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Nerve growth factor stimulates protein tyrosine phosphorylation in PC-12 pheochromocytoma cells

(microtubule-associated protein/kinase/signal transduction/epidermal growth factor)

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ABSTRACT **The cellular actions of nerve growth factor (NGF) and epidermal growth factor (EGF) may be mediated by changes in protein phosphorylation. The tyrosine phosphorylation of two predominant proteins of molecular mass 40 and 42 kDa is seen in PC-12 cells treated with NGF or EGF, correlating with activation of a previously identified serine/threonine protein kinase that phosphorylates microtubule-associated protein (MAP). Stimulation of phosphoprotein (pp) 40 and 42 phosphorylation and MAP kinase activity by NGF but not EGF is selectively attenuated by staurosporine and K-252A. Moreover, the time courses of pp40/42 phosphorylation and MAP kinase activation produced by NGF or EGF are identical. Chromatography of lysates from growth factor-treated cells on ion-exchange or hydrophobic-interaction HPLC resolves MAP kinase into two peaks, neither of which precisely coelutes with pp40 or pp42. One of these peaks (II) exhibits no detectable phosphotyrosine. The other peak (I) has some overlap with pp40. However, the activity residing in both peaks is almost completely inhibited after treatment with alkaline phosphatase, suggesting that, at least, serine/threonine phosphorylation is required for the activity of these enzymes. These data indicate that while tyrosine phosphorylation appears to be a critical early event in NGF action, the role of this modification in activation of MAP kinases remains unclear.**

Although the molecular mechanisms involved in the action of nerve growth factor (NGF) remain poorly understood, numerous studies have indicated a role for protein phosphorylation (1–4). We recently described a serine/threonine protein kinase in PC-12 cells that is stimulated by both NGF and epidermal growth factor (EGF) (3, 4). This protein kinase catalyzes the phosphorylation of microtubule-associated protein (MAP) and appears similar to 42-kDa growth factor-sensitive MAP kinases detected in 3T3-L1 adipocytes (5–7) and Swiss mouse 3T3 fibroblasts (8, 9). Additionally, a 54-kDa cycloheximide- and insulin-sensitive MAP kinase was identified in rat liver (10), and a cDNA encoding a putative insulin-stimulated MAP kinase has been cloned from Rat-1 fibroblasts (11). There is some data to suggest that these kinases are themselves phosphorylated on tyrosine by the action of receptor or nonreceptor tyrosine kinases (12) and may, in turn, serve as intermediates in a phosphorylation cascade induced by certain growth factors. In addition, recent reports (13, 14) suggest that phosphorylation of the enzyme on serine or threonine residues may also contribute to regulation of catalytic activity.

Although numerous tyrosine-phosphorylated proteins have been identified in oncogenically transformed or growth factor-stimulated cells, little is known about the functional significance of these phosphoproteins. A number of reports have described a family of tyrosine-phosphorylated proteins that migrate with an apparent molecular mass of 42 kDa on SDS/PAGE (15–26). Recent evidence suggests that pp42A, one of these proteins phosphorylated on tyrosine in response to a diverse number of mitogens, is "MAP kinase" (13). These observations led us to explore the possibility that activation of MAP kinase in PC-12 cells by NGF might result from the tyrosine phosphorylation of the enzyme.

EXPERIMENTAL PROCEDURES

Materials. All reagents were from Sigma except for tissue culture supplies (GIBCO), 2.5S NGF and EGF (Bioproducts for Science, Indianapolis), [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq) (NEN), ^{125}I-labeled protein A (30 mCi/mg) (Amersham), staurosporine and K-252A (Kamiya Biochemical, Thousand Oaks, CA), okadaic acid (Moana Biochemical, Honolulu), and x-ray film (Kodak). The Mono Q HR 5/5 fast protein liquid chromatography and phenyl Superose fast protein liquid chromatography columns were from Pharmacia LKB Biotechnology. Anti-phosphotyrosine antibodies were prepared as described by Wang (27, 28).

Analysis of Protein Tyrosine Phosphorylation in PC-12 Cells. PC-12 cells were adapted to grow on plastic 100-mm tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM)/10% fetal bovine serum/5% horse serum. Before hormonal treatment, the medium was replaced with serum-free medium and incubated for 1 hr. Unless otherwise indicated, cells were incubated with 4 nM NGF or 20 nM EGF for the indicated time at 37°C. After treatment, the medium was removed, cells were washed three times with 10 ml of ice-cold Ca^{2+}-free phosphate-buffered saline and solubilized in 600 μl of lysis buffer (10 mM Tris·HCl, pH 7.4/1% SDS/1 mM phenylmethylsulfonyl fluoride). Lysed cells were scraped into microcentrifuge tubes, frozen, and subsequently boiled for 5 min, followed by dilution with 400 μl of RIPA buffer (10 mM Tris·HCl, pH 7.4/1% Triton X-100/150 mM NaCl/2 mM EGTA/1 mM sodium orthovanadate/0.1 mM sodium molybdate/2 mM dithiothreitol/leupeptin at 10 μg/ml/aprotinin at 10 μg/ml). Lysates containing 100 μg of protein were subjected to SDS/10% PAGE. Proteins were transferred electrophoretically to a nitrocellulose filter and probed with anti-phosphotyrosine antibody, followed by ^{125}I-labeled protein A (29). Autoradiography was done on x-ray film with an intensifying screen at −80°C for 12 hr.

