Induction of the mitogen-activated protein kinase phosphatase MKP3 by nerve growth factor in differentiating PC12

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Abstract In PC12 sympathetic neurons activation and nuclear translocation of ERK family MAP kinases plays an essential role in processes underlying nerve growth factor (NGF)-dependent differentiation. We have recently cloned MKP-3 as a novel dual specificity phosphatase displaying selectivity towards inactivation of the ERK1 and ERK2 MAP kinases. Here we report that in PC12 cells, MKP-3 undergoes powerful and specific up-regulation by NGF while a number of mitogens and cellular stresses are ineffective. NGF-stimulated MKP-3 expression appears after 1 h, is maximal at 3 h, and is sustained for 5 days. This coincides with a critical period of neurite outgrowth and terminal differentiation. Consistent with a role mediating inhibition of PC12 cell MAP kinases, NGF-stimulated ERK2 activation was suppressed considerably following pre-treatment with fibroblast growth factor and 9-*cis*-retinal, two additional differentiation factors found to induce powerfully MKP-3 expression. Given the clear cytosolic localization of MKP3 in PC12 cells and sympathetic neurons, these results suggest a critical role for inactivating ERK MAP kinases in non-nuclear compartments during essential stages of NGF-mediated PC12 differentiation.

Key words: Dual specificity phosphatase; Mitogen-activated protein kinase phosphatase 3; Mitogen-activated protein kinase; Extracellular signal-regulated kinase; PC12 cell; Neuronal differentiation; Nerve growth factor

1. Introduction

Extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase or stress-activated protein kinase (JNK/SAPK) and p38/CSBP/RK (p38) exemplify three major classes of mitogen-activated protein (MAP) kinase [1–3]. While ERK activation is linked predominantly to cellular stimulation by growth factors, oncogenic $p21^{ras}$ and G-protein coupled receptors, JNK/SAPK and p38 activation follows exposure to cellular stresses and inflammatory cytokines [1–3]. Activated MAP kinases can phosphorylate and regulate many downstream targets including additional kinases, receptors, cytoskeletal proteins, as well as transcription factors such as Elk-1, ATF-2, c-Jun and CHOP [1,4–11]. This, together with observations using specific chemical inhibitors and dominant negative constructs [12–17], highlights a central role for MAP kinases orchestrating a range of rapid and long-term changes in cell function.

Of the known functions for activated MAP kinases, good evidence now supports a key role in processes underlying differentiation, survival and death of neurons. Hence, in the pheochromocytoma cell line PC12, nerve growth factor (NGF) elicits cell cycle arrest, neurite formation and enhanced electrical excitability and this is accompanied by prolonged activation and nuclear translocation of ERK family MAP kinases [14,18–20]. Extended ERK activation by constitutively active upstream kinases is also sufficient to mimic NGF action, while inhibition of this MAP kinase cascade blocks neuronal differentiation [14,15]. In addition, withdrawal of NGF from terminally differentiated PC12 cells leads to apoptosis coinciding with a drastic decrease of ERK activity [13,21]. Moreover, use of mutant kinases functioning upstream of different MAP kinase pathways indicates that not only ERK inhibition, but activation of JNK/SAPK and p38 MAP kinases is critical to processes leading to the induction of apoptosis in growth factor-deprived cells [13]. Despite the importance of MAP kinases in neuronal function, mechanisms responsible for controlling their activation state remain unclear.

Activation of MAPK family members requires dual phosphorylation on tyrosine and threonine residues and specific kinases responsible for this modification have been identified [1–3,14–16,22–28]. Cellular phosphorylation is a reversible process and among the large number of protein tyrosine phosphatases, a new class of dual specificity phosphatase (DSP) has recently been recognized as mediating direct and specific inactivation of MAP kinase activity [29]. Nine DSP genes have so far been identified, and significantly, some appear highly selective for inactivation of different MAP kinases. MKP-3, for instance, is highly selective for inactivating ERK, while the DSP M3/6 is effective only against the JNK/SAPK and p38 MAP kinases [30]. Interestingly, many DSPs display rapid and powerful transcriptional induction by growth factors and/or cellular stresses indicating an important mechanism for MAP kinase control [31–37]. Despite the importance of MAP kinases in neuronal function, their regulation by DSPs during differentiation or programmed cell death

