RESEARCH COMMUNICATION

Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells

Comparison of the effects of nerve growth factor and epidermal growth factor

Sarah TRAVERSE,*‡ Nestor GOMEZ*, Hugh PATERSON,† Chris MARSHALL† and Philip COHEN*
*MRC Protein Phosphorylation Unit, Department of Biochemistry, University of Dundee, Dundee DD1 4HN, Scotland, and †Chester Beatty Laboratories, Institute for Cancer Research, Fulham Road, London SW3 6JB, U.K.

Stimulation of PC12 cells with nerve growth factor (NGF) increased mitogen-activated protein kinase kinase (MAPKK) activity > 20-fold after 5 min to a level that was largely sustained for at least 90 min. MAPKK activity was stimulated to a similar level by epidermal growth factor (EGF), but peaked at 2 min, declining thereafter and returning to basal levels after 60–90 min. Activation of MAPKK by either growth factor occurred prior to the activation of MAP kinase, consistent with MAPKK being the physiological activator of MAP kinase. The results demonstrate that the transient activation of MAPKK by EGF and its sustained activation by NGF underlies the transient and sustained activation of MAP kinase induced by EGF and NGF respectively. NGF or EGF induced the same two forms of MAPKK that were resolved on a Mono Q column. The Peak-1 MAPKK was activated initially and partially converted into the more acidic peak-2 MAPKK after prolonged growth-factor stimulation. The Peak-2 MAPKK was 20-fold more sensitive to inactivation by the catalytic subunit of protein phosphatase 2A. Stimulation with NGF caused a striking translocation of MAP kinase from the cytosol to the nucleus after 30 min, but no nuclear translocation of MAP kinase occurred after stimulation with EGF. The results suggest that sustained activation of the MAP kinase cascade may be required for MAP kinase to enter the nucleus, where it may initiate the gene transcription events required for neuronal differentiation of PC12 cells.

INTRODUCTION

The rat phaeochromocytoma cell line PC12 is a chromaffin-like cell which is responsive to several growth factors whose receptors are protein tyrosine kinases, such as nerve growth factor (NGF) and epidermal growth factor (EGF). Consistent with a common signalling mechanism, NGF and EGF both stimulate a number of processes in PC12 cells within minutes, such as deoxyglucose uptake [1], Na^+ influx via the Na^+–H^+ exchanger [2], induction of transcription factors like Jun and Fos [3] and appearance of ornithine decarboxylase activity [1]. However, a striking difference between NGF and EGF is that only the former is able to promote differentiation of PC12 cells into a sympathetic-neuron-like phenotype. The changes that occur progressively over several days include neurite outgrowth, electrical excitability and cessation of growth [4]. These observations have led to the suggestion that NGF stimulates differentiation via activation of a signal-transduction system distinct from that which underlies the common effects of NGF and EGF [5].

NGF and EGF both trigger the activation of the protein serine/threonine kinase, termed mitogen-activated protein (MAP) kinase, within minutes in PC12 cells [6–9], suggesting that this kinase may mediate some of the rapid actions that are common to both growth factors, and apparently excluding a role for MAP kinase in neuronal differentiation. However, an important difference between these two growth factors is that activation of MAP kinase by NGF is sustained at a high level for at least several hours, whereas activation by EGF is transient [9]. MAP kinase activation requires its phosphorylation at both a threonine and a tyrosine residue (Thr-183 and Tyr-185 in $p42^{mapk}$) [10,11], and evidence obtained in PC12 [7] and other cells [12–14] has shown that a 'dual-specificity' enzyme, termed MAP kinase kinase (MAPKK) phosphorylates both residues. In the present paper we demonstrate that NGF and EGF activate identical MAPKKs and that the transient activation of MAP kinase by EGF is explained by down-regulation of a component in the cascade which is upstream of MAPKK and prior to the point at which signals emanating from the NGF and EGF receptors converge. Further studies of the intracellular localization of MAP kinase have revealed a striking translocation of MAP kinase from the cytoplasm to the nucleus after stimulation by NGF, but not EGF. Our results suggest that there is no need to invoke the existence of a separate signalling pathway for neuronal differentiation and that sustained activation of MAP kinase may explain why NGF, but not EGF, can trigger this process.

