Nerve growth factor stimulates a novel protein kinase in PC-12 cells that phosphorylates and activates mitogen-activated protein kinase kinase (MEK)

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Activation of mitogen-activated protein kinase (MAP kinase) plays an important role in the cellular effects of nerve growth factor (NGF). Although the precise pathway by which NGF activates MAP kinase is not clear, several enzymes have been identified that may form a linear phosphorylation cascade, in which MAP kinase is activated by MAP kinase kinase (MEK). A key enzyme that links the *ras*-GTP complex to MEK is widely believed to be the *raf* kinase. However, immunoprecipitation experiments in PC-12 cells revealed that *raf* is not the major NGF-dependent MEK kinase [Zheng, Ohmichi, Saltiel and Guan (1994) *Biochemistry* 33, 5595–5599]. We have identified a protein kinase from PC-12 cells that catalyses both the phosphorylation and activation of MEK. This activity is stimulated 3-fold in cells treated with NGF. The partial purification on FPLC and characterization of this MEK kinase indicate that it is distinct from *raf*, MEK, MAP kinase and other previously described NGF-stimulated protein kinases. The activity of this enzyme is

unaffected by direct addition to the assay of heparin, staurosporine, K252A and the heat-stable cyclic AMP-dependent kinase peptide inhibitor, but is slightly inhibited by NaF and calcium ions. Comparison of its behaviour on gel permeation and sucrose-density gradients indicates a molecular mass in the region of 50 000 Da. Moreover, isoelectric focusing of the enzyme revealed a pI of approx. 7.3. The kinase activity is specific for ATP as substrate with a K_m of 11 μ M, and requires Mg^{2+} as a cofactor. Analysis of the activation of this enzyme in PC-12 cells transfected with a dominant inhibitory mutant of p21^{ras} suggests that this MEK kinase resides downstream of *ras* in the MAP kinase activation pathway. Moreover, site-directed mutation of the residues on MEK that are phosphorylated by *raf* does not completely abrogate phosphorylation by the MEK kinase, suggesting that this enzyme may share some phosphorylation sites with *raf*, but also phosphorylates MEK on other sites.

INTRODUCTION

Protein phosphorylation plays an important role in the cellular actions of nerve growth factor (NGF). Although a number of serine/threonine kinases activated in response to NGF have been described, the best characterized is the family of enzymes known collectively as the mitogen-activated protein kinases (MAP kinases) [1]. These enzymes, which are stimulated by virtually every known mitogen [1–11], are activated by phosphorylation on tyrosine and threonine residues, catalysed by a specific MAP kinase kinase or MEK [12]. MEK is itself activated by serine phosphorylation. Thus far, three families of enzymes capable of phosphorylating MEK have been described, homologues of the yeast *Saccharomyces cerevisiae* protein kinase STE11 [13], the product of the proto-oncogene *c-mos* [14] and the products of the proto-oncogene *c-* and *B-raf* [15–17]. It has been proposed that STE11 may be activated by a *ras*- or G_i -specific pathway, perhaps mediating the stimulation of MEK by thrombin and other ligands for G_i -associated receptors [13]. The expression of *c-mos* is limited to germ cells [14], suggesting that it is unlikely to play a major role in growth factor-dependent MAP kinase activation.

There is considerable evidence to support an important role for the *raf* kinase in MEK/MAP kinase activation [6,13,15–19]. In *v-raf* transfected cells, both MEK and MAP kinase are constitutively active [15–17]. A *Drosophila* homologue of MEK can rescue a *Drosophila raf* mutant phenotype [20]. Moreover,

baculovirus-expressed *raf* can efficiently phosphorylate MEK *in vitro*, causing its activation [18,19]. However, expression of an activated *raf* in PC-12 cells did not lead to MAP kinase activation [21]. In growth factor-stimulated 3T3 or PC-12 cells, immunoprecipitated *raf* can phosphorylate and activate MEK [22]. However, sequential precipitation with anti-*raf* sera depleted lysates of *raf*, but did not remove the majority of growth factor-sensitive MEK kinase activity, suggesting a second kinase that is separate from *raf*. We report here the characterization of this MEK kinase, and demonstrate that it is activated by NGF in a cascade that is downstream from p21^{ras} activation.