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Abbreviations: MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK/SAPK, c-Jun NH_2-terminal kinase/stress-activated protein kinase; MAPKAPK, MAP kinase activated protein kinase; DSP, dual specificity protein phosphatase; MKP-3, MAP kinase phosphatase 3; MBP, myelin basic protein; EGF, epidermal growth factor; NGF, nerve growth factor; bFGF, basic fibroblast growth factor; RTK, receptor tyrosine kinase; Trk, tyrosine receptor kinase

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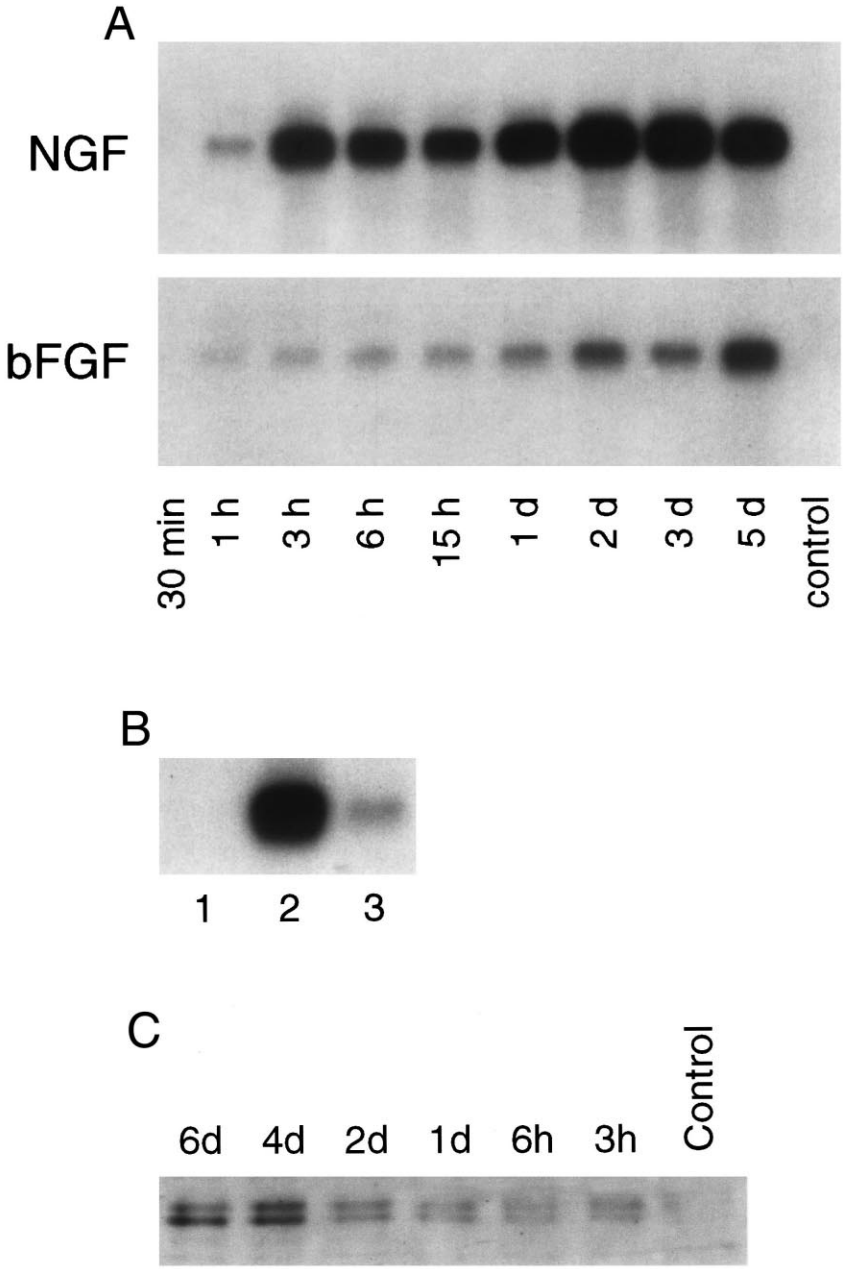