MATERIALS AND METHODS

Materials

Protein phosphatase 2A (PP2A) was purified from rabbit skeletal muscle [15] by Dr. D. Schelling at the University of Dundee. Recombinant murine $p42^{mapk}$ was expressed as a glutathione *S*-transferase fusion protein in *Escherichia coli*, purified by affinity chromatography on GSH–Sepharose and cleaved from glutathione *S*-transferase with thrombin [16]. Microcystin-LR was provided by Professor G. Codd, Department of Biological Sciences, University of Dundee. NGF was purchased

Abbreviations used: NGF, nerve growth factor; EGF, epidermal growth factor; MAP kinase, mitogen-activated protein kinase; MAPKK, MAP kinase kinase; PBS, phosphate-buffered saline; PP2A, protein phosphatase 2A.

‡ To whom correspondence should be sent.

from Gibco/BRL (Paisley, Renfrewshire, Scotland, U.K.) and EGF from Sigma Chemical Co. (Poole, Dorset, U.K.). Sources of other materials are given elsewhere [13].

Assay of MAPKK

MAPKK in PC12 extracts or Mono Q fractions was diluted in 50 mM-Tris/HCl [pH 7.5 (20 °C)]/0.1 mM-Na-EGTA/0.1 % (v/v) 2-mercaptoethanol (Buffer A) containing 1 mg of BSA/ml. An aliquot of diluted MAPKK (5 μl) was incubated for 3 min at 30 °C with 5 μl of 2 μM recombinant MAP kinase (100 units/ml after maximal re-activation by MAPKK) diluted in Buffer A containing 0.04 % (w/v) Brij 35 and 5 μl of 100 mM-Tris/HCl [pH 7.5 (20 °C)]/0.2 mM-Na-EGTA/0.5 mM-sodium orthovanadate/0.1 % (v/v) 2-mercaptoethanol (Buffer B) containing 0.4 μM-okadaic acid. The MAPKK reaction was then initiated by the addition of 5 μl of 40 mM-magnesium acetate/0.8 mM unlabelled ATP. After 20 min at 30 °C the reaction mixtures were diluted 5-fold into ice-cold Buffer A containing 1 mg of BSA/ml and 0.5 mM-sodium orthovanadate, and a 10 μl aliquot was added to 40 μl of Buffer B containing the specific peptide inhibitor of cyclic AMP-dependent protein kinase (1.25 μM) [17], 25 μM-myelin basic protein, 12.5 mM-magnesium acetate and 0.125 mM-[γ-^{32}P]ATP prewarmed to 30 °C. After incubation for 10 min at 30 °C, incorporation of phosphate into myelin basic protein was measured as described previously [6]. Control incubations were carried out in parallel in which either MAPKK or recombinant MAP kinase were omitted, and these reaction 'blanks' were subtracted from the value obtained in the presence of both MAPKK and MAP kinase. A unit of MAP kinase was that amount which catalysed the incorporation of 1.0 nmol of phosphate into myelin basic protein in 1 min. A unit of MAPKK activity was that amount which produced 50 % re-activation of MAP kinase in 1 min. The activation of MAP kinase was linear with time up to 30 % re-activation, and fractions containing MAPKK were therefore diluted appropriately before assay to ensure that initial-rate conditions were met.

Anti-p42^{mapk} antibody

Polyclonal antiserum 121, which only recognizes p42^{mapk} in either immunoblotting or immunoprecipitation experiments (S. J. Leevers, unpublished work), was raised against the *C*-terminal sequence of p42^{mapk} [18]. The antiserum (3 ml) was then affinity-purified by adsorption to 6 ml of Reactigel (Pierce, Rockford, IL, U.S.A.), to which the *C*-terminal peptide had been coupled covalently. After packing the Reactigel into a column and washing with 100 ml of 100 mM-sodium phosphate, pH 8.0, the antibody was eluted with 3.5 M-potassium thiocyanate/0.5 M-ammonium hydroxide, pH 11–12; 2 vol. of 100 mM-sodium phosphate, pH 5.5, were added to the active fractions (identified by e.l.i.s.a.) and dialysed overnight at 4 °C.

