Identification and Characterization of a Nerve Growth Factor-stimulated Mitogen-Activated Protein Kinase Activator in PC12 Cells*

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Nerve growth factor (NGF) and nerve growth factor treatment of PC12 cells results in the rapid activation of MAP kinases. These enzymes are activated through interaction with a protein "activator." The mitogen-activated protein (MAP) kinase activator has been partially purified by ion exchange and gel filtration chromatography. The activator has an apparent molecular mass of 50–90 kDa. The MAP kinase activator is rapidly generated in response to nerve growth factor (NGF) and can be detected within 30 s of exposure, reaching maximal levels within 2 min and then declining to near basal levels by 15–20 min. The activation of MAP kinase is dependent upon the time of incubation with the activator and on activator concentration. The MAP kinase activator is itself a protein kinase that phosphorylates MAP kinases and mediates their activation. The NGF-stimulated MAP kinase activator phosphorylates MAP kinase on serine, threonine, and tyrosine residues, establishing this enzyme as dual specific kinase. The MAP kinase activator is itself a phosphoprotein whose phosphorylation on tyrosine residues is stimulated upon NGF treatment of the cells. The enzyme activity of MAP kinase activator is abolished by treatment with both the tyrosine-specific phosphatase PTP-1 and the serine/threonine-specific phosphatase PP2A. The activator is produced in response to NGF, epidermal growth factor, and fibroblast growth factor. The protein kinase inhibitor K252a selectively inhibits the ability of NGF to generate MAP kinase activator activity. These data suggest that the upstream events governing MAP kinase activation involve the regulated phosphorylation of dual specificity MAP kinase activator as an immediate consequence of receptor activation.

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Nerve growth factor (NGF) plays an important physiological role in the maintenance, survival, and differentiation of neurons (for review, see Ref. 1). Treatment of the rat pheochromocytoma PC12 cell line with NGF promotes the conversion of cells from a chromaffin-like phenotype to that of a sympathetic neuron (2–4). These changes include cessation of cell division, neurite outgrowth, and acquisition of a variety of physiological and biochemical characteristics of neurons. The mechanisms by which NGF supports the survival of these cells and directs their differentiation have been extensively studied but remain poorly understood. NGF initiates its action through binding to the trk proto-oncogene product, activating its intrinsic tyrosine kinase activity (5, 6). Subsequently, a number of serine/threonine kinases are activated, driving a cascade of protein phosphorylation that mediates the specific biochemical events characteristic of hormone action. Among the most prominent members of this cascade are the MAP kinases, also termed extracellular regulated kinases or ERKs (7). In PC12 cells, two related MAP kinases, ERK1 (p44mapk) and ERK2 (p42mapk), are rapidly activated upon NGF treatment of the cells (7–9). In PC12 cells, MAP kinases become phosphorylated on tyrosine, serine, and threonine residues following NGF treatment (7, 10, 11). Initially, it was thought that the MAP kinases were substrates for the hormone receptor kinases and were the most proximal members of a signal transduction cascade (10). Recent findings suggest that this is not the case, and the sequence of the events mediating the activation of these enzymes is considerably more complex than originally envisioned. MAP kinase activation has been shown to require functionally active p21ras, although it remains unclear as to how ras action is coupled to subsequent kinase activation (12, 13).

Earlier studies by Ahn et al. (14) and subsequent by Gomez and Cohen (9) have demonstrated that the MAP kinases are activated through interaction with another protein(s) or "activator." This interaction resulted in the enhanced tyrosine and serine/threonine phosphorylation of the enzyme and subsequent enzymatic activation of the MAP kinases. Phosphorylation at both tyrosine and threonine residues was required for expression of kinase activity, as removal of tyrosine or threonine phosphate with CD45 and PP2A, respectively, inactivated the MAP kinases (15, 16). It has recently been reported that MAP kinases are phosphorylated and activated by a member of the src family of tyrosine kinases, p56lck (17), as well as other yet unidentified protein kinases (18). However, the discovery that the MAP kinases can undergo autophosphorylation on both tyrosine and threonine residues (19–21) raised the possibility that MAP kinases may achieve their active phosphorylated state without the interaction with another protein kinase. A number of reports published since the completion of the present study have conclusively demonstrated that the MAP kinase activator is a kinase that phosphorylates and activates MAP kinases (22–25).

We report here the characterization of a MAP kinase activator that is rapidly generated upon treatment of PC12 cells with NGF. Of particular significance is our finding that...
upon NGF treatment of PC12 cells, a single species of MAP kinase activator is very rapidly detected. The activator is dual specific kinase that becomes tyrosine phosphorylated upon NGF treatment. The MAP kinase activator is inactivated upon treatment with either PP2A or the tyrosine-specific phosphatase PTP-1.