EXPERIMENTAL

Materials

All reagents were from Sigma except for tissue-culture supplies (GIBCO), 2.5 S NGF (Bioproducts for Science, Indianapolis, IN, U.S.A.), Walsh peptide (generous gift of Dr. Harry LeVine), and [γ -³²P]ATP (3000 Ci/mmol) (Amersham Corp.). The Mono Q (HR 5/5 or 10/10) and Superose-12 FPLC columns were from Pharmacia Biotechnology. A PC-12 cell line expressing an inducible dominant inhibitory mutant of *ras*, c-Ha-*ras*(Asn-17) with a substitution of asparagine for serine at position 17 [23], was the generous gift of Dr. J. Blenis (Harvard Medical School) [24]. Antibodies to the C-terminus of *B-raf* were purchased from

Abbreviations used: IEF, isoelectric focusing; MAP kinase, mitogen-activated protein kinase; MAP-2, microtubule-associated protein-2; MBP, myelin basic protein; MEK, MAP kinase kinase; NGF, nerve growth factor; GST, glutathione S-transferase; PMA, phorbol 12-myristate 13-acetate; DEX, dexamethasone; EGF, epidermal growth factor.

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Santa Cruz Biotechnology. A second anti-*raf* serum specific for *raf-1* [25] was a generous gift from Dr. R. Jove (University of Michigan). Anti-STE11 serum [13] was a generous gift from Dr. G. Johnson (National Jewish Center for Immunology and Respiratory Medicine).

Anti-MAP kinase serum was prepared by immunizing three New Zealand White rabbits with a glutathione S-transferase (GST) fusion protein containing 21 C-terminal amino acids (TDEPVAEEPFTFDMELDDLKP) of p44^{mapk} [26,27]. This pooled antiserum recognized pp42^{mapk} and pp44^{mapk} by Western blotting and immunoprecipitation of denatured protein (results not shown).

Cell culture and preparation of cell-free extract

PC-12 cells and a PC-12 cell line expressing the inducible dominant inhibitory mutant of *ras*, c-Ha-*ras*(Asn-17) controlled by a dexamethasone (Dex) promoter [23], were adapted to grow on plastic 150-mm-diam. tissue-culture dishes in Dulbecco's modified Eagle's medium with 10% (v/v) fetal bovine serum. Before hormonal treatment, the medium was replaced with serum-free medium and incubated for 1 h. Dex was dissolved in ethanol and diluted in culture medium to a final concentration of 5 μ M and incubated for 3 h at 37 °C. All treatments with NGF were performed by adding 100 nM NGF directly to the medium for 5 min at 37 °C. After hormonal treatment, the medium was removed, and the cell layer was quickly washed three times with 10 ml of ice-cold Ca²⁺-free PBS. Cells were collected by centrifugation and resuspended in lysis buffer (20 mM Tris/HCl, pH 7.4, containing 1% CHAPS, 1 μ g/ml leupeptin and 1 μ g/ml aprotinin). The cells were vortexed briefly and centrifuged at 20000 *g* for 25 min. The resulting supernatant was loaded on to a Mono Q FPLC column.

MAP kinase assay

Cell-free lysates were prepared as described above. Lysate protein (5 μ g) was used for MAP kinase assay in a 25 μ l reaction mixture containing 20 mM Tris/HCl, pH 7.4, 10 mM MgCl₂, 2 mM EGTA, 50 μ M [γ -³²P]ATP (1 μ Ci), and 10 μ g of microtubule-associated protein-2 (MAP-2) as described previously [28].

Partial purification of the MEK kinase

Cell-free extract was applied at a flow rate of 1 ml/min to a Mono Q column equilibrated with buffer A (20 mM Tris/HCl, pH 7.4, 0.2 mM PMSF, 10 mM MgCl₂, 2 mM EGTA) and eluted with a 30-ml linear gradient of 0–0.3 M NaCl in buffer A. Fractions were immediately assayed for MEK kinase activity.

The Mono Q chromatography-purified MEK kinase preparation was applied to a pH range 1–12 Rotofer agarose isoelectric focusing (IEF) column. Active fractions were dialysed into 9 mM NaCl/1 mM EGTA/10% (v/v) glycerol. Ampholyte (pH 5–8) was added to the sample at a final concentration of 3%. The IEF was carried out at 10 °C for 2.5 h at 12 W of constant power. The MEK kinase activity in each fraction was measured. The pH value of each fraction was also measured.

