatment with NGF, EGF, insulin or PMA.

Protein kinase C-dependent and -independent pathways

The S6 kinase activity stimulated by NGF in PC12 cells, like the S6 kinase activity from CEF (Blenis and Erikson, 1985, 1986), is chromatographically distinct from protein kinase C (J.Blenis and H.-P. Biemann, unpublished observations). However, phorbol ester rapidly activates S6 kinase in PC12 cells and therefore protein kinase C, the only known receptor for PMA (for review see Nishizuka, 1984), apparently is involved in regulating S6 kinase activity. To determine whether NGF stimulation of S6 kinase activity in PC12 cells was mediated by protein kinase C, we made use of the ability to 'down-regulate' protein kinase C as well as S6 kinase activity, by long-term exposure to phorbol ester. As has been demonstrated in CEF (Blenis and Erikson, 1986), PMA-pretreatment of PC12 cells for 12 h resulted in the inability to reactivate the S6 kinase activity upon a second addition of phorbol ester (Figure 7, lanes 3 and 4). However, a significant increase in S6 kinase specific activity was measured in PMA-pretreated PC12 cells subsequently treated with NGF (lane 2). As previously observed in CEF stimulated with pp60^{v-src} or serum growth factors (Blenis and Erikson, 1986), the NGF-stimulated response in the PMA-pretreated PC12 cells was slightly attenuated in comparison to the control (lane 1). In several experiments the attenuation of S6 kinase activity ranged from 10–50% in phorbol ester-pretreated PC12 cells subsequent-

Fig. 5. Regulation of S6 kinase activity in PC12 cells by a variety of agents and the effect of combinations of these agents. (A) Cell-free lysates were prepared from PC12 cells 1 h following the addition of various agents. *In vitro* S6 kinase activity was analyzed as described (Materials and methods). Lane 1, no treatments; lane 2, 100 μ g/ml NGF; lane 3, 100 ng/ml EGF; lane 4, 100 ng/ml insulin; lane 5, 1 mM dbcAMP and 1 mM IBMX; lane 6, 100 ng/ml PMA; lane 7, 1 mM sodium vanadate; lane 8, 100 μ M cycloheximide. In this experiment, the background *in vitro* phosphorylation in lanes 1, 7, 8 was lower than observed in other lanes, however, the results obtained with respect to S6 kinase activity were consistent over several experiments. (B) Cell-free lysates were prepared after 1 h treatment and S6 kinase activity monitored as described (Materials and methods). Lane 1, no treatment (600 c.p.m. S6); lane 2, 100 ng/ml NGF (2700 c.p.m. S6); lane 3, 100 ng/ml EGF (1300 c.p.m. S6); lane 4, 1 mM dbcAMP and 1 mM IBMX (1200 c.p.m. S6); lane 5, 100 ng/ml PMA (1400 c.p.m. S6); lane 6, 100 ng/ml NGF plus 100 ng/ml EGF (3100 c.p.m. S6); lane 7, 100 ng/ml NGF plus 1 mM each dbcAMP and IBMX (2600 c.p.m. S6); lane 8, 100 ng/ml NGF plus 100 ng/ml PMA (3200 c.p.m. S6).

ly treated with NGF, whereas >90% inhibition was observed in pretreated cells rechallenged with PMA.

Discussion

In this report we present evidence supporting the presence of protein kinase activity in PC12 cell-free lysates which is distinct from protein kinase A and C and yet, like these protein kinase activities, may participate in the regulation of specific cellular processes. As measured by phosphorylation of the ribosomal protein S6, the activity is rapidly stimulated following addition of NGF with half maximal activity observed after 7.5 min. This change in activity appears to be regulated at the post-translational level based on the kinetics of activation of enzyme activity, and on the inability of cycloheximide to inhibit stimulation. Thus we have identified previously undescribed protein kinase activity in PC12 cells with the potential to mediate rapidly the transmission of information from the cell membrane to the nucleus in response to the appropriate growth factors. In contrast to several cellular responses which appear transitory in nature, such an expression of the proto-oncogenes *c-fos* and *c-myc* (Greenberg *et al.*, 1985), the S6 kinase activity remains elevated for >10 h following addition of NGF. In addition to NGF, cell lysates from PC12 cells incubated for 1 h with EGF, insulin, PMA, dbcAMP and IBMX, and cycloheximide all exhibit increased phosphotransferase activity. Under the culture conditions described (Materials and methods), the level of stimulation in NGF-treated PC12 cells was greater than observed with various other factors tested here. The