**EXPERIMENTAL PROCEDURES**

**Materials**—NGF was prepared by the method of Smith et al. (28). Epidermal growth factor (EGF) was purchased from Upstate Biotechnology (Stoughton, MA), and basic fibroblast growth factor was from Amgen (Thousand Oaks, CA). Okadaic acid was purchased from Kamya Biochemical Co. (Thousand Oaks, CA), and K252a was a gift of Dr. Y. Matsuda (Tokyo Research Lab, Tokyo, Japan). Recombinant rat brain protein tyrosine-phosphatase-1 (rrbPTP-1) was a gift of Dr. J. Dixon (University of Michigan), and protein phosphatase 2A (PP2A) was from M. M. M. M. University of Texas). Recombinant ERK2 and K252R mutant expressed in *Escherichia coli* were kindly provided by Dr. M. H. Cobb (University of Texas). Radiolabeled ATP was synthesized using *Gamma* Prepa (Promega Biotech, Madison, WI) from ICN (Irvine, CA). All other reagents were purchased from Sigma.

**Cell Culture and Preparation of Extracts**—PC12 cells were grown in Dulbecco's modified Eagle's medium containing 10% horse serum and 5% fetal calf serum in an atmosphere of 10% CO2. The cells were collected by trituration in phosphate-buffered saline containing 1 mg/ml bovine serum albumin and 1 mg/ml glucose, washed once, and then resuspended at a final concentration of 2–3 × 106 cells/ml. The cells were treated with growth factors for 5 min at 37°C at the indicated concentrations. The cells were then collected by centrifugation and resuspended in lysozyme buffer containing 20 mM Tris, pH 7.4, 1 mM MgCl2, 1 mM EDTA, 1 mM DTT, 0.5 mM Na3VO4, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 10 mM p-nitrophenyl phosphate. The cells were sonicated briefly (2 × 15 s at 4°C) with a Kontes Cell Disrupter at a power setting of 5 and then centrifuged at 12,000 × g in a microcentrifuge at 4°C for 5 min. The supernatant was collected and centrifuged for 35 min at 100,000 × g. In each experiment, control and NGF-treated cells were processed in parallel.

**Chromatography**—Extracts were prepared from approximately 1–1.5 × 10^6 cells, which had been treated for 5 min with NGF (50 ng/ml). Anion exchange chromatography was performed on a Pharmacia fast protein liquid chromatography Mono Q HR5/5 column equilibrated in Buffer A (20 mM Tris, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 0.1 mM Na3VO4). Samples were applied to the column at a flow rate of 1 ml/min, and the column was washed with 5 ml of Buffer A containing a 0.1 M NaCl gradient. Fractions were immediately assayed for MAP kinase activity. For cation exchange chromatography, peak fractions from the Mono Q column were dialyzed for 2 h against a buffer containing 20 mM HEPES, pH 7.4, 2 mM EGTA, 1 mM EDTA, and 1 mM DTT at 4°C. The dialyzed samples were applied to a Mono S HR5/5 column preequilibrated in the same buffer. The column was eluted with a linear gradient of 0–0.4 M NaCl (40 ml) in the same buffer at a flow rate of 1 ml/min, and 1 ml fractions were collected.

**Gel filtration chromatography** was performed by first concentrating column fractions using Centricon ultracentrifugation units (Amicon). The proteins were then eluted from the Centricon tubes in 0.1 M NaCl Buffer A containing 2 mM NaCl. The flow rate was 0.25 ml/min, and 0.25-ml fractions were collected. The column was calibrated using IgG (97 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), and cytochrome c (12.4 kDa) as molecular mass standards.

**For time course and ligand activation experiments, the MAP kinase activator was isolated using a stepwise elution method.** PC12 cells (1–2 × 10^5 cells) were treated with growth factors for the indicated times. The cells were centrifuged briefly in a microcentrifuge and then lysed in 1 ml of lysis buffer as described above. The supernatant was mixed with 0.2 ml of DE-52 ion exchange resin (Whatman) in a microcentrifuge tube and incubated for 10 min on ice with occasional shaking. The resin was collected by centrifugation and then washed 3–4 times with 1 ml of Buffer A. The resin was washed with 1 ml of Buffer A containing 0.1 M NaCl and activator eluted with 0.7 ml of Buffer A containing 0.04 M NaCl.

**Alkaline Phosphatase Inactivation of MAP Kinase**—Mono Q fractions containing peak 1 MAP kinase (Fig. 1) were dialyzed overnight at 4°C against 20 mM Tris-HCl, pH 7.4, containing 1 mM EGTA, 1 mM EDTA, 10 mM MgCl2, and 1 mM DTT. The dialyzed enzyme (0.4 ml) was treated with alkaline phosphatase-Sepharose beads (20 units) for 30 min at 30°C to inactivate the enzyme. The reaction mixture was centrifuged in a microcentrifuge, and the supernatant was removed. The alkaline phosphatase treatment reduced the activity of the MAP kinase by 80–90%.