Sucrose-density-gradient analysis

Peak fractions from Mono Q chromatography were concentrated by dialysing against powdered polyethyleneglycol (molecular mass 15000–20000 Da). Aliquots (100 μ l) of concentrated fractions were subjected to sedimentation-equilibrium analysis in 5–20% sucrose gradients (4 ml) in 25 mM Tris/HCl, pH 7.4, containing 2 mM EGTA and 0.25 M NaCl. After centrifugation at 56000 rev./min (100000 *g*) for 16 h in a Beckman SW 60 rotor,

six-drop fractions were collected and assayed for MEK kinase activity. The migrations of BSA (66000 Da) and carbonic anhydrase (29000 Da) were determined by analysis of parallel gradients.

Gel-filtration chromatography of MEK kinase

Peak fractions of partially purified MEK kinase were concentrated and 200 μ l aliquots were chromatographed on a Superose-12 FPLC column. The column was equilibrated with buffer A containing 0.15 M NaCl, and run at a flow rate of 0.5 ml/min.

MEK kinase assays

GST–MEK fusion protein was prepared as described previously [29]. Aliquots (30 μ l) of peak fractions from Mono Q chromatography were incubated with 10 μ g of GST–MEK at 30 °C for 15 min in a final volume of 50 μ l containing 50 mM Tris/HCl, pH 7.4, 2 mM EGTA, 10 mM MgCl₂ and 50 μ M [γ -³²P]ATP (10 μ Ci). The reaction was stopped by the addition of 1 ml of ice-cold PBS containing 20 μ l of glutathione–Sephacrose beads. The suspensions were mixed at 4 °C for 30 min, followed by washing, to isolate GST–MEK from free [γ -³²P]ATP. The isolated GST–MEK was then resolved by SDS/8%–PAGE. Gels were dried for autoradiography. For quantitative measurements, phosphorylated proteins were excised from the gels, and incorporated radioactivity was measured by Čerenkov counting.

MAP kinase kinase (MEK) assay

The MEK kinase-activated GST–MEK was assayed for its ability to phosphorylate a GST fusion protein containing p44^{MAPkinase} (GST–MAPK) in 50 mM Tris/HCl (pH 7.4), 2 mM EGTA, 10 mM MgCl₂ and 50 μ M ATP using 1 μ g of purified GST–MAPK as a substrate. The activated GST–MAPK was assayed by adding 40 μ g of myelin basic protein (MBP) and 1 μ Ci of [γ -³²P]ATP into the reaction for an additional 15 min. The reaction was stopped by the addition of 4 $\times$ Laemmli SDS/PAGE sample buffer [30], and phosphorylated MBP was resolved by SDS/12%–PAGE. The gel was dried and subjected to autoradiography. For quantitative measurements, phosphorylated proteins were excised from the gels, and incorporated radioactivity was measured by Čerenkov counting.

Western blots of *raf* and MAP kinase

Cells were grown in 150-mm-diam. dishes. Before NGF treatment, the medium was replaced with serum-free medium for 12 h. After NGF treatment, the cells were lysed and chromatographed on a Mono Q column as described above. Each fraction was resolved on SDS/8%–PAGE and then transferred to nitrocellulose membrane. The membranes were subjected to immunoblot analysis with anti-MAP kinase, anti-MEK, anti-STE11 homologue, and anti-*raf* sera, as reported before [11,31].

RESULTS

Identification of a novel NGF-stimulated MEK kinase

The phosphorylation and activation of MAP kinase in PC-12 cells by NGF is mediated by the threonine/tyrosine kinase MEK [32]. Although the precise mechanism by which MEK is activated in response to growth factors is not clear, the protein kinase encoded by the *raf* proto-oncogene can phosphorylate and activate MEK *in vitro* [13,15,16,19]. However, in recent studies [22] we have shown that c-*raf* is not the major NGF- or epidermal