Fig. 1. Induction of MKP-3 by NGF and bFGF in PC12 cells. A: PC12 cells were primed overnight in medium containing 1% horse serum and subsequently treated with 100 ng/ml of NGF or bFGF for the times indicated. RNA extraction and Northern blot analysis were performed as described in Section 2. Methylene blue staining to visualize ribosomal RNA revealed equal RNA loading. B: To test cycloheximide sensitivity PC12 cells were stimulated by addition of NGF as indicated above in the presence or absence of cycloheximide (140 μM) followed by incubation for 3 h at 37°C. 5 μg of total RNA was electrophoresed, blotted onto nylon and subjected to Northern blot hybridization, as described. Lane 1: control untreated cells; lane 2: cells treated with NGF for 3 h; lane 3: cells treated with NGF plus cycloheximide for 3 h. C: To analyze MKP-3 protein expression following NGF treatment for the indicated period of time, total cellular proteins were extracted as described in Section 2. 60 μg of the cellular lysates were resolved in a 10% SDS polyacrylamide gel and Western blot analysis performed using affinity purified MKP-3 rabbit antiserum. This figure shows a representative experiment among several with identical results.

has not yet been studied. As part of an investigation of DSP functions in embryonic and adult nervous system, we report here on the regulated expression of the ERK-selective DSP MKP-3 during NGF-dependent differentiation of PC12 cells.

2. Materials and methods

2.1. Chemicals

RPMI 1640 cell culture medium was obtained from Gibco (Basel, Switzerland), protein A-Sepharose 4B was from Pharmacia Biotech Inc. (Uppsala), horse and fetal calf serum were from AMIMED (Bioconcept, Allschwill, Switzerland). 2.5 S NGF was from Promega (Madison, WI, USA). [γ-^{32}P]ATP (5000 Ci/mmol) and [^{32}P]UTP were from DuPont de Nemours International S.A. (Regensdorf, Switzerland). Goat anti-rabbit IgG horseradish peroxidase conjugate was from Bio-Rad Laboratories (Glattbrugg, Switzerland) or Amersham International. 9-*cis*-Retinal was obtained from Sigma (Buchs, Switzerland). All other reagents were obtained from Boehringer Mannheim A.G. (Rotkreuz, Switzerland), Sigma, or local vendors.

2.2. PC12 cell culture

PC12 cells were grown in 10 cm collagen IV coated plates in me-

dium containing 10% (v/v) horse serum and primed overnight in medium containing 1% horse serum before addition of mitogens or NGF as described [38]. In studies on the activation of MAP kinases, cells were seeded at a density of 6×10^4 cells/cm^2 in 60 mm on day 0, primed in 1% horse serum containing medium on day 1 and pretreated with growth factors from day 2 on, together with a further exchange into 0.5% horse serum containing medium.

2.3. RNA extraction and Northern blot analysis

Total RNA extraction, electrophoresis and Northern blot hybridization were performed as previously described using an antisense [^{32}P]UTP-labeled riboprobe specific for MKP3 [38,39].

2.4. MKP3 antibody generation and Western blotting analysis

New Zealand White rabbits were immunized with a keyhole limpet hemocyanin conjugated peptide corresponding to amino acids 95–112 of MKP3. The polyclonal serum was affinity purified using GST-MKP3 coupled to DEAE-Affi-gel Sepharose [39]. For immunodetection of MKP3, PC12 cells plated on 10 cm dishes and treated for different times with NGF were washed twice with ice-cold PBS and extracted in 100 μl of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.7, 150 mM NaCl, 1% (v/v) NP-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM benzamidine, 1 mM PMSF, 10 mM NaF, 1 mM sodium pyrophosphate, 5 mM sodium vanadate and 10 nM calyculin) and immediately frozen in a dry ice-methanol mixture. Cell extracts were thawed and rotary mixed for 1 h at 4°C and insoluble material was removed by centrifugation at $100\,000\times g$ for 20 min at 4°C. 60 μg of soluble proteins were resolved on SDS-polyacrylamide gels (10%) followed by electrotransfer onto nitrocellulose membranes as described [38].