Assay of MAP Kinase Activity. This activity was assayed as described (3). Briefly, 20-μl aliquots of cell lysates were incubated with MAP at 0.2 mg/ml for 10 min at 30°C in a final

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Abbreviations: NGF, nerve growth factor; EGF, epidermal growth factor; MAP, microtubule-associated protein.

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Table 1. Effect of various protein kinase modulators on activation of MAP kinase by NGF and EGF in PC-12 cells

Addition	Modulators, (MAP kinase units/mg) × 100				
	None	Staurosporine	K-252A	Okadaic acid	PMA
None	1.26 ± 0.11	ND	ND	3.83 ± 0.01	1.41 ± 0.12
NGF	5.00 ± 1.17	1.00 ± 0.05	1.20 ± 0.14	9.76 ± 1.20	3.54 ± 0.11
EGF	4.08 ± 1.72	6.31 ± 0.41	4.67 ± 0.48	3.65 ± 0.91	ND

PC-12 cells were treated for the indicated conditions in Fig. 1. Aliquots (20 μl) of the cell-free lysates were assayed for MAP kinase activity as described. Results are the means of duplicate determination ± SD and are representative of two independent experiments. PMA, phorbol 12-myristate 13-acetate.

volume of 50 μl containing 50 mM Tris (pH 7.4), 2 mM EGTA, 10 mM $MgCl_2$, and 40 μM [γ-^{32}P]ATP (10 μCi). The reaction was stopped by the addition of 5× Laemmli SDS sample buffer (30), and phosphorylated MAP was resolved by SDS/7.5% PAGE (30). Coomassie blue-stained bands containing phospho-MAP were excised from the gels, and incorporated radioactivity was measured by Cerenkov counting.

Chromatography. Lysates prepared from ten 100-mm dishes with RIPA buffer (200 μl per dish) were partially purified on DEAE-cellulose (6). Peak fractions were concentrated by immersible-CX ultrafiltration (Millipore) to ≈1.5 ml and filtered through a 0.22-μm Millipore filter. One milliliter of the concentrated fraction was chromatographed on an HR 5/5 Mono Q column, equilibrated with buffer A (25 mM Tris, pH 7.4/2 mM EGTA/2 mM dithiothreitol/0.2 mM phenylmethylsulfonyl fluoride). The column was eluted at a flow rate of 1 ml/min, washed with 3 ml of buffer A, and eluted with a 30-ml linear gradient of 0–0.5 M NaCl in buffer A. Lysates were also analyzed by hydrophobic-interaction HPLC. One milliliter of the concentrated lysate was chromatographed on a phenyl Superose fast protein liquid chromatography column at a flow rate of 0.25 ml/min. The column was equilibrated with buffer A containing 0.25 M NaCl, washed with 6 ml of buffer A, and eluted with a 12-ml gradient of decreased concentrations of NaCl (250–25 mM) and simultaneously increased concentrations of ethylene glycol (0–60%). Fractions were immediately assayed for MAP kinase activity or subjected to immunoblot analysis as described above.

Treatment with Alkaline Phosphatase. In some experiments, 1 ml of concentrated HPLC fractions was diluted with 500 μl of 1.5 M Tris·HCl, pH 8.3, and treated with or without 62.5 units of bovine intestinal alkaline phosphatase at 37°C for 60 min. After treatments, the sample was passed through a 0.22-μm Millipore filter, followed by chromatography as described above.

RESULTS

Both NGF and EGF Stimulate Protein-Tyrosine Phosphorylation in PC-12 Cells. A number of studies have suggested that the hormonal regulation of a 42-kDa MAP kinase activity requires phosphorylation of the protein on tyrosine residues (5–13). To explore whether NGF and EGF stimulate the tyrosine phosphorylation of proteins of a similar molecular mass in PC-12 cells, cells were exposed to the growth factors, and activation of MAP kinase (Table 1) was compared with appearance of tyrosine-phosphorylated proteins, identified by immunoblotting of one-dimensional SDS gels with anti-phosphotyrosine antibody (Fig. 1). Exposure of cells to NGF for 5 min resulted in the appearance of two predominant tyrosine-phosphorylated proteins of 40 and 42 kDa detected by immunoblotting, as well as a more diffuse band migrating at ≈120 kDa (Fig. 1, lane 2). Addition of EGF to cells for 5 min caused the appearance of the 40/42-kDa doublet, as well as an additional band, migrating at 180 kDa. As described previously, NGF and EGF stimulated activity of MAP kinase under these conditions (Table 1).