Figure 1 Mono Q chromatography of an NGF-stimulated MEK kinase

PC-12 cells were treated with (□) or without (●) 100 nM NGF for 5 min. Cell lysates were prepared and chromatographed on a Mono Q column, as detailed in the Experimental section. Fractions (1 ml) were collected and assayed for the phosphorylation of a GST-MEK fusion protein. Following the incubation, GST-MEK was isolated on glutathione-Sepharose beads, and phospho-GST-MEK was analysed on SDS/PAGE (a). Phosphorylated protein was excised from gels and counted. Results are expressed as c.p.m. of incorporated ^{32}P . (b) Autoradiograph showing the phosphorylation of GST-MEK by column fractions on SDS/PAGE. (c) Immunoblot analysis of these column fractions for c-*raf* and MAP kinase. Arrows indicate eluting positions of the MEK kinase as well as MAP kinase and *raf* on this column. MEK itself does not bind to the column. A representative experiment that was repeated more than 10 times is shown.

growth factor (EGF)-stimulated MEK activator, since the majority of MEK-activating activity was detected in the anti-*raf* supernatant. To identify other kinases that may catalyse the phosphorylation of MEK, PC-12 cells were treated with or without NGF. Following exposure to the growth factors, cell lysates were chromatographed on a Mono Q column and each fraction was assayed for phosphorylation of a GST-MEK fusion protein. The incorporation of ^{32}P into GST-MEK was evaluated by SDS/PAGE, followed by autoradiography (Figure 1b) and Čerenkov counting (Figure 1a). The major peak of MEK kinase activity was detected in fractions that eluted at about 0.16 M NaCl. NGF treatment consistently caused a 3-fold increase in this kinase activity compared with that of control cells. This kinase activity could be resolved from MEK itself, which does not bind to the column. A second peak of activity was detected after elution with 1 M NaCl. The activity in this peak, which co-eluted with the *raf*-1 kinase as detected by immunoblotting (Figure 1c), was only slightly sensitive to NGF treatment. Moreover, B-*raf* was also detected in this second peak, probed with a polyclonal antibody that specifically detects this form of the kinase (results not shown). Both *raf* kinases and the MEK kinase were resolved from MAP kinase (Figure 1c). In addition, Western blotting of the column fractions with a polyclonal antibody to the STE11 homologue failed to reveal any signal,

Figure 2 The NGF-stimulated MEK kinase activates GST-MEK

Ten 100-mm-diam. plates of PC-12 cells were treated with 100 nM NGF for 5 min. Cell lysate was prepared and chromatographed on a Mono Q (HR 10/10) column as described in Figure 1. Active fractions eluting at 0.16 M NaCl were pooled, dialysed into buffer A for 12 h and chromatographed on a Mono Q (HR 5/5) column. The active fractions from the second chromatography were pooled and concentrated. An aliquot of this MEK kinase preparation was incubated with 10 μg of GST-MEK in the presence of 50 μM ATP/10 mM MgCl_2 . The activated GST-MEK was isolated with glutathione-Sepharose beads and assayed for the activation of a GST-MAP kinase fusion protein, assayed by directly adding 40 μg of MBP and 1.0 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ to the reaction. The phospho-MBP was subjected to SDS/PAGE followed by autoradiography.

consistent with previous observations that this kinase is not significantly expressed in these cells [22]. The mammalian Ste11 homologue has been found in other PC-12 cell lines [13] although cloned PC-12 lines frequently differ regarding phenotype and protein levels.

To explore whether the phosphorylation of GST-MEK by the major NGF-dependent kinase resulted in activation of the enzyme, we evaluated the activation of a GST-MAPK fusion protein by the recombinant MEK fusion protein after treatment with the kinase fractions (Figure 2). Fractions containing the kinase activity eluting at 0.16 M NaCl from control or NGF-treated cells were pooled and reconstituted with the GST-MEK in the presence of ATP/ MgCl_2 . Following a 15 min incubation, the MEK fusion protein was immobilized on glutathione-Sepharose beads, followed by extensive washing to remove the unbound kinase and ATP. The purified GST-MEK was eluted from the beads with 10 mM reduced glutathione and then incubated with a recombinant MAP kinase fusion protein in the presence of ATP/ MgCl_2 . Following a further 15 min incubation, 40 μg of MBP and 1.0 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ were added directly into the reaction for an additional 15 min incubation, and the phosphorylation of MBP was evaluated on SDS/PAGE followed by autoradiography. Preincubation of GST-MEK with the fractions isolated from control cells produced only a small increase in MEK activity. However, the pooled fractions from NGF-treated cells caused a substantial increase, reflecting approximately a 40-fold stimulation by NGF. The phosphorylation of MBP detected in this reconstitution assay was entirely catalysed by the recombinant MAP kinase, since MEK itself produces no phosphorylation of MBP (results not shown).