Fig. 6. Effect of cAMP-dependent protein kinase inhibitor on S6 kinase activity. Cell-free lysates were prepared from PC12 cells incubated for 1 h with various agents. Equal amounts of lysate protein were assayed in the absence (–) and presence (+) of the heat stable inhibitor of the cAMP-dependent protein kinase (PKI, Walsh *et al.*, 1971). The concentration of PKI used completely inhibited *in vitro* S6 phosphorylation by purified catalytic subunit of cAMP-dependent protein kinase (activity of protein kinase A towards S6 approximately equalling the S6 phosphotransferase activity in the cell-free lysates). Lane 1, no addition; lane 2, 100 ng/ml NGF; lane 3, 100 ng/ml EGF; lane 4, 100 μ g/ml insulin; lane 5, 1 mM dbcAMP and 1 mM IBMX; lane 6, 100 ng/ml PMA; lane 7, *in vitro* assays with cAMP-dependent protein kinase. Migration of mol. wt standards shown on right. Arrow indicates position of a 57-kd phosphoprotein sensitive to *in vitro* phosphorylation in all cell-free lysates by the heat stable inhibitor of protein kinase A.

significance of the rapid post-translational regulation of the S6 kinase activity to NGF-stimulated PC12 neuronal differentiation is presently unknown. However, our data provides new information which should lead to the design of several experiments aimed at specifically addressing this question. For example, it will be necessary to compare the long-term kinetics of activation of S6 kinase by NGF to that stimulated by other agents to determine if differences in time course of activation may be related to the inability of the latter growth factors to induce the differentiation process. It is also of interest that two distinct peaks of NGF-stimulated S6 kinase activity are observed to elute from cation-exchange (f.p.l.c. Mono S) columns, whereas in quiescent CEF induced to proliferate with serum, PMA or by expression of pp60^{v-src}, only a single peak of stimulated S6 kinase activity is observed (Blenis and Erikson, 1985). The identity of this second peak of stimulated S6 kinase activity and any potential relationship to the first peak or the enzyme stimulated in CEF is currently under investigation. Furthermore, it will be important to analyze S6 kinase activity in PC12 cells stimulated by the various other non-differentiation-inducing factors by cation-exchange chromatography and compare these results to the NGF-regulated enzyme elution profile. It may be relevant that two S6 kinase activities are stimulated during NGF-induced PC12 differentiation, as two distinct S6 protein kinase activities from *Xenopus* eggs have also been observed after chromatography on DEAE–Sephacel (Erikson and Maller, 1986). A comparison of these enzymes to the NGF-induced S6 kinase activities in PC12 cells and chicken embryo fibroblasts is necessary to improve our understanding of these processes.

Fig. 7. Effect of PMA pretreatment on stimulation of S6 kinase activity by NGF. Cell-free lysates were prepared from PC12 cells incubated with 100 ng/ml PMA (0.05% DMSO) or 0.05% DMSO for 12 h followed by a 1-h incubation with NGF or PMA as described below. S6 kinase activity was monitored as described (Materials and Methods). Lane 1, DMSO-pretreated PC12 cells plus NGF for 1 h (2100 c.p.m. S6); lane 2, PMA-pretreated PC12 cells plus NGF for 1 h (2000 c.p.m. S6); lane 3, DMSO-pretreated PC12 cells plus PMA for 1 h (1700 c.p.m. S6); lane 4, PMA-pretreated PC12 cells plus PMA for 1 h (1000 c.p.m. S6); lane 5, DMSO-pretreated PC12 cells (900 c.p.m. S6); lane 6, PMA-pretreated PC12 cells (1000 c.p.m. S6).