**Assay of MAP Kinase Activator**—Inactive MAP kinase obtained from the untreated PC12 cells or alkaline phosphatase treatment of the NGF-stimulated MAP kinase (10 μl) and/or recombinant MAP kinase (ERK2) (1 μg) were mixed with MAP kinase activator (10 μM). Activation of the MAP kinase was then initiated by addition of 15 μl of buffer containing 25 mM Tris-HCl, pH 7.4, 2 mM MnCl2, 10 mM MgCl2, 1 mM DTT, 1 mg/ml BSA, 0.1 mM Na3VO4, 1 mM EGTA, 10 mM p-nitrophenyl phosphate, and 50 μM ATP (all concentrations are final). In control incubations, MAP kinase activator was replaced by buffer. After 15 min, the protein substrate, myelin basic protein (MBP, 1 μg), and [γ-32P]ATP (5 μCi) were added to the reaction mixture and incubated further for 20 min at 25°C. After incubation, aliquots from the reaction were removed and pipetted onto a Whatman P-81 paper (2 × 2 cm). The P-81 paper was washed 3 × 5 min in 1 ml of 10 mM Tris-HCl, 1 mM EDTA, and 0.1% sodium metaperiodate, and radioactivity was measured by Cerenkov counting. In some experiments, the phosphorylation reaction was stopped by addition of Laemmli sample buffer, and the samples were then boiled for 5 min. The reaction mixtures were then separated by SDS-PAGE, and the bands were excised from the gel and counted for radioactivity. 

**MAP Kinase Phosphorylation**—MAP kinase activator (20 μl) was mixed with inactive MAP kinase (20 μl) obtained from untreated cells, recombinant ERK2 (1.5 μg), or a kinase-defective ERK2 mutant-K52R (1.5 μg) in a buffer containing 25 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 1 mM DTT, 1 mM EGTA, 0.1 mM Na3VO4, and 15 mM ATP (20 μg of [γ-32P]ATP). After 30 min at 30°C, the reaction was terminated by boiling in Laemmli sample buffer. The proteins were separated by 10% SDS-polyacrylamide gel electrophoresis, dried, and exposed to x-ray film (Kodak, XAR 5). The phosphorylated MAP kinases were visualized as 42 kDa bands on the autoradiogram.

**Phosphorylation**—Phosphorylation of MBP was measured as described previously (27). Labeled proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to Immobilon P membranes. The phosphorylated bands at 42 kDa were cut from the membrane and digested in 50 μl of 0.7 M HCl at 106°C for 1 h. Water (0.5 ml) was then added, and the solution was transferred to a microcentrifuge tube. The tube was dried under vacuum in a SpeedVac, and the residue was resuspended in 3 μl of a solution containing phosphoserine, phosphothreonine, and phosphotyrosine (10 mg/ml). The samples were then applied to a thin layer cellulose plate and subjected to electrophoresis (pyridine:acetic acid:HOAc, 1:1:1) for 45 min at 200 V, followed by a 45 min, air-dried, and autoradiographed after ninhydrin staining.

**Inactivation of MAP Kinase Activator by Phosphatases**—MAP kinase activator obtained from Mono Q fractions from NGF-treated PC12 cells were pooled and dialyzed overnight at 4°C against a buffer containing 25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT specifically to reduce the orthovanadate concentration to levels that would not inhibit the phosphotyrosine phosphorylation. Dialyzed MAP kinase activator (0.4 ml, 0.1 mg of protein) was treated with either alkaline phosphatase-Sepharose (20 units) or with rrbPTP-1 (300 units/ml). The protein tyrosine phosphatase 1 is a rat brain protein tyrosine phosphatase-1 expressed in E. coli (28). The MAP kinase activator was incubated at 30°C with the various phosphatases. At the indicated times, aliquots (10 μl) were removed from the reaction, and the PTP-1 was inactivated by the addition of 2 mM Na3VO4 or by simply removing the alkaline phosphatase-Sepharose resin by centrifugation. PP2A treatment was performed by incubating this phosphatase (10 milliunits/ml) with Mono Q fractions (0.4 ml) containing MAP kinase activator, which had been dialyzed against 25 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 2 mM MgCl2, and 0.5 mM BSA. At the indicated times, aliquots (10 μl) were removed from the reaction and assayed for MAP kinase activity. The supernatant was mixed with 0.2 ml of DE-52 ion exchange resin (Whatman) in a microcentrifuge tube and incubated for 10 min on ice with occasional shaking. The resin was collected by centrifugation and then washed 3–4 times with 1 ml of Buffer A. The resin was washed with 1 ml of Buffer A containing 0.1 M NaCl and activator eluted with 0.7 ml of Buffer A containing 0.04 M NaCl.
MAP Kinase Activator in PC12 Cells