These results suggest that the protein eluting from the Mono Q column at 0.16 M NaCl was capable of stimulating both the

Figure 3 Phosphorylation of site-directed mutants of MEK by MEK kinase and *raf*

The MEK kinase preparation described in Figure 2 was used to assay MEK phosphorylating activity using site-specific mutants of GST-MEK and wild-type GST-MEK as substrates (a). The indicated mutant MEK and wild-type MEK fusion proteins were incubated with the purified kinase in the presence of [³²P]ATP as described in the Experimental section. After the reaction, MEK fusion proteins were isolated with glutathione-Sepharose beads and subjected to SDS/PAGE followed by autoradiography. (b) This shows the quantification of results from (a). In (c), immunoprecipitated *c-raf* from NGF-treated PC-12 cells evaluated in the phosphorylation of MEK mutants. Results shown were reproduced in six independent experiments.

phosphorylation and activation of MEK. However, it is possible that the increased phosphorylation of MEK reflected auto-phosphorylation of the enzyme secondary to activation. To evaluate directly whether the activator was an authentic MEK kinase, we used a site-directed mutant of MEK in which Lys⁹⁷ was substituted with Arg (R⁹⁷), rendering the kinase catalytically inactive (Figure 3a). The pooled active fractions catalysed the phosphorylation of both the wild-type and catalytically inert MEK almost to the same extent, although phosphorylation of the R⁹⁷ mutant was approximately 20% lower than that observed with wild-type enzyme, possibly due to increased auto-phosphorylation in the wild-type protein (Figure 3a, lane 8 versus 10). Thus, these column fractions contain a protein kinase that can both activate and directly phosphorylate MEK.

To characterize the properties of the NGF-stimulated MEK kinase further, the activity of the enzyme was assayed in the presence of various agents known to modulate protein kinases. Heparin and the cyclic AMP-dependent protein kinase inhibitor peptide had no effect on the kinase activity, suggesting that the MEK phosphorylation is neither due to casein kinase, which is inhibited by low concentrations of heparin [32a], nor to cyclic

Figure 4 Isoelectric focusing of MEK kinase

An aliquot of MEK kinase preparation described in Figure 2 was applied to a pH 4–9 rotator IEF column. Fractions were assayed for MEK kinase activity. The autoradiograph of a representative assay is shown in (a). The pH curve determined by pH measurements of the fractions is shown in (b). Similar results were obtained from three separate experiments. The arrow indicates the fraction containing the MEK kinase activity.

AMP-dependent protein kinase. Direct addition of NaF, H-7, staurosporine or K252A did not significantly inhibit the kinase activity. This observation, in addition to the lack of requirement of the enzyme activity for Ca²⁺ ions, indicated that this activity was not due to protein kinase C or a calmodulin-sensitive kinase.

The effects of bivalent cations on the kinase activity were also evaluated. Both Mg²⁺ and Mn²⁺ supported MEK kinase activity, although Mg²⁺ was the preferred cofactor. In the presence of MgCl₂ addition of 10 mM CaCl₂ to the incubation caused an 80% inhibition of the enzyme activity. ZnCl₂ also caused a marked reduction in MEK kinase activity, possibly due to the Zn²⁺-catalysed reduction of an essential sulphhydryl.

To characterize further the MEK kinase, we employed preparative IEF (Figure 4). The peak fraction of the NGF-stimulated MEK kinase purified by Mono Q chromatography was applied to an IEF column for 750 V/h IEF. Fractions were collected and assayed for MEK kinase activity (Figure 4a). In a parallel lane, pH was measured (Figure 4b). The MEK kinase was detected in a single peak, with its pI estimated to be 7.3.

