FEBS Letters 353 (1994) 9–12

FEBS 14613

# Rapid deactivation of MAP kinase in PC12 cells occurs independently of induction of phosphatase MKP-1

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Received 12 July 1994; revised version received 25 August 1994

**Abstract** Growth factors or serum can induce transcription and translation of a dual specificity MAP (mitogen-activated protein) kinase phosphatase, MKP-1 (MAP kinase phosphatase-1). The role of induction of MKP-1 (formerly 3CH134) in the rapid phase of MAP kinase deactivation was studied in rat pheochromocytoma (PC12) cells. MAP kinase was nearly completely deactivated in PC12 cells by 10 min after stimulation with epidermal growth factor (EGF) whereas MAP kinase activity remained elevated at 30% of the maximal response after stimulation with nerve growth factor. Protocols for treating cells with actinomycin D and cycloheximide were established that eliminate detection of MKP-1 mRNA and protein in PC 12 cells. Treatment of PC12 cells with actinomycin D and cycloheximide did not affect the rapid deactivation of MAP kinase. Thus, the rapid phase of MAP kinase deactivation in PC12 cells is not dependent on the induction of the MAP kinase phosphatase MKP-1.

*Key words:* PC12 cell; MAP kinase; Phosphatase; Actinomycin D; Epidermal growth factor; Transcription

## 1. Introduction

MAP kinases (also known as ERKs) are activated by dual tyrosine and threonine phosphorylations in response to diverse extracellular signals in many cell types and contexts (reviewed in [1]). Two closely related, and so-far apparently redundant, MAP kinase isoforms $p42^{mapk}$/ERK2 and $p44^{mapk}$/ERK1 have been studied most intensely. Each MAP kinase is activated by phosphorylations of the regulatory threonine and tyrosine residues in a conserved TEY motif, catalyzed by MAP kinase kinases [2]. The mechanisms that determine the kinetics of activation and deactivation of MAP kinase are incompletely understood. Some growth factors or hormones, such as insulin in adipocytes [3,4], or EGF in PC12 cells ([5]; this report), produce a rapid and transient activation which peaks at 2–10 min. A biphasic response with a rapid, transient activation phase followed by a second, more prolonged activation phase was observed in lung fibroblast CCL39 cells stimulated with thrombin [6]. In contrast, serum-stimulated NIH 3T3 fibroblasts contain elevated MAP kinase activity which does not significantly decline for up to 60 min ([7]; data not shown). These differences in activation kinetics reflect differences in usage of the multiplicity of operative activation and deactivation pathways.

Deactivation of MAP kinase can be accomplished by dephosphorylation of either the regulatory phosphothreonine residue or the phosphotyrosine residue, or both. Two protein phosphatases that are effective in vitro are the protein-serine/threonine phosphatase 2A and the protein-tyrosine phosphatase CD45 [8]. Recently, several laboratories have identified a family of inducible protein phosphatases with dual protein-tyrosine/threonine specificity and selectivity for MAP kinase [7,9–11]. MKP-1 was identified as the product of an immediate early gene *3CH134* induced by serum in mouse Balb/c 3T3 cells [10]. The human homolog of MKP-1 (97% identity) was found independently as the product of a stress-induced mRNA (CL100) in fibroblasts [11]. PAC-1, a distinct but MKP-1 related enzyme, has been characterized as a T-cell specific, inducible MAP kinase phosphatase localized to the nucleus [12]. Induction of MKP-1 is likely to be an important physiological mechanism for deactivation of MAP kinase [7,13]. Not only does MKP-1 exhibit specificity for MAP kinase as a substrate but MKP-1 interacts sufficiently strongly with MAP kinase to permit co-immunoprecipitation [7]. Treatment of cells with cycloheximide prolonged activation of MAP kinase in quiescent NIH 3T3 cells treated with serum [7]. However, serum causes a prolonged activation of MAP kinase in NIH 3T3 cells, without a rapid inactivation phase ([7]; data not shown). Whether the phosphatase could be induced rapidly enough to account for the initial, rapid deactivation of MAP kinase that occurs within 10–15 min in other cells and contexts seemed doubtful to us. We report results of critical tests of this hypothesis for MAP kinase deactivation in PC 12 cells using actinomycin D and cycloheximide in combination to inhibit transcription and translation, respectively, of inducible protein phosphatases. The results demonstrate that induction of MKP-1 plays no role in the rapid, first phase of MAP kinase deactivation in PC12 cells.