We previously described the selective attenuation of the NGF-dependent activation of MAP kinase with the kinase inhibitor staurosporine (3). Interestingly, the activation of MAP kinase by EGF was unaffected by staurosporine, leading us to explore whether the growth factor stimulation of pp40/42 tyrosine phosphorylation was differentially affected by these agents. The addition to cells of staurosporine (100

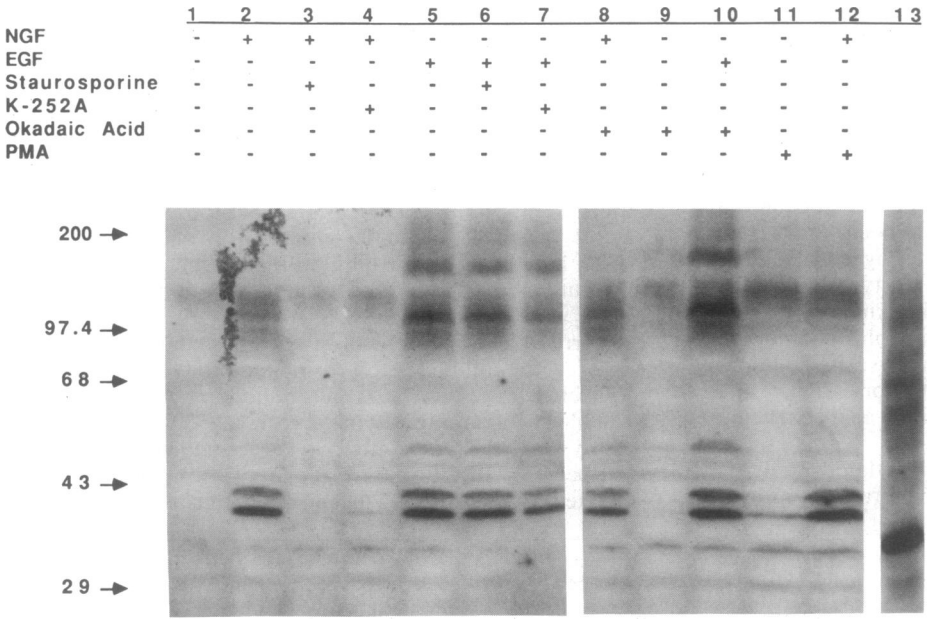

FIG. 1. Regulation of protein-tyrosine phosphorylation in PC-12 cells. After preincubation with or without staurosporine (100 nM) or K-252A (100 nM) for 10 min, PC-12 cells were treated for 5 min with 4 nM NGF or 20 nM EGF (lanes 1–7). After preincubation with okadaic acid (500 nM) and phorbol myristate acetate (100 nM) for 60 min, PC-12 cells were treated for 5 min with 4 nM NGF or 20 nM EGF (lanes 8, 10, and 12). PC-12 cells were treated for 60 min with okadaic acid (500 nM) (lane 9) and phorbol myristate acetate (100 nM) (lane 11). Stock solutions of all protein kinase modulators were diluted with dimethyl sulfoxide in the proper concentrations, and 10 μl of diluted solution was directly added to the medium. One hundred micrograms of protein from each cell lysate was analyzed by immunoblotting with anti-phosphotyrosine antibody as described. Lane 13 contains 25 μg of protein from a cell lysate of Rous sarcoma virus-transformed rat 3Y1 cells. Molecular mass markers (in kDa) are at left.

nM) or K-252A (100 nM) completely inhibited tyrosine phosphorylation of both pp42 and pp40 (Fig. 1, lanes 3 and 4), as well as the activation of MAP kinase caused by NGF (Table 1). Interestingly, staurosporine did not inhibit EGF-stimulated pp40/42 tyrosine phosphorylation (Fig. 1, lane 6) or MAP kinase activation (Table 1), although K-252A slightly reduced the tyrosine phosphorylation induced by this growth factor (Fig. 1, lane 7). Addition to the cells of the specific serine/threonine protein phosphatase inhibitor okadaic acid, or the protein kinase C activator phorbol myristate acetate, alone or in combination with NGF or EGF, had no effect on tyrosine phosphorylation of these proteins (Fig. 1, lanes 8–12). In contrast, as described (4), okadaic acid alone modestly activated MAP kinase and potentiated the effect of NGF but was without effect on activation by EGF (Table 1).