Cell culture and measurement of protein concentration

PC12 cells were cultured [6], incubated with 50 ng of NGF/ml or 100 ng of EGF/ml, and then lysed [7]. The protein concentration of the extracts was measured as described by Bradford [19].

Immunofluorescence localization of MAP kinase

PC12 cells were seeded on to glass coverslips 48 h prior to stimulation, then treated with NGF or EGF as described above. At various times the cells were fixed for 20 min in 4 % formaldehyde, washed in phosphate-buffered saline (PBS) and permeabilized for 1 h with 0.05 % saponin containing 0.2 % BSA in PBS. All subsequent steps were carried out in this solution. Coverslips were incubated for 1 h with affinity-purified rabbit anti-p42^{mapk} (1 μg/ml), and after washing were incubated with fluorescein isothiocyanate-conjugated goat anti-(rabbit IgG) antiserum (Pierce) used at a 1:1250 dilution. After mounting in Moviol containing 0.1 % *p*-phenylenediamine, preparations were examined on a Bio-Rad MRC 600 confocal imaging system in conjunction with a Nikon Optiphot fluorescent microscope and an X60 planapo objective. Nuclear fluorescence of p42^{mapk} was quantified by using the histogram program of the confocal imaging system, which measures the average pixel brightness of any defined area.

RESULTS

Activation of MAP kinase and MAPKK by NGF and EGF

A method was developed for the simultaneous quantification of MAPKK and MAP kinase activities in PC12 cell extracts. In the MAPKK assay, the apparent K_m for MAP kinase was found to be 0.15 μM, and maximal activity was obtained at 0.5–1.0 μM. The optimal concentrations of Mg^{2+} and ATP were 10 mM and 0.2 mM respectively, and the activity at pH 7.5 was higher than at pH 7.0. The effects of NGF and EGF on MAPKK and MAP kinase activity in cell extracts are shown in Fig. 1. NGF activated MAP kinase with a half-time of about 3 min, activation reaching a maximum after 15 min and only declining by 15–20 % after 90 min (Fig. 1*a*). EGF caused a similar, but even more rapid ($t_{0.5}$ = 1–2 min) activation of MAP kinase than NGF, which peaked after 5 min and then declined sharply to only 10 % of the peak value after 90 min (Fig. 1*b*).

NGF activated MAPKK with a half-time of 1–2 min, activation reaching a plateau value after 5 min that was sustained

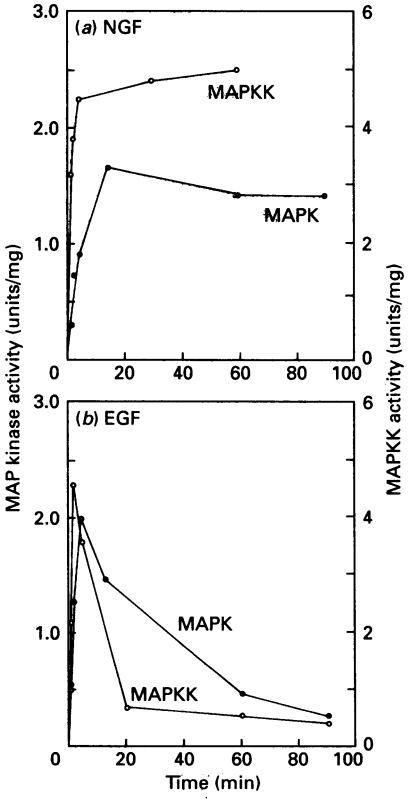

Fig. 1. Activation of MAP kinase and MAPKK in PC12 cells following stimulation by NGF or EGF

PC12 cells were stimulated for the indicated times with either NGF (*a*) or EGF (*b*) and the specific activities of MAP kinase (MAPK) (●) and MAPKK (○) measured after cell lysis as described in the Materials and methods section.