2.5. ERK2 immunoprecipitations and immunocomplex kinase assay

For immunoprecipitation of *endogenous* ERK2, cell extracts were prepared as mentioned above. 150 μg of soluble proteins were diluted to a final volume of 900 μl with lysis buffer and mixed with 2 μl of anti-ERK2 polyclonal antibody (#122; generous gift from Professor C.J. Marshall, Institute of Cancer Research) prebound to a 50% suspension (v/v) of protein A/G-Sepharose beads. The complex was rotary mixed overnight at 4°C and beads were then sedimented by centrifugation at $10\,000\times g$, washed once in 1 ml of buffer, three times in 1 ml of Tris-HCl 10 mM, pH 7.5 and finally resuspended in 30 μl of Tris-HCl 10 mM, pH 7.5. Immunocomplex kinase assays were performed as described, using MBP as substrate [30,38]. Every experiment shown in this publication is representative of at least four additional experiments showing similar or identical results.

3. Results and discussion

Northern blot analysis revealed that MKP-3 mRNA was undetectable in undifferentiated PC12 cells growing in the absence of NGF (Fig. 1A). However, addition of NGF led to a powerful induction of MKP-3 mRNA which was maximal at 3 h and sustained throughout the following 5 days in the continual presence of neurotrophic factor (Fig. 1A). It is of note that the expression of MKP-3 mRNA parallels extensive neurite outgrowth during this period of NGF-dependent differentiation [18] (data not shown). Interestingly, basic FGF (bFGF) and 9-*cis*-retinal are additional factors known to promote neuronal differentiation or expression of genes characteristic of differentiated PC12 cells [40–42], and both of these agents also result in induction of MKP-3 mRNA. While bFGF-stimulated MKP3 mRNA expression displays a time course which parallels responsiveness to NGF (Fig. 1A), induction in response to 9-*cis*-retinal is slightly delayed insofar that maximal expression appears after 6 h of exposure (Fig. 3). In contrast to these observations, MKP-3 mRNA expression was not induced by any other mitogen or cellular stress tested including serum, IGF-II, EGF, phorbol esters, dibutyryl cyclic AMP, ultraviolet light, anisomycin, hydrogen peroxide, osmotic or heat shock (data not shown). Together, these

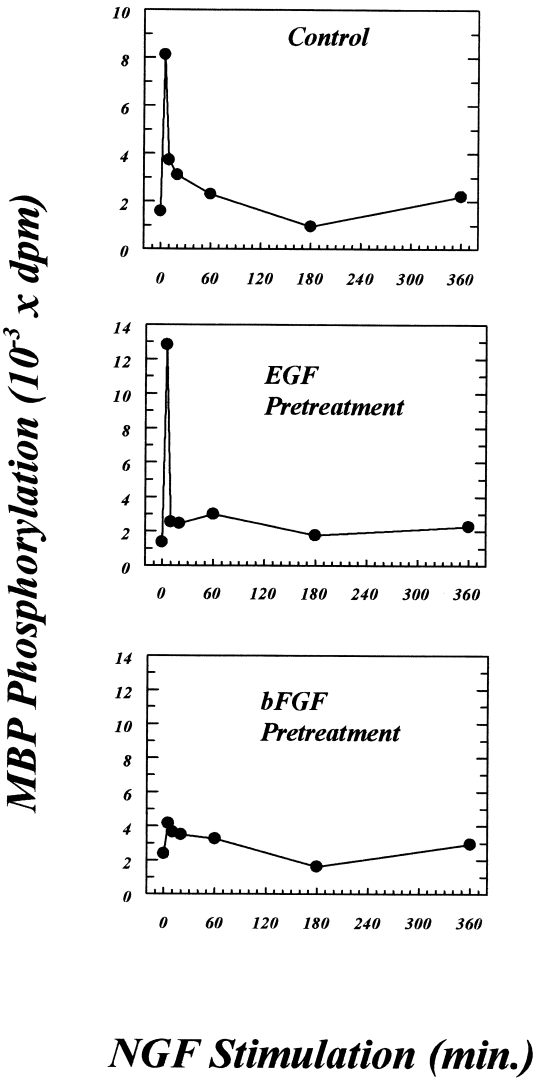

Fig. 2. Effect of bFGF and EGF pretreatment on NGF stimulation of ERK2. PC12 cells were primed for 24 h in medium containing 1% horse serum after which bFGF (100 ng/ml), EGF (150 ng/ml) or vehicle was added following a further exchange into medium containing 0.5% horse serum as indicated in the figure. Cells were then incubated for 48 h prior to stimulation with NGF (100 ng/ml), after which ERK2 activity was measured by immunocomplex assay with MBP as substrate, at the indicated times.