The MEK kinase shares one *raf* phosphorylation site on MEK

The sites on MEK that are phosphorylated by the *raf* kinase have been mapped to Ser²¹⁸ and Ser²²² [33]. Mutation of these residues to alanine blocks the *raf*-catalysed phosphorylation and activation of the enzyme [33]. In order to determine whether the MEK kinase described here phosphorylates similar or different sites, we evaluated phosphorylation of the corresponding mutant GST-MEK fusion proteins (Figure 3). GST fusion proteins were constructed containing an Ala substitution for Ser at position 218 (A²¹⁸) and 222 (A²²²), plus the Lys-Arg substitution at position 97, as well as a triple mutation at sites 218, 222 and 97. The phosphorylation of these mutant proteins was evaluated by both immunoprecipitated *raf* (Figure 3c) and the purified kinase

Figure 5 Gel-filtration chromatography of MEK kinase

An aliquot of MEK kinase preparation from Figure 2 was chromatographed on a Superose-12 FPLC column as detailed in the Experimental section. Fractions (0.5 ml) were collected and assayed for MEK kinase activity. Inset: Stokes' radius was determined by comparison with standards: aldolase (48 Å) [46], BSA (36 Å) [46], carbonic anhydrase (CA, 20 Å) [47].

(Figures 3a and 3b) from cells treated with NGF. As described above for the MEK kinase, the phosphorylation of R⁹⁷ MEK by immunoprecipitated *raf* is equivalent to that seen with wild-type MEK [33]. A single substitution of either Ser²¹⁸ or Ser²²² to alanine in the catalytically inert enzyme did not significantly block *raf*-catalysed phosphorylation. However, substitution of both serines for alanine in the catalytically inert MEK greatly reduced phosphorylation by *raf*. Interestingly, mutation of Ser²²² had little effect on phosphorylation induced by the MEK kinase (Figure 3a, lane 4 versus lane 8). However, substitution of Ala for Ser²¹⁸ reduced by approximately 75%, but did not eliminate, the phosphorylation catalysed by this kinase. This reduction was observed in both the double Arg⁹⁷/Ala²¹⁸ as well as in the triple Arg⁹⁷/Ala²¹⁸/Ala²²² mutants. Thus, these data indicate that the MEK kinase may share one *raf* phosphorylation site, Ser²¹⁸, but also exhibits a distinct recognition pattern for MEK.

Physicochemical and kinetic properties of the NGF-stimulated MEK kinase

The apparent molecular mass of the NGF-stimulated MEK kinase was determined by gel-filtration chromatography and sucrose-density-gradient sedimentation. The peak fractions of NGF-stimulated MEK kinase activity purified by Mono Q chromatography were applied to a Superose-12 FPLC column (Figure 5). MEK kinase activity was detected primarily in a peak eluting at 26 min, just prior to the carbonic anhydrase standard. The Stokes' radius was estimated to be 32 Å, by comparison with standards.

The peak fractions of NGF-stimulated MEK kinase activity purified by Mono Q chromatography were concentrated and applied to a 5–20% sucrose-density gradient. Gradients were subjected to equilibrium sedimentation, and MEK kinase activity was assayed in the collected fractions (Figure 6). A sedimentation coefficient of 3.55 S was calculated by comparison with standards. Analysis of data from Superose-12 gel filtration and density-gradient-sedimentation equilibrium yielded an apparent molecular mass of 45000 Da (sucrose gradient) or 50000 Da (gel filtration).

The dependence of the partially purified MEK kinase on substrate concentration was evaluated. In assays using GST–

Figure 6 Sucrose-density-gradient centrifugation of MEK kinase

An aliquot of MEK kinase preparation from Figure 2 was subjected to density-gradient centrifugation on a 5–20% sucrose gradient. Six-drop fractions were collected from the bottom of the tube and assayed for MEK kinase activity as described in the Experimental section. Inset: Sedimentation coefficient of the enzyme was determined by comparison with standards: carbonic anhydrase (CA, 3.2 S) [46] and BSA (4.3 S) [45].

MEK fusion protein as substrate in the presence of 10 mM MgCl₂, ATP was the preferred phosphate donor. GTP (up to 0.5 mM) did not reduce ³²P labelling of the substrate when added to the standard assay containing 50 μM [γ-³²P]ATP, suggesting that it was a poor phosphoryl donor for this enzyme (results not shown). Evaluation of the dependence of the NGF-stimulated kinase on ATP concentration revealed that the K_m for ATP was approx. 11 μM under these conditions. To determine the dependence of the partially purified MEK kinase on GST–MEK, the affinity of the MEK kinase towards a GST–MEK fusion protein was evaluated by adding increasing concentrations of the fusion protein. Phosphorylation of GST–MEK was dose-dependent, with a K_d of approx. 1 μM under these conditions.