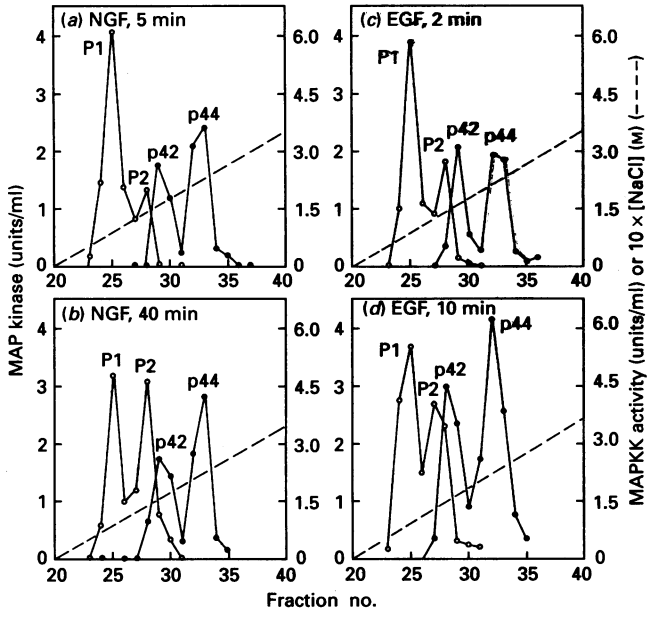

Fig. 2. Resolution of two isoforms of MAP kinase and two peaks of MAPKK activity by chromatography on Mono Q

PC12 cells were stimulated with NGF for 5 min (*a*) or 40 min (*b*) or with EGF for 2 min (*c*) or 10 min (*d*). The cells were then lysed as described in the Materials and methods section and 10 ml was applied to 5 cm × 0.5 cm columns of Mono Q equilibrated in 50 mM-Tris/HCl[pH 7.3(20 °C)]/2 mM-Na-EDTA/2 mM-Na-EGTA/0.1% (v/v) 2-mercaptoethanol/5% (v/v) glycerol/0.03% (w/v) Brij 35/0.3 mM-sodium orthovanadate/1 mM-benzamidine/leupeptin (4 μg/ml). After washing with 10 ml of equilibration buffer, the columns were developed with 40 ml linear salt gradients to 0.7 M-NaCl. The flow rate was 1.0 ml/min, and fractions (1.0 ml) were assayed for MAP kinase (●) and MAPKK (○) activities. The two peaks of MAPKK activity are denoted as P1 and P2 and the two isoforms of MAP kinase, termed $p42^{mapk}$ and $p44^{mapk}$, by 'p42' and 'p44' respectively. MAP kinase and MAPKK activities are undetectable in cells that have not been stimulated by NGF or EGF [6,7].

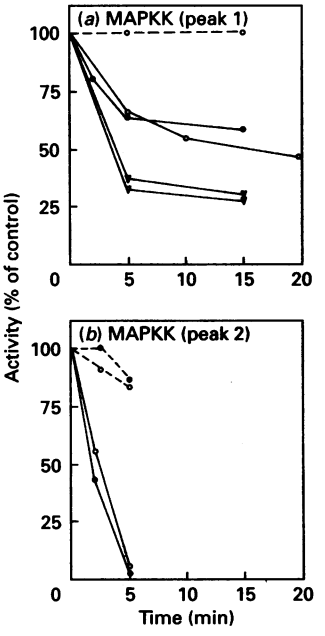

Fig. 3. Inactivation of the two forms of MAPKK by PP2A

The Peak-1 (*a*) and Peak-2 (*b*) forms of MAPKK from Fig. 2 were dialysed against 50 mM-Tris/HCl [pH 7.0 (20 °C)]/1.0 mM-Na-EDTA/0.1% (v/v) 2-mercaptoethanol (Buffer C) containing 5% (v/v) glycerol, 1.0 mM-benzamidine and leupeptin (4 μg/ml). Aliquots (5 μl) were then incubated at 30 °C with 5 μl of PP2A catalytic subunit (20 munits/ml, ○,●; 100 munits/ml, ▽,▼) in Buffer C containing 1.0 mg of BSA/ml (see [15] for a definition of units of PP2A activity). At the times indicated the PP2A reaction was stopped by the addition of 5 μl of Buffer B containing 3 μM (○,●) or 9 μM (▼,▽) microcystin-LR and 2 μM recombinant MAP kinase. The MAPKK assay was initiated with 5 μl of 40 mM-magnesium acetate and 0.8 mM-ATP and then performed as described in the Materials and methods section. The open symbols show experiments with MAPKK from NGF-treated cells and the closed symbols experiments with MAPKK from EGF-treated cells. The broken lines show control experiments in which microcystin-LR was included together with PP2A.

for at least 1 h (Fig. 1*a*) EGF caused a similar, but even more rapid, activation of MAPKK ($t_{0.5}$ ~ 1 min) than NGF. However, activation peaked after 2 min and declined to < 20% of the peak value after 20 min and to about 10% after 90 min (Fig. 1*b*).