RESULTS

Chromatographic Characterization of MAP Kinase Activator—To characterize the NGF-stimulated MAP kinase activity in PC12 cells, cytosolic extract prepared from PC12 cells treated with 50 ng/ml NGF for 5 min was chromatographed on a Mono Q column. The Mono Q chromatography resolved two major peaks of NGF-stimulated kinase activities. As shown in Fig. 1A, NGF-stimulated MAP kinase activity (p42\textsuperscript{MAP}) designated peak I which eluted from the Mono Q column at 0.09–0.1 M NaCl, and the MAP 2 kinase (p44\textsuperscript{MAP}) designated peak II) eluted at 0.18–0.2 M NaCl. Both peaks exhibited almost equal levels of kinase activity when assayed with MBP and consistently showed a >16-fold increase, as compared with control cells. The chromatographic behavior and MBP phosphorylation activity of these two peaks of MAP kinase activity are identical with that previously reported in these cells and many other cell types in response to NGF or other growth factors (14, 15, 29).

In order to ascertain whether NGF treatment of PC12 cells generated a species that is able to activate MAP kinase (a MAP kinase activator), we screened the Mono Q fractions for the ability to reactivate the peak I MAP kinase that has been previously inactivated by alkaline phosphatase treatment. Although we have routinely used alkaline phosphatase-inactivated MAP kinase, identical results were obtained in these assays whether we employed peak I MAP kinase obtained from untreated control cells or following alkaline phosphatase inactivation of peak I activity obtained from NGF-treated cells. Upon combination of Mono Q fractions from NGF-treated PC12 cells with inactive MAP kinase, we detected reactivation of the enzyme confined to a peak including fractions 14–15, which eluted between 0.05–0.06 M NaCl. A 5–6-fold stimulation in the rate of MBP phosphorylation above control levels was observed. No MAP kinase-activating activity was found in any other fractions, nor in corresponding fractions obtained from untreated control cells (Fig. 1B), indicating that this activator was specifically generated upon NGF treatment of the cells.

The peak activator fractions from the Mono Q column were further characterized by cation exchange chromatography using a Mono S column. Fractions were examined for MAP kinase-activating activity by preincubation with inactive MAP kinase prior to addition of MBP as previously described.

A single MAP kinase activator peak was eluted at 0.1 M NaCl (Fig. 2).

The apparent molecular weight of the NGF-stimulated MAP kinase activator was determined by gel filtration chromatography. Fractions from Mono Q columns corresponding to MAP kinase-activating activity were concentrated 10-fold and then applied to a Superose 12 gel filtration column. MAP kinase-activating activity was detected in a peak that eluted with an apparent \( M_r = 50-60,000 \) (Fig. 3). A 26-fold increase in MAP kinase activity was observed when peak fractions from Superose 12 column were incubated with inactive MAP kinase (Fig. 3).

The capacity of the MAP kinase activator to stimulate the MAP kinase activity was time- and dose-dependent. MAP kinase activity increased as a function of incubation times and was evident within 5 min, reaching a maximum enhancement after 25 min. The rate of MAP kinase activation was linear for up to 20 min and was directly proportional to the amount of MAP kinase activator added to the reaction mixture (data not shown).

Time Course of Activation of MAP Kinase Activator—We investigated the time course of stimulation of MAP kinase-activating activity by NGF (Fig. 4). Exposure of PC12 cells to 50 ng/ml NGF resulted in a rapid but transient rise in the activator levels that was first detected after 30 s, reaching a
maximum level at 2 min, and subsequently declining to basal levels by 20-22 min. It is interesting to note that the appearance of the MAP kinase activator preceded that of the NGF-stimulated MAP kinase activity in PC12 cells. The activity of MAP kinase is maximal after 5 min and then returns to basal level within 30 min (8). The development of MAP kinase-activating activity was correlated with the time course of tyrosine phosphorylation of MAP kinase in PC12 cells (11). The tyrosine phosphorylation of MAP kinase preceded the activation of MAP kinase activity and was apparent within 1 min of NGF exposure, with the peak tyrosine phosphorylation occurring after 2 min and then subsequently declining significantly over the next 30 min.

**Ligand Specificity of MAP Kinase Activator**—PC12 cells possess receptors for a variety of growth factors, including EGF (29, 30) and fibroblast growth factor (31), all of which have been shown to activate MAP kinase (32). MAP kinase activity is also stimulated by exposure of the PC12 cells to phorbol esters (12-0-tetradecanoylphorbol-13-acetate (TPA, 1 µM) for 5 min. In experiments where K252a was used, cells were preincubated for 20 min with 25 nM of K252a before the addition of growth factors. Cells were pelleted by brief centrifugation, and the activator was isolated by absorption to DEAE-cellulose as described under "Experimental Procedures." MAP kinase activity was determined as described in the legend to Fig. 4. The values represent means (±S.D.) of three experiments performed in duplicate and normalized for protein concentration.