The time courses of protein-tyrosine phosphorylation by NGF and EGF were compared with the effects of the growth factors on MAP kinase activation (Fig. 2). Exposure of cells to NGF rapidly caused the appearance of both pp42 and pp40. Maximal levels were reached by 5 min, followed by a steady decline, although after 60 min tyrosine phosphorylation could still be detected (Fig. 2*A*). MAP kinase was activated over an identical time course by NGF, reaching maximal levels at 5 min and declining to steady-state levels by 30 min. Tyrosine phosphorylation of these proteins in response to EGF was detected earlier than that seen with NGF, reaching a maximum at 2 min and rapidly declining thereafter (Fig. 2*B*). After 60-min incubation with EGF, the levels of phosphotyrosine-containing proteins were indistinguishable from control. Similarly, the activation of MAP kinase by EGF was maximal at 2 min, followed by rapid decline of enzyme activity.

The concentration-dependence of the phosphorylation of pp42 and pp40 in PC-12 cells in response to NGF, EGF, and insulin was evaluated (Fig. 3). Phosphorylation of both proteins depended on the concentration of NGF or EGF. Half-maximal concentration for stimulation of MAP kinase (EC_{50}) by NGF in PC-12 cells was 0.3 nM, with a maximally effective concentration of 1 nM (3). In contrast, the EC_{50} for stimulation of pp40/42 phosphorylation by NGF was 1 nM (Fig. 3*A*). Identical EC_{50} values (2 nM) were seen for stimulation of the kinase (Fig. 3*B*) and induction of pp40/42 by EGF. Insulin also stimulated the phosphorylation of pp40/42, although the extent of phosphorylation was negligible compared to that seen with NGF or EGF.

Growth Factor-Stimulated Tyrosine-Phosphorylated Proteins Do Not Coelute with MAP Kinase on HPLC. To explore in greater detail the relation between the growth factor-sensitive phosphoproteins and MAP kinase, their chromato-

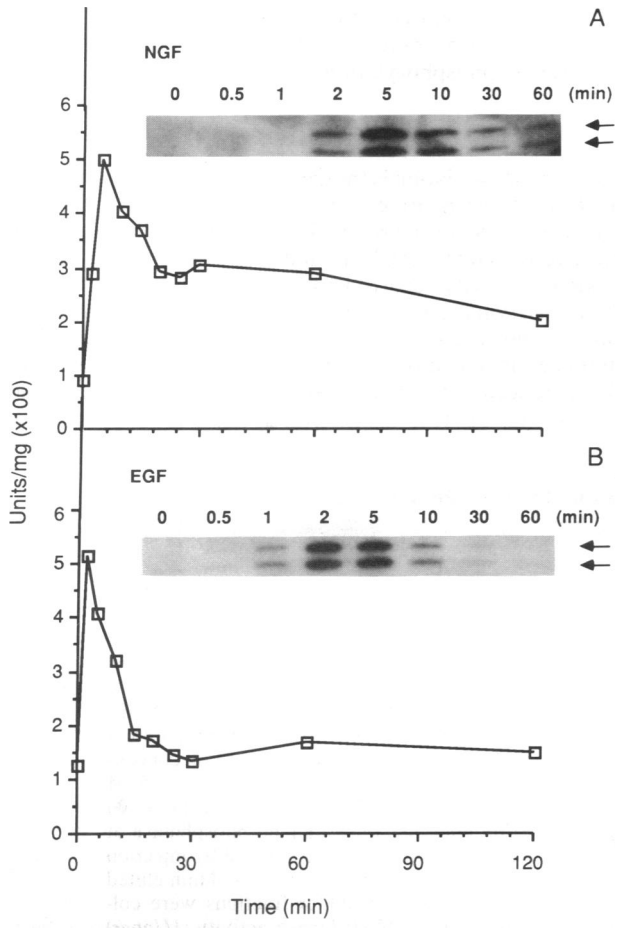

FIG. 2. Time course of protein-tyrosine phosphorylation by NGF and EGF. PC-12 cells were treated for the indicated times with 4 nM NGF (*A*) or 20 nM EGF (*B*). One hundred micrograms of protein from each cell lysate was analyzed by immunoblotting with anti-phosphotyrosine antibody as described. Arrows indicate positions of pp40/42. PC-12 cells were treated with NGF or EGF for the indicated times, as described above, and MAP kinase activities were assayed as described.