The role for protein kinase A (cAMP-dependent protein kinase) in NGF-regulated phosphorylations and neuronal differentiation in PC12 cells has received considerable attention and yet remains controversial (for review see Greene and Tischler, 1982). Although it has been suggested that protein kinase A mediates the phosphorylation of ribosomal protein S6 in PC12 cells (Halegoua and Patrick, 1980), claims that cAMP can play a second messenger role for NGF in PC12 cells (Schubert and Whitlock, 1977; Schubert *et al.*, 1978) have not been confirmed (Hatanaka *et al.*, 1978; Connolly *et al.*, 1984; Greene *et al.*, 1986). In this regard, it has been shown that stimulation of intracellular levels of cAMP in Swiss 3T3 cells has little effect on S6 phosphorylation (Thomas *et al.*, 1980). In fact, raising cAMP levels attenuates serum-stimulated S6 phosphorylation (Thomas *et al.*, 1980) as well as S6 kinase activity (J. Blenis, unpublished observations). In addition, *in vitro*, protein kinase A incorporates only 1–2 mol phosphate/mol S6 as determined by direct quantitation and two-dimensional gel electrophoresis (Traugh *et al.*, 1981; Wettenhall and Cohen, 1982, and unpublished observations). As we have shown, PC12 cells incubated with NGF for 2 h resulted in the incorporation of up to 5 mol phosphate/mol S6 (Figure 1). The results presented here suggest that incubation of cells with dbcAMP and IBMX activates protein kinase A and that these cells exhibit elevated levels of S6 phosphotransferase activity. Greater than 50% of this activity is sensitive to the heat stable inhibitor (PKI) of protein kinase A whereas the S6 kinase activity in cells treated with NGF or various other factors is unaffected by PKI. It is important to note that the PKI-resistant activity in PC12 cells treated with dbcAMP/IBMX is

still slightly elevated compared to controls which might account for the small increase in S6 phosphorylation observed by Halegoua and Patrick (1980). These data may be interpreted to indicate that activated protein kinase A in extracts of dbcAMP/IBMX-treated cells phosphorylates S6, as does the purified enzyme *in vitro*, but that it may be an inefficient S6 kinase *in vivo*. Alternatively protein kinase A may phosphorylate some but not all of the available serine sites as has been previously suggested.

Previous data suggest that regulation of the S6 kinase in fibroblasts involves protein kinase C-dependent and -independent pathways. Here we have demonstrated that PMA-pretreatment also abolishes the stimulation of S6 kinase activity in PC12 cells by subsequent treatments with phorbol ester whereas only a partial attenuation is observed in NGF-treated cells. Thus at least two mechanisms are also involved in regulating S6 kinase activity in NGF-treated PC12 cells. It remains to be determined if down-regulation of protein kinase C differentially affects NGF-stimulated peak I or peak II activities, or the effects of PMA pretreatment on insulin, EGF, or dbcAMP-stimulated activities.

Protein phosphorylation has been proposed as a mechanism for controlling cell proliferation and differentiation processes. In this study we have carried out an analysis of the regulation of protein kinases potentially involved in the signal transduction process(es) in rat pheochromocytoma cells following addition of NGF to the culture medium. It is of interest that agents which initially act on very different receptors lead, at an early stage of signal transduction, to an activation of a common S6 kinase. Thus, the identification of other substrates for this serine-specific enzyme may further define the biochemical pathways leading to cell proliferation and/or cell differentiation.

Materials and methods

Cell cultures

CEF were prepared from virus-free 11- to 12-day-old chicken embryos (Spafas) and were routinely cultured at 41.5°C in Richter's Improved MEM with Insulin (Irvine Scientific, Santa Ana, CA) containing 5% calf serum (HyClone Laboratories, Logan, UT) and 10% tryptose phosphate broth (Difco). Culture conditions with CEF for experiments have been described (Blenis *et al.*, 1984; Blenis and Erikson, 1985). Rat pheochromocytoma cells (PC12) were cultured in 100-mm Corning cultured dishes in DME medium containing heat inactivated 10% fetal bovine serum and 5% horse serum (HyClone labs). For all experiments, PC12 cells were transferred to 100-mm or 150-mm culture dishes previously coated with bovine collagen (type 1, Collaborative Research, Lexington, MA) at $1-2 \times 10^6$ or $4-5 \times 10^6$ cells/dish, respectively. After 24-48 h, S6 phosphorylation or S6 kinase activity stimulated by a variety of agents was analyzed (details of experimental conditions are given in figure legends). 2.5S-NGF and EGF (receptor grade) were purchased from Collaborative Research. Insulin was obtained from Calbiochem. PMA was from Pharmacia P-L Biochemicals. Dibutyl cAMP and IBMX were from Sigma. Cycloheximide was obtained from Boehringer Mannheim.