NGF activates the two different isoforms of MAP kinase [6–8], termed $p42^{mapk}$ (or ERK2) and $p44^{mapk}$ (or ERK1), which are resolved by chromatography on Mono Q (Figs. 2*a* and 2*b*), and the same two isoforms were found to be activated by EGF (Figs. 2*c* and 2*d*). We have also reported that two peaks of MAPKK activity can be resolved by chromatography on Mono Q after stimulation for 15 min with NGF [7]. In the present study the peak of MAPKK that is the first to be eluted from Mono Q (Peak-1 MAPKK) was found to be activated by NGF initially, and after a 5 min stimulation accounted for > 80% of the activity (Fig. 2*a*). After stimulation by NGF for 40 min, Peak-1 MAPKK and Peak-2 MAPKK each contributed about 50% of the activity (Fig. 2*b*). Similar observations were made with EGF. After stimulation for 2 min the Peak-1 MAPKK was the dominant activity (Fig. 2*c*), the Peak-2 activity becoming more prominent after stimulation for 10 min (Fig. 2*d*).

MAPKK from PC12 cells [7], rabbit skeletal muscle [13,14] or *Xenopus* oocytes [20] is inactivated by preincubation with protein serine/threonine phosphatases, implying that this enzyme is dependent on serine/threonine phosphorylation for activity. The effect of PP2A on the activity of the Peak-1 and Peak-2 forms of MAPKK is shown in Fig. 3. The Peak-2 form obtained after stimulation by either NGF or EGF was found to be far more sensitive to inactivation by PP2A than the Peak-1 form. The peak-2 MAPKK was inactivated almost completely by incubation for 5 min with 10 munits of PP2A/ml (Fig. 3*b*), whereas the Peak-1 MAPKK was only inhibited by 30% after 15 min (Fig. 3*a*). Even with 50 munits of PP2A/ml, the Peak-1 MAPKK was only inhibited 50% after 5 min and 70% after 15 min. The Peak-1 MAPKK from NGF- or EGF-stimulated cells responded similarly to PP2A (Fig. 3*a*).

Stimulation with NGF, but not EGF, results in nuclear translocation of MAP kinase

Chen *et al.* [21] have reported that stimulation of Hela cells with serum leads to the translocation of $p42^{mapk}$ and $p44^{mapk}$ from the cytoplasm to the nucleus after 10 min. We were therefore interested to determine whether the difference in kinetics of MAP kinase activation by NGF and EGF in PC12 cells was accompanied by differences in nuclear translocation. Fig. 4(*a*) shows that unstimulated PC12 cells contain little nuclear $p42^{mapk}$. No significant increase in nuclear $p42^{mapk}$ was observed 15 min after stimulation by NGF (Fig. 5), but after 30 min many of the nuclei became brightly fluorescent (Fig. 4*b*). Nuclear fluorescence peaked at 60 min after NGF stimulation (Fig. 5) and was largely maintained at 90 min (Fig. 4*d* and Fig. 5) and 180 min (results