**Fig. 2.** Cation exchange chromatography of MAP kinase activator. The peak fractions containing MAP kinase-activating activity from a Mono Q column were pooled and loaded on a Mono S column as described under "Experimental Procedures." The column was developed with a linear gradient (dotted line) from 0-0.4 M NaCl. One-ml fractions were collected, and alternate fractions were assayed for MBP phosphorylation by preincubating with peak 1 MAP kinase obtained from untreated PC12 cells (○) or buffer (C) as described in the legend to Fig. 1. Similar results were obtained in at least three different experiments.

**Fig. 3.** Gel filtration chromatography of MAP kinase activator. Mono Q fractions corresponding to MAP kinase activator were pooled and concentrated (10-fold). The sample (200 µl) was loaded on Superose 12 as described under "Experimental Procedures." Aliquots (10 µl) of eluting fractions were preincubated with equal volumes of inactive peak 1 MAP kinase (○) or buffer (C) in the presence of Mg-ATP for 15 min prior to addition of MBP and [γ-32P]ATP. MBP phosphorylation was then measured after a further 20-min incubation. The position of void volume (V0) and elution of the protein standards are indicated by arrows. OA, ovalbumin; CYC, cytochrome c.

**Fig. 4.** Time course of induction of MAP kinase-activating activity. PC12 cells (1-2 × 10^6) were incubated with NGF (50 ng/ml) for the indicated time periods. The cells were collected by brief centrifugation (15 s), and the cells were lysed. The clarified lysate was incubated with DE-52, and the MAP kinase activator was eluted from the resin by buffer A containing 0.34 M NaCl as described under "Experimental Procedures." Aliquots (10 µl) of each fraction were preincubated with equal volumes of inactive MAP kinase in the presence of Mg-ATP for 15 min, followed by 20-min incubation with MBP (1 µg) and [γ-32P]ATP (5 µCi). Phosphorylation of MBP was measured as described under "Experimental Procedures." Values were normalized for protein concentration. Data are mean (±S.D.) of triplicate determinations.

**Fig. 5.** Effect of various growth factors and kinase activators on stimulation of MAP kinase-activating activity. PC12 cells (1-2 × 10^6) were incubated with NGF (50 ng/ml), EGF (100 ng/ml), basic fibroblast growth factor (bFGF, 100 ng/ml), and 12-O-tetradecanoylphorbol-13-acetate (TPA, 1 µM) for 5 min. In experiments where K252a was used, cells were preincubated for 20 min with 25 nM of K252a before the addition of growth factors. Cells were pelleted by brief centrifugation, and the activator was isolated by absorption to DEAE-cellulose as described under "Experimental Procedures." MAP kinase activity was determined as described in the legend to Fig. 4. The values represent means (±S.D.) of three experiments performed in duplicate and normalized for protein concentration.
and 12-O-tetradecanoylphorbol-13-acetate (1 μM). NGF was the most potent stimulus, resulting in a 7-fold stimulation of the MAP kinase activator, whereas EGF and basic fibroblast growth factor stimulated the activator by 4- and 3-fold, respectively. In general, the magnitude of stimulation of the PC12 MAP kinase activator was correlated with that of MAP kinase activity and was consistent with that reported earlier by Ahn et al. (14) in EGF-stimulated 3T3 cells. However, 12-O-tetradecanoylphorbol-13-acetate was able to produce only a modest increase in activator activity. This correlates with the modest stimulation of MAP kinase activity by this agent in PC12 cells (35).

We tested the effect of K252a on the growth factor-stimulated MAP kinase-activating activity. K252a is a protein kinase inhibitor which has been shown to specifically inhibit NGF-mediated responses in PC12 cells at nanomolar concentrations, while having no effect on the EGF- and fibroblast growth factor-stimulated responses (36, 37), presumably by selective inhibition of the trk kinase (38). Pretreatment of the PC12 cells with 25 nM K252a specifically inhibited the production of the NGF-stimulated MAP kinase activator by more than 60%. K252a did not have any inhibitory effect on EGF- and fibroblast growth factor-stimulated levels of MAP kinase activator. Paradoxically, K252a was found to have a stimulatory effect on fibroblast growth factor-mediated stimulation of the MAP kinase activator.