Preparation of cell-free lysates and chromatographic fractionation of the protein kinase activity

Cell-free lysates from PC12 cells were prepared essentially as described for CEF (Blenis and Erikson, 1985). Briefly, monolayers were gently washed with cold lysis buffer (10 mM potassium phosphate/1 mM EDTA/5 mM EGTA/10 mM $MgCl_2$ /100 mM glycerol 2-phosphate/1 mM sodium vanadate/1 mM dithiothreitol (DTT)/40 μ g of phenylmethylsulfonyl fluoride per ml, pH 6.5), cells were then scraped off the dish in 1 ml of lysis buffer and homogenized in a 1 ml Dounce homogenizer with 25 strokes of a tightly-fitting pestle. Use of protease and phosphatase inhibitors was required for maintenance of activity. Lysates were prepared at 4°C. After centrifugation at 10 000 g for 15 min, the resulting supernatant was adjusted to 10% (vol/vol) glycerol and centrifuged at 150 000 g for 1 h. The supernatant (cell-free lysate) was stored at -5°C. Cells or cell-free lysates were never frozen before analysis of S6 kinase activity as such treatment resulted in alterations of biochemical properties as determined by changes in chromatographic properties (J.Blenis and C.Kuo, unpublished results). Storage at -5°C was used to prevent the influence of proteolysis on S6 kinase activity

and its chromatographic properties. Under these conditions, activity was maintained for several days. However, all assays were completed within 6-18 h of preparation of cell-free lysates. S6 kinase activity in cell-free lysates was fractionated by cation-exchange chromatography (Mono S, Pharmacia f.p.l.c. system) followed by anion-exchange chromatography (Mono Q, Pharmacia) as described previously using only Mono S column buffer containing 0.05% Brij 40 (Blenis and Erikson, 1985). Partially purified enzyme was concentrated by dialysis against 10 mM potassium phosphate/1 mM EDTA/5 mM EGTA/10 mM $MgCl_2$ /2 mM DTT/0.05% Brij 40/50% (vol/vol) glycerol, pH 6.5, and stored at -20°C.

Assay of S6 phosphotransferase activity

Unphosphorylated 40S ribosomal subunits were prepared from *Xenopus laevis* ovaries as described by Erikson and Maller (1985). S6 phosphotransferase activity in the various lysates was determined with equal amounts of lysate protein (generally 2-5 μ g) (Bradford, 1976) in 5 μ l of lysis buffer, 0.16 A_{260} unit of 40S ribosomal subunits, 10 mM $MgCl_2$, 50 μ M ATP (10-20 μ Ci of [γ - 32 P]-ATP) and 20 mM Tris-HCl, pH 7.5. Reaction mixtures (25 μ l) were incubated for 15 min at 30°C and were stopped by the addition of 6.5 μ l of five times concentrated gel electrophoresis sample buffer followed by boiling for 5 min. Under these standard assay conditions, incorporation of 32 P into S6 was linear from 0 to 45 min.

Gel electrophoresis

40S ribosomal proteins used in protein kinase assays were resolved by SDS/12% polyacrylamide gel electrophoresis as described by Laemmli (1970). For qualitative analysis of *in vitro* S6 phosphorylation, Coomassie Blue-stained gels were dried and exposed to Du Pont Cronex-4 film or Kodak XAR-5 X-Omat film with Du Pont Lightning Plus intensifying screens. For quantitation, radiolabeled S6 was identified and excised, and associated radioactivity determined by liquid scintillation spectrometry.

Ribosomes were isolated from [35 S]methionine-labeled PC12 cells as described by Thomas *et al.* (1977). Cells, seeded at 5×10^6 cells per 150-mm collagen-coated dish, were labelled in methionine-free DME medium containing non-dialyzed fetal bovine serum (10% vol/vol) and horse serum (5%, v/v) for 24 h prior to cell lysis. The extent of S6 phosphorylation in cultured PC12 cells was analyzed by measuring its change in electrophoretic mobility on two-dimensional alkali- and acid-urea polyacrylamide gels (Kaltschmidt and Wittman, 1970; Thomas *et al.*, 1982).

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