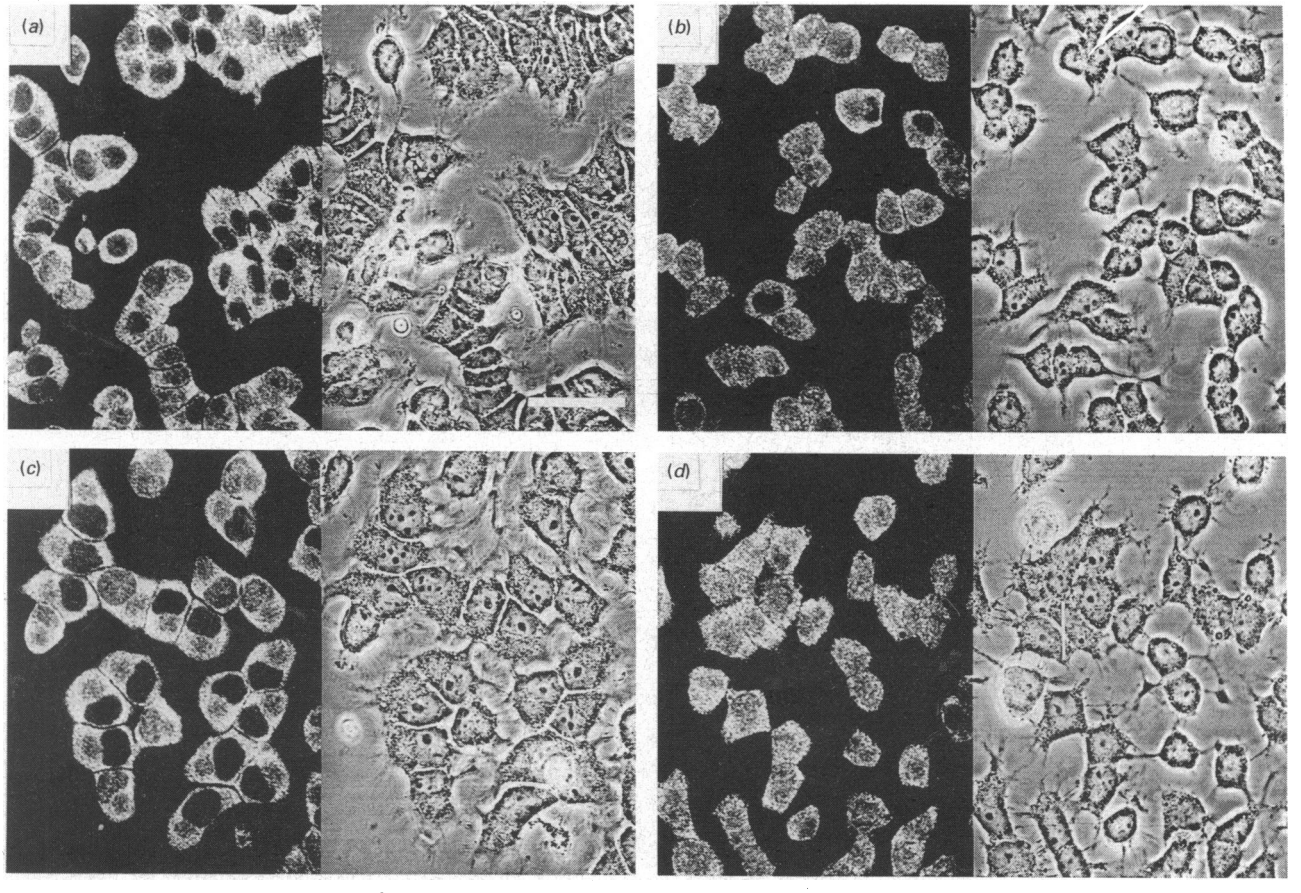

Fig. 4. Immunofluorescence staining of $p42^{mapk}$ in NGF- and EGF-stimulated PC12 cells

(*a*) Control unstimulated cells; (*b*) 30 min after NGF stimulation; (*c*) 90 min after EGF stimulation; (*d*) 90 min after NGF stimulation.

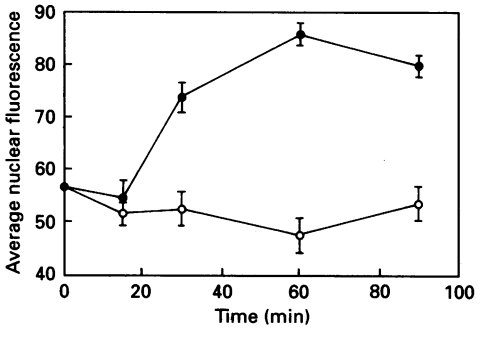

Fig. 5. Quantification of fluorescence in nuclei stained with antiserum against $p42^{mapk}$

The average pixel brightness was measured in 25–30 nuclei at each time point for cells treated with either EGF (○) or NGF (●).

not shown). In contrast with NGF, incubation with EGF caused no increase in nuclear $p42^{mapk}$ at any time (Fig. 4*c* and Fig. 5). Thus NGF and EGF not only have different effects on the kinetics of MAP kinase activation, but have markedly different effects on the cellular localization of this enzyme.