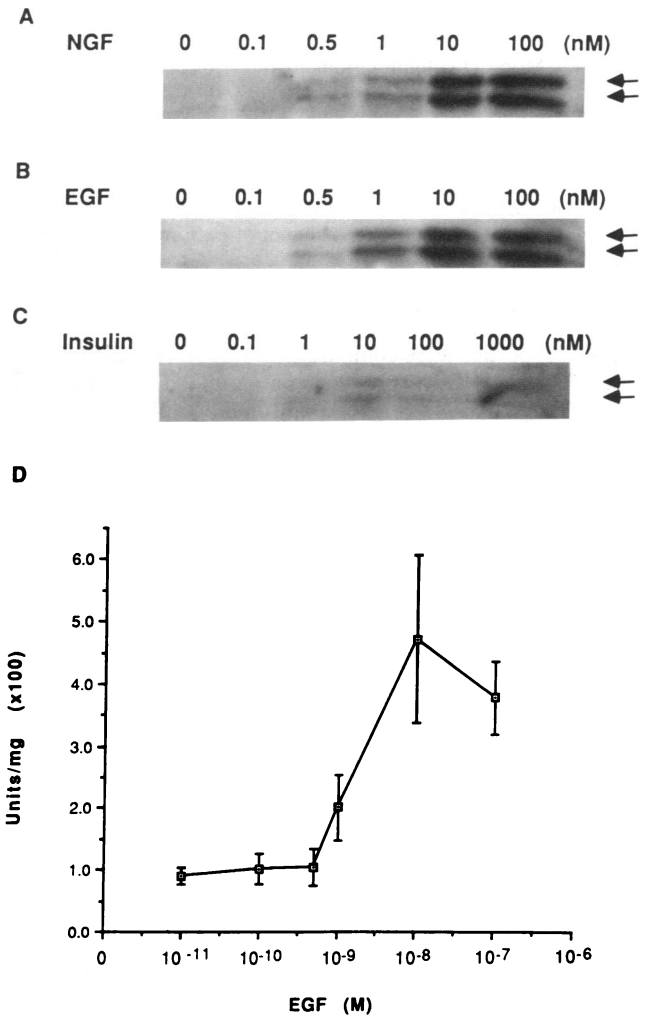

FIG. 3. Concentration-dependence of growth factor-stimulated tyrosine phosphorylation. PC-12 cells were treated with the indicated concentrations of hormones [NGF for 5 min (*A*), EGF for 5 min (*B*), and insulin for 10 min (*C*)]. One hundred micrograms of protein from each cell lysate was analyzed by immunoblotting with anti-phosphotyrosine antibody as described. Arrows indicate positions of pp40/42. PC-12 cells were treated with EGF for 5 min in the same way as described above, and MAP kinase activities were assayed as described in text.

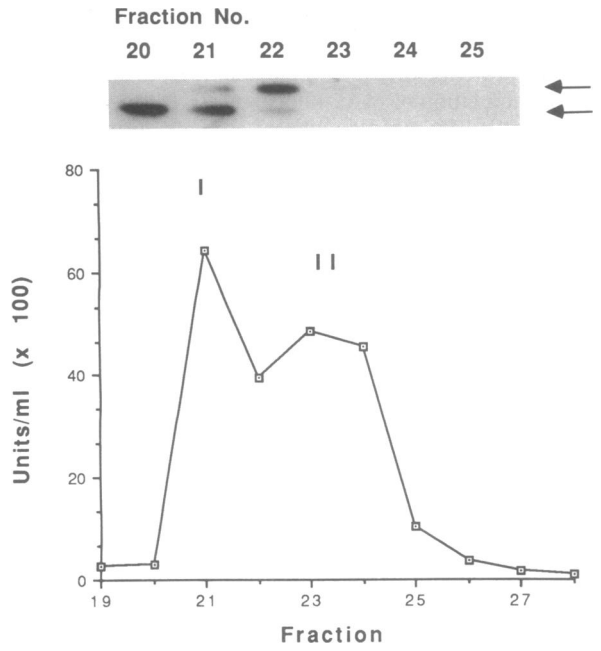

FIG. 4. Mono Q column chromatography of MAP kinases and pp40/42. (*Lower*) PC-12 cells (five plates) were treated with 4 nM NGF for 5 min. Extracts were prepared with 1% Triton X-100 RIPA buffer (200 μl per dish) and partially purified by DEAE-cellulose column; peak fractions were then concentrated. One milliliter of concentrated fractions was chromatographed on Mono Q as described. One-milliliter fractions were collected and assayed for MAP kinase activity. (*Upper*) A 200-μl aliquot of each fraction was also analyzed by immunoblotting with anti-phosphotyrosine antibody as described. Arrows indicate positions of pp40/42. Identical peaks were identified in cells treated with 20 nM EGF.

graphic properties were compared. Lysates from cells treated with NGF or EGF were chromatographed on Mono Q ion-exchange HPLC (Fig. 4). Chromatography of lysates from both NGF- and EGF-treated cells resulted in the resolution of two kinase activities (I and II) eluting at 21 and 23 min, respectively (Fig. 4 *Lower*). Interestingly, immunoblotting of these column fractions with anti-phosphotyrosine antisera revealed that neither pp40 nor pp42 precisely coeluted with peak MAP kinase activities. Lysates from NGF-treated cells were also chromatographed on a phenyl-Superose column, eluted with a simultaneous descending gradient of 250–25 mM NaCl and ascending gradient of 0–60% ethylene glycol (Fig. 5). As observed with Mono Q HPLC, two peaks of NGF-stimulated MAP kinase were resolved. Sequential chromatography of the Mono Q-purified fractions revealed that peak II from Mono Q eluted at 19 min on phenyl Superose, and peak I eluted at 32 min. Both peaks were similarly increased by NGF and EGF (data not shown). As described for Mono Q chromatography, an immunoblot analysis of pp40/42 elution from this column again revealed that these phosphoproteins did not coelute with MAP kinase activity. pp42 eluted in a broad peak at 10–15 min, completely resolved from the MAP kinase peaks. pp40 eluted in a broad peak between 29–33 min. Although pp40 exhibited some overlap with MAP kinase peak I, the peaks of activity were not identical.