observations indicate that induction of MKP-3 in PC12 cells is a highly specific response to agents promoting or facilitating neuronal differentiation. This highly selective MKP-3 induction by neurotrophic agents is in contrast to several other DSPs (CL100/MKP-1, MKP-2, hVH5) which appear equally well induced by mitogenic agents, cellular stresses or NGF (unpublished, [31,37,43]). One additional observation relating to NGF-stimulated MKP-3 mRNA induction is its sensitivity to inhibition by the protein synthesis inhibitor cycloheximide. Indeed, cycloheximide completely inhibited the NGF-mediated expression of MKP-3 mRNA (Fig. 1B), indicating that MKP-3 is not an immediate early gene and its delayed expression in PC12 cells reflects molecular events dependent on NGF-stimulated protein synthesis. In this respect also, MKP-3 is distinct from several other DSPs studied in PC12 cells where, for instance, CL100/MKP-1, MKP-2 and hVH5 all

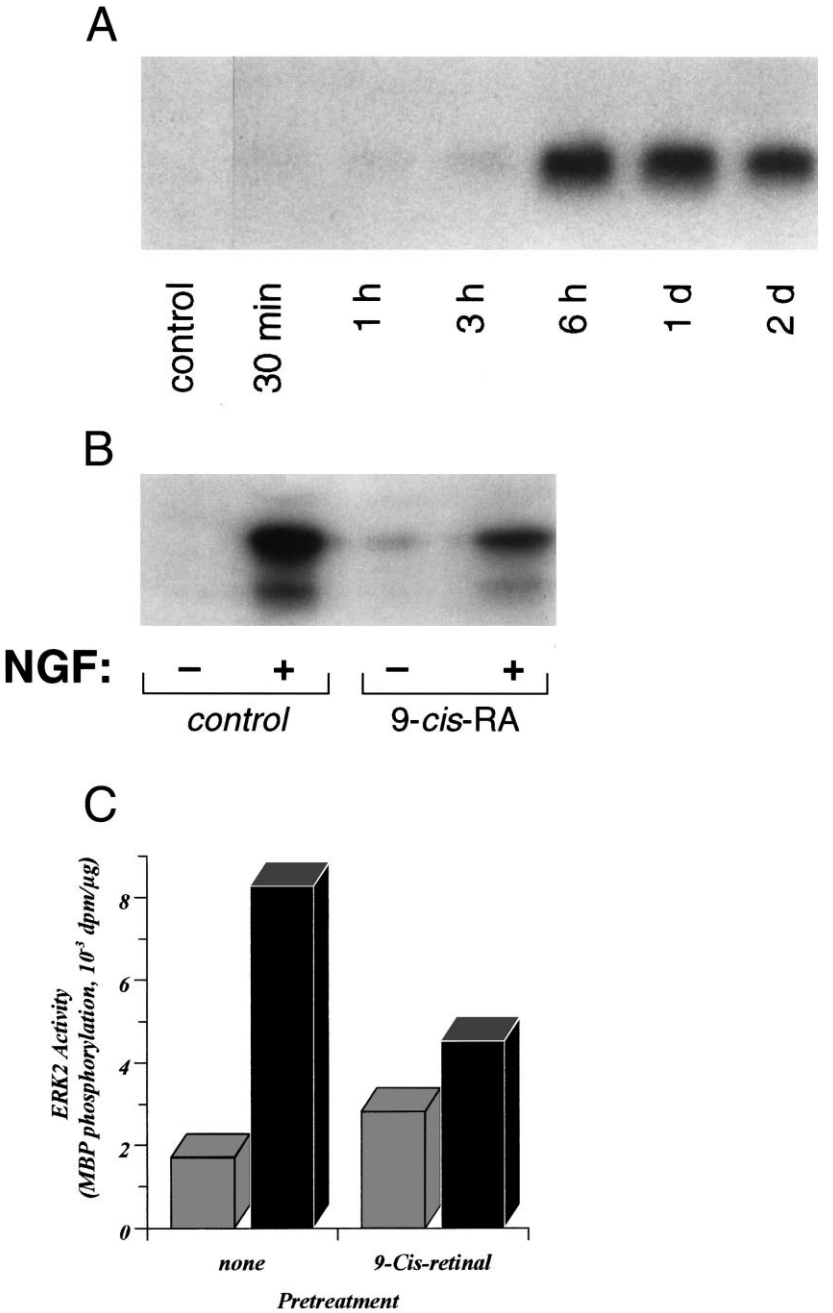

Fig. 3. Induction of MKP3 mRNA by 9-*cis*-retinal and blockade of NGF-stimulated ERK2 activity by 9-*cis*-retinal pretreatment. A: PC12 cells were primed in medium containing 1% horse serum for 24 h after which they were stimulated with 9-*cis*-retinal (10 μM) in medium containing 0.5% horse serum. At the indicated times total cellular RNA was extracted and 5 μg analyzed by Northern blot hybridization as described in Section 2. B,C: PC12 cells were primed in medium containing 1% horse serum for 24 h before addition of vehicle (control) or 9-*cis*-retinal (10 μM) (9-cis-RA) in medium