DISCUSSION

Several lines of evidence presented here demonstrate that NGF and EGF activate the same MAPKK activities in PC12 cells. The two peaks of MAPKK activity detected after stimulation by either growth factor were eluted at identical positions from Mono Q (Fig. 2), were activated in the same order (Peak-1 before Peak-2) and were affected by preincubation with PP2A in a similar manner (Fig. 3). The finding that the rate of inactivation of the Peak-2 MAPKK by PP2A is at least 20-fold higher than the rate of inactivation of the Peak-1 enzyme is an intriguing observation, but the molecular basis underlying this difference is unclear. The two peaks of activity could represent different isoforms of MAPKK, but it is also possible that they are products of the same gene. The large increase in the proportion of the peak-2 MAPKK between 5 and 40 min after NGF stimulation, over which period there is almost no overall increase in total MAPKK activity (Fig. 1*a*), suggests that the Peak-2 MAPKK is formed from Peak-1. Its elution from Mono Q at higher concentrations of NaCl than for Peak 1 could be explained by a further post-translational modification, such as phosphorylation of an additional residue(s). It is possible that phosphorylation of MAPKK at a second position may alter its conformation in such a way as to facilitate the dephosphorylation of the primary residue responsible for activation. This might be a device for increasing the rate at which the effects of NGF can be reversed after growth-factor stimulation has terminated.

MAPKK is activated more rapidly than MAP kinase in response to either NGF or EGF (Fig. 1), consistent with the activity measured in extracts being catalysed by the physiologically relevant MAPKK. Furthermore, the sustained activation of MAPKK by NGF and transient activation by EGF (Fig. 1) strongly suggests that it is alterations in MAPKK activity that underlie the sustained activation of MAP kinase by NGF and transient activation of MAP kinase by EGF (Fig. 1),

although the possibility that alterations in MAP kinase phosphatase activity also take place is not excluded. Nevertheless, the transient activation of MAPKK and MAP kinase by EGF implies that a mechanism exists for rapidly terminating the action of this growth factor on the MAP kinase cascade, even in the continued presence of EGF. Since EGF does not block the effect of NGF on MAPKK or MAP kinase (results not shown) or the effect of NGF on neuronal differentiation [2], this implies that down-regulation of the action of EGF is most unlikely to occur at a step in this protein kinase cascade that is common to both NGF and EGF. The rapid inactivation of the EGF response is more likely to take place at a step that is very near to (or at) the level of the EGF receptor.

The finding that NGF and EGF both activate MAP kinase rapidly suggests that this protein kinase may be involved in mediating the common actions of these growth factors that can be observed within minutes (see the Introduction). Furthermore, the sustained activation of MAP kinase and/or another signal-transduction pathway stimulated in parallel by NGF, and transient activation of MAP kinase (or another pathway stimulated in parallel) by EGF could explain why the increased rate of Na^+ influx induced by NGF is sustained for 1–2 h, whereas the activation of Na^+ influx by EGF peaks after 15 min and has almost returned to basal levels after 1 h [2]. It could also explain why NGF causes a 4-fold greater induction of ornithine decarboxylase activity than EGF after a 6 h stimulation [1].

The failure of EGF to stimulate neuronal differentiation has led to the suggestion that this process may be triggered by a distinct signal-transduction pathway that is switched on by NGF, but not by EGF [5]. The results presented here suggest a different interpretation, namely that neuronal differentiation may be triggered by sustained activation of MAP kinase, because it is only after prolonged (30 min) activation that marked translocation of MAP kinase to the nucleus takes place (Figs. 4 and 5). EGF may be unable to mimic the action of NGF because activation of the MAP kinase cascade is too transient to permit significant nuclear translocation of MAP kinase to take place. Since MAP kinase has been implicated in the phosphorylation and activation of the transcription factor c-Jun [22], it may well activate other nuclear factors that stimulate the transcription of genes whose protein products are essential for neuronal differentiation. The diverse effects of different growth factors on a given cell may therefore depend on the level and duration of activation of protein kinases in particular signal-transduction pathways, and does not necessarily imply that distinct signal-transduction pathways are involved.