As described before (3), treatment of lysates of NGF-treated cells with alkaline phosphatase reduced the MAP kinase activity. To further explore the role of tyrosine phosphorylation of pp40/42 in MAP kinase activation, lysates from NGF-treated cells were chromatographed on phenyl Superose, and the effects of alkaline phosphatase on MAP kinase and pp40/42 tyrosine phosphorylation were compared. Phosphatase treatment caused almost an 80% decrease in MAP kinase activity residing in both peaks I and II (Fig. 5). In contrast, under these conditions alkaline phosphatase caused only a modest reduction in pp40 and pp42 tyrosine phosphorylation (Fig. 5).

DISCUSSION

Protein phosphorylation is thought to play a critical role in the growth and differentiative response of cells to growth factors. PC-12 cells contain a protein kinase (MAP kinase) that is rapidly activated by both NGF and EGF. Chromatography of this kinase activity resolved two forms of the enzyme with identical enzymatic properties. Interestingly, although both forms of the kinase were stimulated by NGF and EGF, differences in the growth factor responses were detected. EGF produced a faster activation of the kinases than did NGF. Moreover, the serine/threonine protein phosphatase

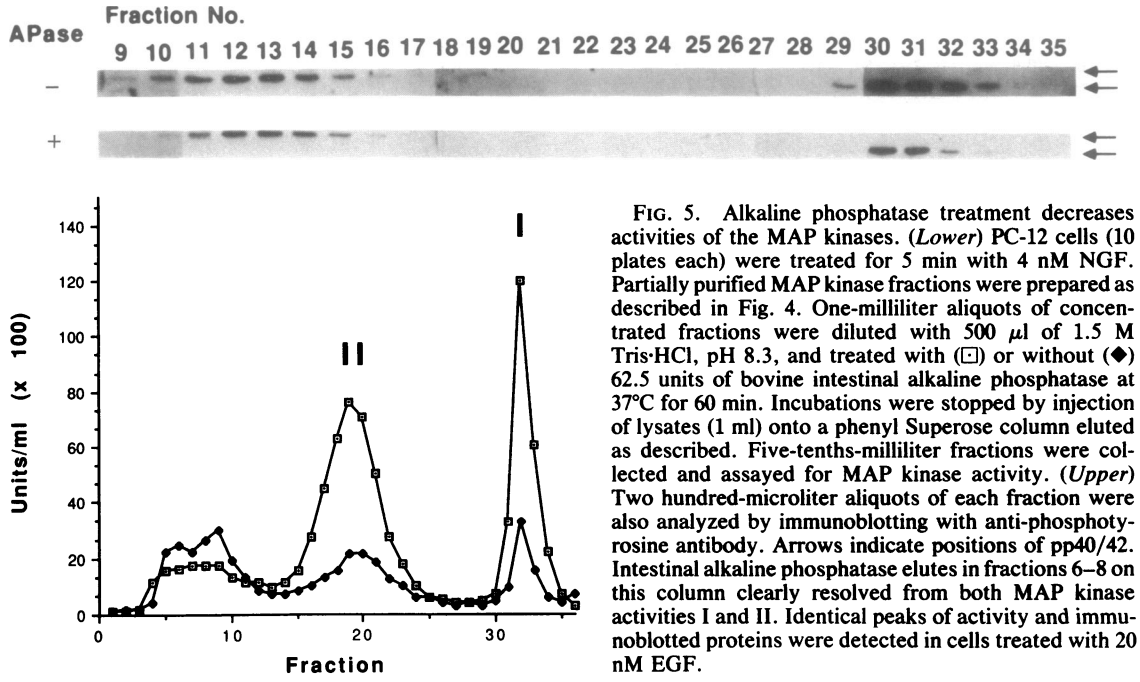

FIG. 5. Alkaline phosphatase treatment decreases activities of the MAP kinases. (*Lower*) PC-12 cells (10 plates each) were treated for 5 min with 4 nM NGF. Partially purified MAP kinase fractions were prepared as described in Fig. 4. One-milliliter aliquots of concentrated fractions were diluted with 500 μl of 1.5 M Tris·HCl, pH 8.3, and treated with (□) or without (◆) 62.5 units of bovine intestinal alkaline phosphatase at 37°C for 60 min. Incubations were stopped by injection of lysates (1 ml) onto a phenyl Superose column eluted as described. Five-tenths-milliliter fractions were collected and assayed for MAP kinase activity. (*Upper*) Two hundred-microliter aliquots of each fraction were also analyzed by immunoblotting with anti-phosphotyrosine antibody. Arrows indicate positions of pp40/42. Intestinal alkaline phosphatase elutes in fractions 6–8 on this column clearly resolved from both MAP kinase activities I and II. Identical peaks of activity and immunoblotted proteins were detected in cells treated with 20 nM EGF.

inhibitor okadaic acid potentiated the NGF activation but did not modulate the effect of EGF. In addition, the kinase inhibitors staurosporine and K-252A selectively blocked only NGF activation.