containing 0.5% horse serum. After incubation for 48 h, cells were stimulated with (+) or without (−) NGF (100 ng/ml) for 5 min and MBP phosphorylation measured in ERK immunocomplex assays. B: Autoradiography of the SDS-PAGE gel showing NGF-stimulated MBP phosphorylation by ERK2 immunprecipitates after 9-*cis*-retinal pretreatment. C: Graphical representation of ERK2 activation measured as incorporation of radioactivity into the band corresponding to MBP upon stimulation with vehicle (gray bars) or NGF for 5 min (black bars). This figure shows the results of a representative experiment among several with identical results.

display a rapid and transient induction characteristic of the expression of immediate early genes [31,36,37]

To confirm that induction of mRNA leads to the synthesis of MKP-3 protein, PC12 cell extracts were subjected to Western analysis using an affinity purified polyclonal antibody. Similar to the pattern of MKP-3 mRNA induction, MKP-3 protein can first be detected 3 h following challenge with NGF and thereafter levels increase in parallel with mRNA levels for up to 6 days (Fig. 1C). As we reported previously for MKP-3 transfected into COS-7 cells [38], endogenous MKP-3 is detected in PC12 extracts as a doublet running as a protein of 42–44 kDa (Fig. 1C). This doublet may be attributed to alternative translational initiation at two in-frame 5′ methionines [38]. Previously, MKP-3 has been shown by us and others to be localized exclusively in cytosolic compartments of a number of cell types [38,46]. We have also confirmed this distri-