**Activation and Phosphorylation of MAP Kinases by MAP Kinase Activator**—We tested whether the NGF-stimulated MAP kinase activator would directly phosphorylate MAP kinase and mediate its activation. Phosphotransferase activity toward MBP was measured as an index of MAP kinase activity in the absence or presence of MAP kinase activator (Fig. 6A). Incubation of normal wild type (WT) MAP kinase obtained from PC12 cells, recombinant p42\textsuperscript{mapk} (ERK2) and/or ERK2-K52R mutant (KM) with Mg-\([\gamma\textsuperscript{32}P]ATP\) alone did not result in detectable activation of MAP kinase activity, indicating little or no autoactivation of the catalytically active ERK2 occurred. However, in the presence of MAP kinase activator, WT and ERK2 were both activated and to equal levels. The ERK2-K52R mutant, in which lysine 52 in the ATP-binding site had been changed to arginine, rendering it catalytically inactive, did not exhibit any phosphotransferase activity toward MBP when incubated with activator under similar conditions. We also tested the capacity of MAP kinase activator to phosphorylate WT, ERK2, and KM in parallel experiments. As shown in Fig. 6B, MAP kinase activator phosphorylated WT, ERK2, and KM, as indicated by detection of radioactivity incorporated into the 42-kDa protein bands. Under similar conditions, no phosphorylation of these proteins was observed if MAP kinase activator was omitted. The phosphorylation of ERK2-K52R mutant, as well as WT and ERK2, provided direct evidence that MAP kinase activator is itself a protein kinase.

**Phosphoamino Acid Analysis of in Vitro Phosphorylated WT, ERK2, and K52R Mutant MAP Kinase**—We carried out phosphoamino acid analysis of MAP kinases after in vitro phosphorylation by MAP kinase activator. The autoradiograms reveal the presence of phosphoserine, phosphothreonine, and phosphotyrosine residues. The relative amount of the phosphoamino acids observed was identical in WT, ERK2, and KM (Fig. 7). After the in vitro phosphorylation by MAP kinase activator, all three 42-kDa phosphoproteins contained predominantly phosphotyrosine with lesser amounts of phosphothreonine and phosphoserine. The finding that the catalytically inactive ERK2-K52R mutant was phosphorylated at all residues established that the NGF-stimulated PC12 cell

![Fig. 6. Activation and phosphorylation of wild type (WT), recombinant (ERK2), and kinase-defective (KM) MAP kinases by MAP kinase activator. A, the peak MAP kinase activator fraction (ACT) from Mono Q chromatography was incubated with WT, ERK2, and KM in the presence of Mg-ATP. After 15 min, MBP (1 μg) and [\(\gamma\textsuperscript{32}P\)]ATP (5 μCi) were added, and incorporation of the radioactivity in MBP was determined after 20 min as described under “Experimental Procedures.” B, the WT, ERK2, and KM MAP kinases were phosphorylated in the presence or absence of MAP kinase activator as described under “Experimental Procedures.” The phosphorylated proteins were subjected to SDS-PAGE, dried, and autoradiographed. The arrow indicates the position of phosphorylated 42-kDa protein bands representing the various MAP kinases.

MAP kinase activator is a dual specificity kinase.

**Phosphatase Inactivation of MAP Kinase Activator**—The MAP kinase activator was detected following activation of trk receptor kinase, and it was possible that activator was itself regulated by phosphorylation. To test this possibility, we treated the MAP kinase activator with alkaline phosphatase, the serine/threonine specific protein phosphatase 2A (PP2A), and tyrosine-specific recombinant rat brain tyrosine phosphatase-1 (rrbPTP-1) for different time periods and then tested the ability of the activator to stimulate the activity of MAP kinase (Fig. 8). The treatment of MAP kinase activator with alkaline phosphatase immobilized on agarose resulted in the inactivation of MAP kinase activator. More than 80% inactivation was observed within 10 min, and a complete loss in activator activity was achieved within 30 min. This observation indicated that the activator was itself a phosphoprotein, and its capacity to stimulate MAP kinase was exhibited only by the phosphorylated MAP kinase activator species. Alkaline phosphatase acts primarily as serine/threonine phosphatase but also exhibits tyrosine phosphatase activity (39). The specific involvement of the serine/threonine phosphorylation of the activator was tested by incubation of MAP kinase
Phosphotyrosine were analyzed as described under "Experimental Procedures." After electrophoresis on thin layer cellulose, phosphorylated amino acids were located by autoradiography. The position of phosphotyrosine (p-Tyr), phosphothreonine (p-Thr), phosphoserine (p-Ser), inorganic phosphate (Pi), and the point of sample application (Origin) are indicated. Other labeled spots represent partially hydrolyzed phosphopeptides.

**Inactivation of MAP kinase activator by phosphatase.** The peak MAP kinase activator fraction from Mono Q chromatography was dialyzed and then incubated for the indicated times at 30 °C with 10 milliunits/ml PP2A, 300 units/ml PTP-1, or 50 units/ml alkaline phosphatase as described under "Experimental Procedures." Phosphatases were inactivated by okadaic acid (PP2A, ■), Na3VO4 (PTP-1, ○) or by centrifugation (alkaline phosphatase, ▲). MAP kinase activator was then tested for its ability to reactivate the inactive MAP kinase. In control experiments, activator was incubated with PP2A + okadaic acid (Δ) or PTP-1 + Na3VO4 (○) simultaneously.

with PP2A (10 milliunits/ml). PP2A inhibited the ability of the activator to stimulate MAP kinase activity. More than 50% of activity was lost within 5 min of incubation, with a complete loss of activity within 30 min. In control experiments, approximately 90% of the activity remained when activator was treated with PP2A in the presence of okadaic acid. These data demonstrate that serine/threonine phosphorylation is required for the expression of MAP kinase activator activity.