The NGF-stimulated MEK kinase activity is not attenuated by dephosphorylation

To determine whether protein phosphorylation plays a role in activation of the MEK kinase, extracts from NGF-treated cells were treated with calf intestinal alkaline phosphatase for 30 min. Following this treatment, control and alkaline phosphatase-treated extracts were chromatographed on Mono Q columns to separate the phosphatase (which elutes in the breakthrough) from the MEK kinase. The active fractions were collected and assayed for MEK kinase activity as described above. A control assay was carried out in which no GST–MEK was added. Following the assay, the whole reaction was subjected to SDS/PAGE (Figure 7). In the control experiment without addition of GST–MEK (see Figures 7a and 7b), alkaline phosphatase caused a dramatic reduction in the phosphorylation of endogenous cellular proteins in these fractions (Figure 7a versus Figure 7b). However, evaluation of GST–MEK phosphorylation revealed that alkaline phosphatase did not cause any significant change in the MEK kinase activity detected in eluted fractions (Figures 7c versus 7d). Although we can not rule out the possibility that the MEK kinase was not dephosphorylated by alkaline phosphatase treatment, these results suggest that phosphorylation may not be critical to activation of the MEK kinase.

Figure 7 Alkaline phosphatase does not reduce MEK kinase activity

Four plates of PC-12 cells were treated with 100 nM NGF for 5 min. Cell-free lysates were prepared and treated with or without 100 units of calf intestinal alkaline phosphatase (CIAP) for 30 min at 37 °C. Incubations were stopped by loading on to a Mono Q column. Active fractions were assayed for MEK kinase activity in the absence (**a** and **b**) or presence (**c** and **d**) of GST-MEK. Calf intestinal alkaline phosphatase elutes in breakthrough. (**a**) and (**b**) were expressed five times longer than (**c**) and (**d**).

MEK kinase activation by NGF is protein kinase C-independent, but is downstream of *ras*

To characterize the cellular activation of the enzyme further, we evaluated the role of protein kinase C in its stimulation by NGF. NGF can activate the ϵ isoform of protein kinase C [34]. Moreover, stimulation of protein kinase C by phorbol ester can lead to MAP kinase activation [11,35], although the stimulation of MAP kinase activity by NGF in PC-12 cells is protein kinase C-independent [1]. PC-12 cells were exposed to phorbol 12-myristate 13-acetate (PMA) for 5 min or 12 h, prior to addition of NGF. Lysates were fractionated on Mono Q and MEK kinase was assayed. Treatment for 5 min of cells with PMA alone produced only a slight stimulation of MEK kinase, consistent with previous observations that phorbol esters do not cause a significant stimulation of MAP kinase activity in this PC-12 cell line [1]. Prolonged exposure to PMA did not block the effect of NGF, suggesting that protein kinase C does not contribute to NGF-induced MEK kinase phosphorylation in PC-12 cells. The down-regulation of protein kinase C by prolonged PMA treatment was confirmed by enzyme assay and immunoblot analysis [34].

NGF-dependent activation of MAP kinase has been shown to require p21^{ras} activation, since expression of a dominant inhibitory mutant of *ras* protein can block activation of this enzyme [24]. In order to determine whether the MEK kinase described here is downstream from *ras* in the NGF-stimulated phosphorylation cascade, we utilized a PC-12 cell line expressing an inhibitory mutant of *ras* controlled by the MMTV promoter [23], in which the mutant *ras* protein can be induced with Dex.

Figure 8 Induction of dominant inhibitory mutant of *ras* blocks the NGF-stimulated MEK kinase activation

Four 150-mm-diam. plates of PC-12 cells transfected with a inducible dominant inhibitory mutant of *ras* were treated with or without Dex for 3 h followed by 5 min incubation with or without 100 nM NGF. MEK kinase from each plate was purified by Mono Q chromatography. The active fractions were pooled and assayed for MEK kinase activity. Results are representative of two independent experiments.

Cells were treated with or without Dex to induce the dominant negative *ras*, followed by a 5 min exposure to NGF. Lysates were then prepared and the MEK kinase was purified by Mono Q chromatography and assayed. The induction of the dominant inhibitory *ras* with Dex for 3 h significantly reduced (> 90%) NGF-stimulated MAP kinase activity, although treatment of control cells with Dex under these conditions did not reduce MAP kinase or MEK activity (results not shown). However, preincubation of the transfected cells with Dex lowered the basal MEK kinase activity and completely blocked its stimulation by NGF (Figure 8). These data suggest that the enzyme resides downstream of *ras* in the NGF-stimulated phosphorylation cascade.