The similarities and differences in the activation of MAP kinase by NGF and EGF led us to explore the possible role of tyrosine phosphorylation. In confirmation of a previous report (2), tyrosine phosphorylation was detected in response to NGF, although the patterns were different, possibly due to different antibodies or subclones of PC-12 cells. Two proteins (pp40 and pp42) were identified by immunoblotting of SDS gels with anti-phosphotyrosine antibodies. The kinetics of the tyrosine phosphorylation of pp40/42 and activation of MAP kinases in response to NGF were indistinguishable. Similarly, the time courses of EGF action on these two processes were identical, although the responses to EGF were observed earlier than those to NGF. As described above for activation of MAP kinase, staurosporine and K-252A selectively blocked the tyrosine phosphorylation of pp40/42 by NGF, but not by EGF. Despite the striking similarities in the kinetics of activation by both NGF and EGF, sensitivities to staurosporine, and apparent molecular mass, chromatographic characterization of pp40/42 and the MAP kinases revealed that these proteins were not identical. One of the forms of the enzyme, MAP kinase II, was completely separated from phosphotyrosine-containing pp40 and 42. The peak of MAP kinase I activity did not precisely coelute with phosphotyrosine-containing protein, although there was some overlap with pp40. Conversely, fractions containing pp42 had no detectable kinase activity, although pp40 eluted in a broad peak that exhibited some overlap with MAP kinase I. These data strongly suggest that at least one of the isoforms of MAP kinase (II) is not a phosphotyrosine-containing protein, although the MAP kinase I may overlap with a heterogeneous peak of the 40-kDa phosphoprotein. The relationship between these phosphoproteins and previously identified forms of MAP kinase (10, 11) is unknown.

What is the mechanism by which the MAP kinases are activated? Clearly both forms of the enzyme require phosphorylation because treatment with alkaline phosphatase reversed the activation by growth factors. Additionally, the serine/threonine phosphatase inhibitor okadaic acid activated both forms of the enzyme (4), although no effect on pp40/42 tyrosine phosphorylation was detected. Taken together, these results suggest (*i*) that the two forms of MAP kinase may be activated by different mechanisms. Although there is no evidence that MAP kinase II is activated by tyrosine phosphorylation, serine phosphorylation may be required. Reports have indicated that both the phosphotyrosine-specific protein phosphatase CD45 and the serine/threonine-specific phosphatase 2A inhibit an enzyme that exhibits the properties of the MAP kinase I described here, suggesting that MAP kinase I may require both tyrosine and serine phosphorylation for activation (13, 14). (*ii*) pp40 or pp42 may serve as regulatory subunits of MAP kinase I. In this model, analogous to that proposed for regulation of phosphatase I by inhibitor 2 (31), the tyrosine phosphorylation of pp40/42 may induce its dissociation from the serine/threonine kinase, allowing expression of its activity, perhaps by facilitating serine/threonine phosphorylation of the catalytic subunit. Conversely, dephosphorylation of pp40/42 may induce its reassociation with and subsequent inhibition of MAP kinase. This issue may be clarified by the functional reconstitution of the relevant components.

The observation that NGF causes tyrosine phosphorylation in PC-12 cells suggests further similarities in the mechanisms of action of NGF and insulin. As noted (32), NGF and insulin share a number of functional similarities, despite gross structural differences in their receptors. The most likely possibility is that the NGF receptor is itself coupled to a nonreceptor tyrosine kinase, in analogy to the interaction of CD4 or CD8 with the src family kinase $p56^{lck}$ (33) or the T-cell receptor with the src family kinase $pp59^{fyn}$ (34). Moreover, like $pp60^{v\text{-}src}$, the putative NGF-sensitive tyrosine kinase is potently inhibited by staurosporine. Indeed, a link between cellular *src* activity and NGF has been proposed (35–37). However, elucidation of the role of tyrosine phosphorylation in NGF action will critically depend upon the molecular identification of the relevant tyrosine kinases and their substrates.