## 2. Materials and methods

### 2.1. Materials

Rat pheochromocytoma cells (PC12 cells) were obtained from M. Cox and P. Maness (University of North Carolina, Chapel Hill, NC) who obtained them directly from L. Greene (Columbia University, New York, NY). Receptor grade mouse EGF was purchased from Collaborative Research, Bedford, MA; NGF was from Upstate Biotechnology (Lake Placid, NY). Anti-MAP kinase antibody (TR10) recognizing $p42^{mapk}$ was provided by M. Weber (Department of Microbiology). RPMI 1640 medium and fetal calf serum were from Gibco-BRL (Gaithersburg, MD). Actinomycin D, cycloheximide, and human placental type IV collagen (Sigma C-7521) were from Sigma-Aldrich

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*Abbreviations* MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; NGF, nerve growth factor; EGF, epidermal growth factor; MKP-1, MAP kinase phosphatase-1.


*SSDI* 0014-5793(94)01000-5

(St. Louis, MO). Affinity-purified anti-peptide antibody recognizing MKP-1 was a generous gift from N. Tonks (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Sources of other reagents and proteins have been given [14].

*2.2. Cell culture and kinase assays*

PC12 cells were seeded equally in 60 mm tissue culture dishes coated with collagen and grown to confluence in RPMI 1640 media containing 10% (v/v) horse serum (JRH Biosciences, Lenexa, KS), 5% (v/v) fetal calf serum, penicillin G (20 U/ml), and streptomycin (20 mg/ml). NIH 3T3 cells were grown as described [7]. Prior to growth factor stimulation, cells were washed 2 times with RPMI 1640 and serum-starved for 3–3.5 h in the same media. Following stimulation with NGF or EGF, plates were placed on ice, the media was rapidly aspirated, and the plates were washed with cold (4°C) phosphate buffered saline. Individual plates were then processed for immune-complex kinase assays of MAP kinase as described [14]. MAP kinase kinase activity in the initial supernatants was assayed using homogeneous K52R protein as substrate as described [14].

Where used, actinomycin D (2 $\mu$M, final) was added at the onset of serum-serum starvation (3.5 h prior to NGF or EGF treatments). Cycloheximide (140 $\mu$M, final) was added during the last 30 min of serum-starvation.

*2.3. Northern analysis*

Total cellular RNA was isolated from individual 100 mm plates of PC12 or NIH 3T3 cells by a single-step method [15] using RNA STAT-60 reagent (Tel-Test 'B' Inc., Friendswood, TX). Total RNA (20 $\mu$g) from each sample was separated by electrophoresis on formaldehyde-agarose gels (1% gel) and transferred to nitrocellulose for probing with the full-length [$^{32}$P]cDNA for MKP-1 (3CH134) [10], labeled by the random primer method. Hybridization and washing conditions were as described [16].

*2.4. $^{35}$S-Labeling and immunoprecipitation of MKP-1*

Cells were washed in serum-free Dulbecco's modified Eagle's media containing no methionine, cystine, cysteine, or glutamine (ICN, Costa Mesa, CA) and then serum-starved in the same media for 3 h. [$^{35}$S]Methionine/[$^{35}$S]cysteine (Tran$^{35}$S-label (ICN)) was added (0.2 mCi/ml, final) after 1 h of serum-starvation. Additions of actinomycin D and cycloheximide were made as above. After growth-factor treatment, cells were lysed and processed for immunoprecipitation of MKP-1 with an antibody raised against a synthetic peptide from the carboxyl-terminus [7], or with an antibody raised against an *E. coli* expressed recombinant MKP-1 [10] according to the protocol described [7,10]. Immunoprecipitated proteins were separated by SDS/polyacrylamide gel electrophoresis (10% gel) and detected by fluorography using Amplify (Amersham) according to the manufacturer's instructions.