DISCUSSION

The activation of the MAP kinase pathway by NGF is thought to play an important role in the cellular actions of this growth factor [1,6,37]. Although many of the details remain incomplete, a model has emerged in which a linear pathway links the high-affinity pp140^{src} receptor to the kinase [6]. It is believed that the tyrosine kinase activity of the receptor undergoes auto-phosphorylation upon NGF binding, resulting in increased phosphorylation of some intracellular substrates [38]. Among these are the *shc* protein, which upon phosphorylation forms a complex with the adapter protein Grb2, via an interaction with the SH2 domain of the latter molecule [39]. This complexed protein can then target the *ras* nucleotide exchange factor sos-1, inducing GTP binding of p21^{ras} and subsequent activation [38]. The activation of *ras* can then lead to the initiation of a cascade of kinase activations, including stimulation of MEK and its substrate MAP kinase. In this report we describe the identification of a novel protein activator of MEK, the activity of which is stimulated by exposure of cells to NGF. This activator is itself a

protein kinase, capable of phosphorylating and subsequently activating recombinant MEK.

The NGF-sensitive MEK kinase appears not to be an already identified protein kinase. Its chromatographic behaviour and apparent molecular mass indicate that it is not one of the enzymes previously shown to phosphorylate MEK, including the *raf* kinases, *c-mos*, STE11, MAP kinase or MEK itself. Moreover, the unique kinetic and physicochemical properties of the NGF-sensitive MEK kinase described here, in addition to its susceptibility to various agents, indicate that it is distinct from previously identified NGF-sensitive kinases. The MEK kinase differed from kinase N in molecular mass, suitability of GTP as substrate, preference for Mg^{2+} as cofactor and chromatographic behaviour [40]. The MEK kinase also does not appear to be protein kinase C, since it was not inhibited by direct addition of protein kinase C inhibitors to the assay and not down-regulated by prolonged incubation with PMA. Moreover, the inhibition of MEK kinase by calcium ions rules out some forms of protein kinase C, as well as other Ca^{2+} -dependent kinases. The cyclic AMP-dependent protein kinase is ruled out by lack of sensitivity to the Walsh peptide [41]. Additionally, the MEK kinase differs from both casein kinase and ribosomal S6 kinases on the basis of its chromatographic behaviour and sensitivity to heparin [32a,42]. This kinase is similar to a partially characterized MEK kinase recently described in insulin-stimulated adipocytes [43].

Although the biochemical mechanism by which the MEK kinase is activated remains unknown, the enzyme may not require phosphorylation, since treatment with alkaline phosphatase did not cause inactivation. Moreover, the total blockade of NGF-dependent activation of the MEK kinase in PC-12 cells induced expression of a dominant inhibitory mutant of *ras* protein suggests that, like the *raf* kinase, this MEK kinase is downstream of *ras*. MEK kinase appears to share at least one phosphorylation site on MEK with *raf*, Ser²¹⁸. This site appears to be required for activation [33], although other phosphorylation sites may play important roles. Taken together, these data suggest that the *raf* kinase and the MEK kinase may operate synergistically, and perhaps in parallel, to fully activate MEK. Indeed, expression of a dominant negative *raf* protein in COS-M6 and COS-1 cells appears to block growth factor-dependent MAP kinase activation [44,45], indicating an essential role for *raf*. However, expression of a constitutive form of *raf* did not lead to an appreciable increase in MAP kinase [24], suggesting that, *in vivo*, *raf* may be necessary, but not sufficient, for MEK activation.

Although the MEK kinase described here appears to be a new enzyme, it is not unique to PC-12 cells. The activity of this kinase is also increased by exposure of 3T3 cells to EGF and platelet-derived growth factor (L. Pang, C.-H. Zheng, K.-L. Guan and A. R. Saltiel, unpublished work), suggesting that it may play an important role in activation of MEK by many growth factors. However, it is not yet clear whether this enzyme is necessary for MEK activation. The molecular characterization of this protein will hopefully allow the elucidation of its precise role in the control of cellular growth and differentiation.

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