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1. Matsuda, U. & Guroff, G. (1987) *J. Biol. Chem.* **262**, 2832–2844.
2. Maher, P. A. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 6788–6791.
3. Miyasaka, T., Chao, M. V., Sherline, P. & Saltiel, A. R. (1990) *J. Biol. Chem.* **265**, 4730–4735.
4. Miyasaka, T., Miyasaka, J. & Saltiel, A. R. (1990) *Biochem. Biophys. Res. Commun.* **168**, 1237–1243.
5. Ray, L. B. & Sturgill, T. W. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1502–1506.
6. Ray, L. B. & Sturgill, T. W. (1988) *J. Biol. Chem.* **263**, 12721–12727.
7. Sturgill, T. W., Ray, L. B., Erickson, E. & Maller, J. L. (1988) *Nature (London)* **334**, 715–718.
8. Rossomando, A. J., Payne, M. D., Weber, J. M. & Sturgill, T. W. (1988) *Proc. Natl. Acad. Sci. USA* **86**, 6940–6943.
9. Ahn, N. G., Weiel, J. E., Chan, C. P. & Krebs, E. G. (1990) *J. Biol. Chem.* **265**, 11487–11494.
10. Kyriekis, J. M. & Avruch, J. (1990) *J. Biol. Chem.* **265**, 17355–17363.
11. Boulton, T. G., Yancopoulos, G. D., Gregory, J. S., Slaughter, C., Moomaw, C., Hsu, J. & Cobb, M. H. (1990) *Science* **249**, 64–67.
12. Ray, L. B. & Sturgill, T. W. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 3753–3757.
13. Anderson, N. G., Maller, J. L., Tonks, N. K. & Sturgill, T. W. (1990) *Nature (London)* **343**, 651–653.
14. Gomez, N., Tonks, K. N., Morrison, C., Harmar, T. & Cohen, P. (1990) *FEBS Lett.* **271**, 119–122.
15. Cooper, J. A., Sefton, B. M. & Hunter, T. (1984) *Mol. Cell. Biol.* **4**, 30–37.
16. Cooper, J. A. & Hunter, T. (1985) *Mol. Cell. Biol.* **5**, 3304–3309.
17. Bishop, R., Martinez, R., Nakamura, K. B. & Weber, M. J. (1983) *Biochem. Biophys. Res. Commun.* **115**, 536–543.
18. Gilmore, T. & Martin, G. S. (1983) *Nature (London)* **306**, 487–490.
19. Kohno, M. (1985) *J. Biol. Chem.* **260**, 1771–1779.
20. Nakamura, K. D., Martinez, R. & Weber, M. J. (1983) *Mol. Cell. Biol.* **3**, 380–390.
21. Hunter, T. & Cooper, J. A. (1984) *Cancer Cells* **2**, 61–69.
22. Martinez, R., Nakamura, K. D. & Weber, M. J. (1982) *Mol. Cell. Biol.* **2**, 653–665.
23. Cooper, J. A., Nakamura, K. D., Hunter, T. & Weber, M. J. (1983) *J. Virol.* **46**, 15–28.
24. Cooper, J. A. & Hunter, T. (1981) *Mol. Cell. Biol.* **1**, 165–178.
25. Haystead, T. A. J., Weiel, J. E., Litchfield, D. W., Tsukitani, Y., Fischer, E. H. & Krebs, E. G. (1990) *J. Biol. Chem.* **265**, 16571–16580.
26. Ely, C. M., Oddie, K. M., Litz, J. S., Rossomando, A. J., Kanner, S. B., Sturgill, T. W. & Parsons, S. J. (1990) *J. Cell Biol.* **110**, 731–742.
27. Wang, J. Y. J. (1985) *Mol. Cell. Biol.* **5**, 3640–3643.
28. Wang, J. Y. J. & Baltimore, D. (1985) *J. Biol. Chem.* **260**, 64–71.
29. Hamaguchi, M., Grandori, C. & Hanafusa, H. (1988) *Mol. Cell. Biol.* **8**, 3035–3042.
30. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
31. Hemmings, B. A., Resink, T. J. & Cohen, P. (1982) *FEBS Lett.* **150**, 319–324.
32. Chan, B. L., Chao, M. V. & Saltiel, A. R. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 1756–1760.
33. Veillelte, A., Bookman, M. A., Horek, E. M. & Bolen, J. B. (1988) *Cell* **55**, 301–308.
34. Samuelson, L. E., Philips, A. F., Luong, E. T. & Klausner, R. D. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 4358–4362.
35. Alema, S., Casalbore, P., Agostini, E. & Tato, F. (1985) *Nature (London)* **316**, 557–559.
36. Eveleth, D. D., Hanecak, R., Fox, G. M., Fan, H. & Bradshaw, R. A. (1989) *J. Neurosci. Res.* **24**, 67–71.
37. Rausch, D. M., Dickens, G., Doll, S., Fujita, K., Koizumi, S., Rudkin, B. B., Tocco, M., Eiden, L. E. & Guroff, G. (1989) *J. Neurosci. Res.* **24**, 49–58.