The molecular mechanism which leads to nuclear translocation of MAP kinase is a subject for future investigation. One possibility is that the active phosphorylated form of MAP kinase enters the nucleus more rapidly than the inactive dephosphorylated enzyme, or that it leaves the nucleus more slowly. Alternatively, MAP kinase (or another protein kinase that is activated by MAP kinase [16,23]) may phosphorylate a protein(s) of the nuclear membrane/pore which facilitates nuclear translocation of MAP kinases (and perhaps other protein kinases).

S.T. is the recipient of a postgraduate studentship from the U.K. Medical Research Council and N.G. of a long-term EMBO Fellowship. The work was supported by grants from the U.K. Medical Research Council and The Royal Society (to P.C.) and by the Cancer Research Campaign (to C.M.).

REFERENCES

1. Huff, K., End, D. & Guroff, G. (1981) J. Cell Biol. **88**, 189–198
2. Boonstra, J., Moolenaar, W. H., Harrison, P. H., Moed, P., van der Saag, P. T. & Laat, S. W. (1983) J. Cell Biol. **97**, 92–98
3. Greenberg, M. E., Greene, L. A. & Ziff, E. B. (1985) J. Biol. Chem. **260**, 14101–14110
4. Greene, L. A. & Tischer, A. S. (1976) Proc. Natl. Acad. Sci. U.S.A. **73**, 2424–2428
5. Chao, M. (1992) Cell **68**, 995–997
6. Gomez, N., Tonks, N. K., Morrison, C., Härmer, T. & Cohen, P. (1990) FEBS Lett. **271**, 119–122
7. Gomez, N. & Cohen, P. (1991) Nature (London) **353**, 170–173
8. Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radziejewska, E., Morgenbesser, S. D., DePinho, R. A., Panayotatos, N., Cobb, M. H. & Yancopoulos, G. D. (1991) Cell **65**, 663–675
9. Gotoh, Y., Nishida, E., Yamashita, T., Hoshi, M., Kawakami, M. & Sakai, H. (1990) Eur. J. Biochem. **193**, 661–669
10. Anderson, N., Maller, J. L., Tonks, N. K. & Sturgill, T. W. (1990) Nature (London) **343**, 651–653
11. Payne, D. M., Rossomondo, A. J., Martino, P., Erickson, A. K., Her, J. H., Shabanowitz, J., Hunt, D. F., Weber, M. J. & Sturgill, T. W. (1991) EMBO J. **10**, 885–892
12. Ahn, N. G., Seger, R., Bratlien, R. L., Diltz, C. D., Tonks, N. K. & Krebs, E. G. (1991) J. Biol. Chem. **266**, 4220–4227
13. Nakielny, S., Cohen, P., Wu, J. & Sturgill, T. W. (1992) EMBO J. **11**, 2123–2129
14. Nakielny, S., Campbell, D. G. & Cohen, P. (1992) FEBS Lett. **308**, 183–189
15. Cohen, P., Alemany, S., Hemmings, B. A., Stralfors, P. & Tung, H. Y. L. (1988) Methods Enzymol. **159**, 390–408
16. Stokoe, D., Campbell, D. G., Leevers, S., Marshall, C. J. & Cohen, P. (1992) EMBO J. **11**, 3985–3994
17. Scott, J. D., Glaccum, M. B., Fischer, E. H. & Krebs, E. G. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 1613–1616
18. Leevers, S. J. & Marshall, C. J. (1992) EMBO J. **11**, 569–574
19. Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
20. Matsuda, S., Kosako, H., Takenaka, K., Moriyama, K., Sakai, H., Akiyama, J., Gotoh, H. & Nishida, E. (1992) EMBO J. **11**, 973–982
21. Chen, R. H., Sarnecki, C. & Blenis, J. (1992) Mol. Cell. Biol. **12**, 915–927
22. Pulverer, B. J., Kyriakis, J. M., Avruch, J., Nikolakaki, E. & Woodgett, J. R. (1991) Nature (London) **353**, 670–674
23. Sturgill, T. W., Ray, L. B., Erikson, E. & Maller, J. L. (1988) Nature (London) **334**, 715–718

Received 7 September 1992/25 September 1992; accepted 2 October 1992