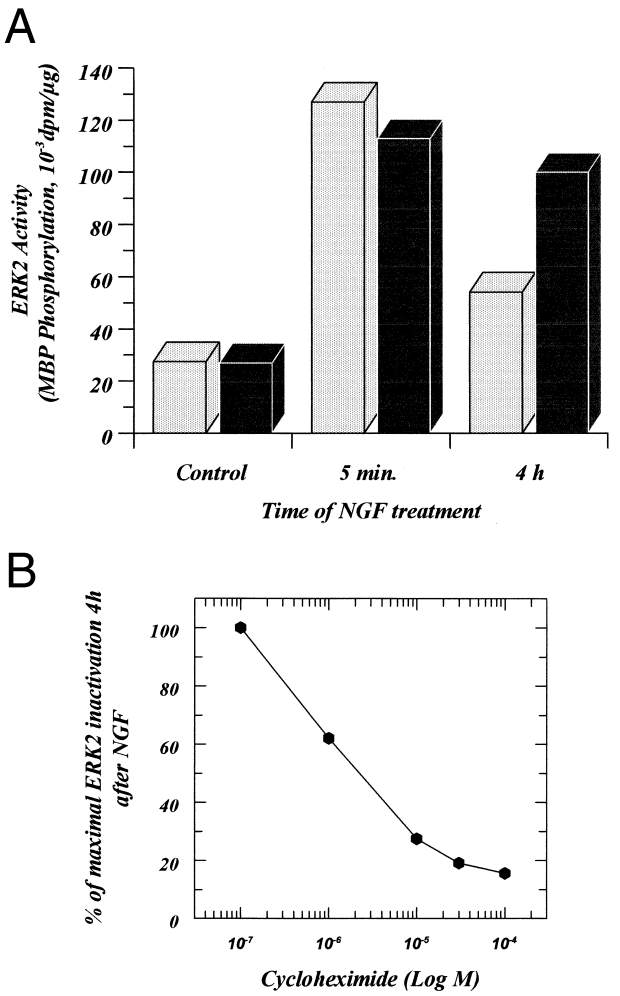

Fig. 4. Effect of the protein synthesis inhibitor cycloheximide on ERK2 activation state following stimulation by NGF. A: PC12 cells were primed for 24 h in medium containing 0.5% horse serum before addition of vehicle (open bars) or 100 μM of cycloheximide (filled bars), after which cells were stimulated with NGF (100 ng/ml) and further incubated at 37°C for 5 min or 4 h. Results are plotted as MBP phosphorylation by immunoprecipitated ERK2 in response to NGF. B: Concentration-dependent inhibition of ERK2 inactivation naturally occurring following 4 h exposure to NGF. Cells were primed as described in A and different concentrations of cycloheximide were added 1 h prior to stimulation with NGF (100 ng/ml). Results represent the percent of maximal ERK2 inactivation following 4 h exposure to NGF in the presence of the cycloheximide concentration indicated.

bution in PC12 cells by performing cellular fractionation with subsequent Western blot analysis (data not shown). In this respect MKP-3 appears distinct from other dual specificity phosphatases which are clearly nuclear in a range of cell types [35,44,45].

Experiments using purified proteins as well as expression in COS cells show that MKP-3 displays clear selectivity for inactivation of ERK MAP kinase family members [30,38,46,47]. To test for a similar role in PC12 cells, we assessed NGF-stimulated activation of ERK2 following treatment with agents inducing MKP-3. Hence, under conditions where bFGF leads to a robust expression of MKP-3 after 48 h (Fig. 1A), NGF-stimulated ERK2 activation was completely abolished (Fig. 2). No inhibition of NGF-stimulated ERK2 was observed following a 48 h exposure to EGF which fails to induce detectable MKP-3 (Fig. 2). Similarly, treatment with 9-*cis*-retinal for 48 h leads to a powerful induction of MKP-3 (Fig. 3A) and this also results in a substantial inhibition of NGF-stimulated ERK2 activity (Fig. 3B,C). These results indicate that MKP-3 induction is an important factor in processes inactivating ERK2 enzymatic activity in PC12 cells. Consistent with this, we found that inhibition of NGF-stimulated MKP-3 expression using cycloheximide (Fig. 1B) abolished the down-regulation of NGF-mediated ERK2 activation, normally occurring 3–4 h following NGF stimulation (Fig. 4). These experimental approaches provide strong circumstantial evidence consistent with the notion that MKP-3 induction in PC12 cells mediates, at least in part, inactivation of ERK family MAP kinases following stimulation with NGF. Such an idea is also consistent with a previous report showing that in vitro dephosphorylation of ERK2 by extracts of PC12 cells pretreated with NGF for 4 h, but not earlier times, is blocked by vanadate (a potent inhibitor of MKP-3) but not by the serine/threonine phosphatase inhibitor microcystine [48].

In this study we demonstrate powerful induction of the dual specificity protein tyrosine phosphatase MKP-3 in PC12 cells exclusively by agents promoting neuronal differentiation. We also describe observations consistent with a functional role for MKP-3 in suppressing NGF-mediated ERK2 stimulation during NGF-mediated differentiation. This is in agreement with highly selective inactivation of ERK1 and ERK2 compared to JNK/SAPK and p38 MAP kinases seen by in vitro enzymatic characterization following MKP-3 expression in COS cells [31,49]. This, together with its late induction by NGF at a time when ERK2 is known to be phosphorylated and translocated into the nucleus [20], could indicate an important role inactivating selectively cytosolic ERK during neurite outgrowth. Such compartmentalized regulation of ERK MAP kinases following long-term exposure to NGF may be of fundamental importance in molecular processes underlying neuronal differentiation.

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