Treatment of the activator with the tyrosine-specific phosphatase rrbPTP-1 also led to its inactivation. Only 4% of the MAP kinase activator activity remained after 30 min of incubation with PTP-1 (300 units/ml). In control experiments in which the activity of PTP-1 was inhibited by the presence of 2 mM Na3VO4, there was no loss in the activity of activator (Fig. 8). The specificity of PTP-1 for phosphotyrosine indicated that the expression of MAP kinase activator activity involved tyrosine phosphorylation. Taken together, these results indicate that the MAP kinase activator must be phosphorylated at serine/threonine and tyrosine for expression of enzyme activity.

**Detection of Phosphotyrosine Phosphorylation of the MAP Kinase Activator.** To determine whether MAP kinase activator was tyrosine-phosphorylated in response to NGF in PC12 cells, we prepared the Mono Q fractions from the NGF-treated or untreated control cells. Fractions containing the MAP kinase activator activity were separated on 10% SDS-PAGE and then transferred to an Immobilon P membrane and immunoblotted with monoclonal anti-phosphotyrosine antibodies. The anti-phosphotyrosine antibodies strongly recognized a protein band whose molecular mass on SDS-PAGE was ~50 kDa in Mono Q fractions containing MAP kinase activator activity (Fig. 9B). The anti-phosphotyrosine immunoreactivity of the protein was dramatically stimulated on NGF treatment of the cells. The peak immunoreactivity was detected in fraction 14, coincident with the peak MAP kinase activator activity as determined by MBP phosphorylation in the presence of ERK2 (Fig. 9A). The observation of the NGF-stimulated tyrosine phosphorylation of an appropriately sized protein in a position coincident with MAP kinase activator activity, coupled with the rrrPTP-1 sensitivity of the activator, provides strong evidence for the critical involvement of tyrosine phosphorylation in MAP kinase activator activation.

**DISCUSSION**

The cellular mechanisms through which NGF elicits its biological actions has recently been the focus of much attention following the discovery that the trk proto-oncogene was capable of binding NGF, resulting in activation of its intrinsic tyrosine kinase activity (5, 6). NGF triggers a signal transduction cascade in which MAP kinases are a prominent component. The potential downstream targets or effectors of MAP kinase action include Raf-1, S-61I kinase, and c-jun (40–42). It is now clear that p21<sup>ras</sup> is an obligatory intermediate in this pathway, as ras-dominant negative mutants abolish the ability of NGF to stimulate MAP kinase activity (12, 13). Kremer et al. (43) have provided evidence that p60<sup>crk</sup> is a potential upstream regulator of p21<sup>ras</sup> action in PC12 cells in response to NGF, at least with respect to the capacity of these cells to extend neurites.

The molecular mechanisms subserving the activation of the MAP kinases have come under increasing scrutiny (44) and have been proven to be substantially more sophisticated than originally believed. It was initially presumed that a very limited number of intervening steps might exist between the hormone receptor and MAP kinase activation. However, the recognition that expression of enzymatic activity required that the enzyme be phosphorylated on both tyrosine and threonine residues was interpreted as being indicative of the convergence of multiple signal transduction pathways acting upon MAP kinase (16). Although this interpretation was conceptually appealing, the available data are not wholly consistent with this hypothesis. Subsequently, it was found...
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that a soluble MAP kinase “activator” was generated on hormone treatment of 3T3 cells (14). Further, the discovery that MAP kinases had the capacity to autophosphorylate on both threonine and tyrosine residues has contributed to the controversy surrounding the mechanisms through which these enzymes are activated.

The original observation that a MAP kinase “activator” could be detected was that of Ahn et al. (14). They found that MAP kinase could be activated upon combination of inactive MAP kinase with other fractions from hormone-treated cells. Moreover, inactivation of MAP kinase by removal of either threonine or tyrosine residues was reversed with the regeneration of the doubly phosphorylated species. Subsequently, Gomez and Cohen (9) have reported similar findings in NGF-treated PC12 cells. After completion of the present studies, a number of reports describing isolation of MAP kinase activator that possess as an intrinsic protein kinase activity have been reported (22–25).

The partial purification of the activator isolated from NGF-treated PC12 cells by ion exchange and gel filtration chromatography reported here are, in general, consistent with previously reported findings. When Mono Q column fractions from NGF-treated PC12 cells were screened for the activities able to reactivate inactive MAP kinase I (Fig. 1, peak I), the activator consistently eluted before the MAP kinase I peak as a sharp peak at 0.03–0.05 M NaCl. We have never detected any MAP kinase activating activity in any other fractions nor in fractions derived from untreated control cells. The MAP kinase activator described by Gomez and Cohen (9) eluted as a broad peak on a Mono Q column and eluting immediately before and overlapping with the MAP kinase I peak. We did not detect activator activity eluting in the flow-through fractions, as reported by Ahn et al. (22, 14) using PC12 or 3T3 cells. The apparent $M_r = 50–60,000$ for the MAP kinase activator reported here is similar to that reported by both Ahn et al. (14) and Gomez and Cohen (9). The recently characterized MAP kinase activator exhibits similar chromographic behavior, but has an apparent size of 45 kDa (23, 24, 45, 52).

The MAP kinase activator is itself a phosphoprotein whose activity is regulated through phosphorylation. We report here that treatment of the MAP kinase activator with PP2A or rrbPTP-1 results in the complete loss of activity. These findings demonstrate that the activator requires phosphorylation at both serine/threonine and tyrosine residues. It is notable that these data differ from those of Gomez and Cohen (9), who found that PP2A was effective in inactivating the MAP kinase activator, whereas the three protein tyrosine phosphatases, CD45, T-cell phosphatase, and LAR, were without effect. The PP2A sensitivity of the MAP kinase activator was also verified by others (25, 45). The capacity of rrbPTP-1 to inactivate the MAP kinase activator may be a consequence of a restricted substrate specificity exhibited by this enzyme. Alternatively, rrbPTP may express low levels of serine/threonine phosphatase activity, although this has not been reported and we consider it unlikely. Our observation of rrbPTP inactivation of the activator was unexpected, and serial repetitions of this experiment lead us to conclude that tyrosine phosphorylation is a critical regulator of the MAP kinase activator. This finding is further supported by our finding that NGF treatment of the PC12 cells resulted in an increased tyrosine phosphorylation of a protein band having a molecular mass of 50–60 kDa eluting in fractions coincident with MAP kinase activator activity on Mono Q chromatography. Indeed, MAP kinase activator has been shown to undergo autophosphorylation on tyrosine, serine, and threonine residues in EGF-stimulated A431 cells (23) and from Xenopus oocytes (45), suggesting the importance of tyrosine phosphorylation in expression of MAP kinase activator activity. MAP kinase was not reactivated following incubation with Mg-ATP alone, indicating that the autophosphorylation of tyrosine and serine/threonine residues occurs at levels that are insufficient to result in significant elevation of the enzymatic activity.

NGF very rapidly stimulated the generation of MAP kinase activator that was detected within 30 s of NGF exposure. Importantly, our results are consistent with an in vivo role of the activator, as its activation precedes the development of MAP kinase activity, which was maximal at 5 min (8, 29, 46) and is closely correlated with the tyrosine phosphorylation of the enzyme (11). Activator levels have returned to near basal levels after 15 min of NGF treatment. These results are consistent with those of Seger et al. (23) but differ from those reported by Gomez and Cohen (9). The latter study found MAP kinase activator in PC12 cells following 15 min of NGF treatment. It is unclear whether the activity they have characterized represents the same species or if multiple activator
species are generated. It is also possible that the activator is progressively modified as a function of time of exposure to NGF, resulting in different susceptibilities to phosphatase inactivation.

The MAP kinases are members of a newly discovered class of dual specificity protein kinases (47). Dual specificity kinases undergo autophosphorylation at serine/threonine, as well as at tyrosine residues, and can phosphorylate other substrates at the same residues (48, 49). These enzymes share sequence homology with the p34*cdc2* protein kinase (18, 47–50). We have clearly demonstrated that the MAP kinase activator is a protein kinase that directly phosphorylates the MAP kinases. However, we can not rule out the possibility that there are other species that also serve to activate the MAP kinases. The existence of an “autokinase-enhancing factor” was suggested by L’Allemain et al. (51), which causes the phosphorylation of the wild type p42*msk* but not of a kinase-defective mutant K52R (Fig. 6A). Furthermore, phosphokinase kinase as indicated by the phosphorylation of the defective K52R mutant, suggesting the possible existence of tyrosine residues. Similarly, Ettehadieh et al. (52) who reported similar findings. We have also screened the activator-containing fractions for activities that react with a monoclonal antibody to MEK1. Activity in these fractions was only present when MAP kinase activator isolated from PC12 cells cross-reacts with a monoclonal antibody to MEK1.2

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Addendum—While this manuscript was under review, Erikson et al. (50–55) reported the purification and cloning of MAP kinase/ERK kinase (MEK). MAP kinase activator isolated from PC12 cells cross-reacts with a monoclonal antibody to MEK1.2

REFERENCES

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MAP Kinase Activator in PC12 Cells