## 3. Results and discussion

When PC12 cells were serum-starved and stimulated with either EGF or NGF, rapid activation of MAP kinase kinase and MAP kinase were observed (Fig. 1). Deactivation of MAP kinase kinase and MAP kinase in response to either growth factor also occurred rapidly. Deactivation of MAP kinase kinase preceded deactivation of MAP kinase. Both MAP kinase and MAP kinase kinase activities were reduced by more than 50% of the peak value within 10 min. Whereas MAP kinase and MAP kinase kinase activities returned to basal values following EGF-treatment, MAP kinase and MAP kinase kinase activities persisted at ~20–30% of the maximal response following NGF-treatment. These differences between EGF and NGF responses are qualitatively similar to previous reports [5,17]. Because deactivation of EGF-stimulated MAP kinase activity was nearly complete, further experiments were focused on the EGF response.

To investigate the contribution of inducible protein phosphatases to the rapid deactivation of MAP kinase in PC12

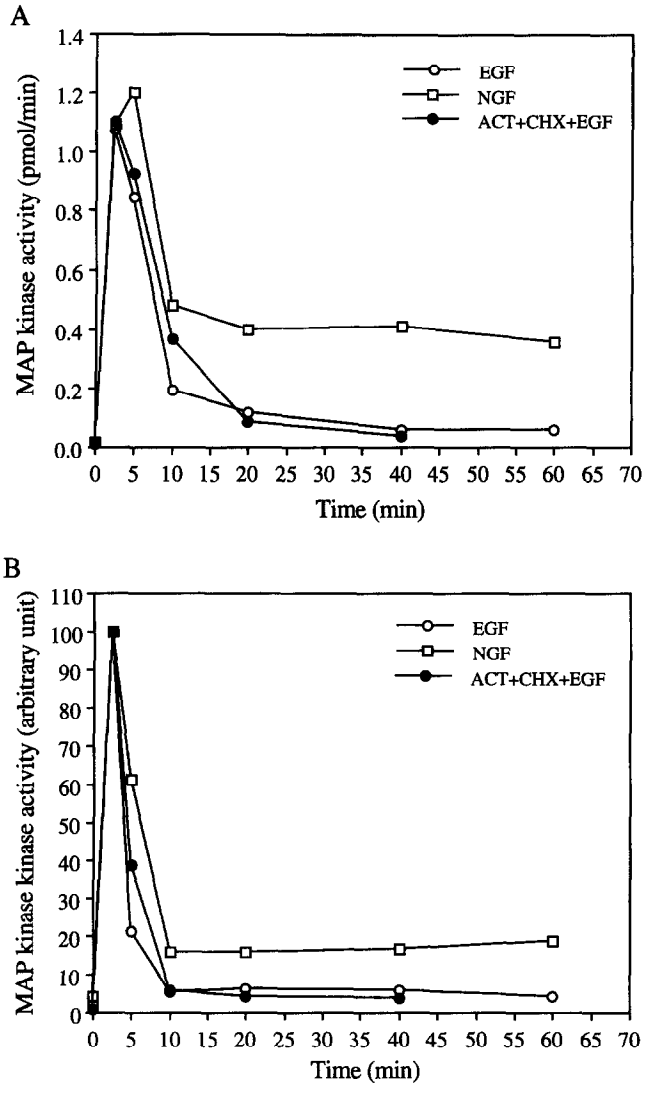


Fig. 1. Time courses of activation of MAP kinase kinase and MAP kinase in PC12 cells. PC12 cells were starved of serum and treated with or without actinomycin D (ACT) and cycloheximide (CHX) as described in section 2. Cells were stimulated with either EGF (150 ng/ml) or NGF (75 ng/ml) for the indicated times and processed for determination of MAP kinase activity by an immune-complex kinase assay method and for determination of MAP kinase kinase activity by phosphorylation of a kinase-defective mutant (K52R) of p42$^{MAPK}$. (A) MAP kinase activities in PC12 cells. The data represent the means of triplicate samples which differed by less than 10%. (B) MAP kinase kinase activities in PC12 cells. MAP kinase kinase activities were measured by phosphorylation of K52R protein and were quantitated with a PhosphorImager (Molecular Dynamics). Data were presented as relative activity in reference to the maximum MAP kinase kinase activity of each treatment.

cells, conditions were used to inhibit transcription and translation of MKP-1. Both MKP-1 mRNA [18] and protein [10] have been demonstrated to undergo rapid degradation in the absence of new synthesis. The half-lives of MKP-1 mRNA and protein determined in mouse Balb/c 3T3 fibroblasts were ~15–30 min and ~40 min, respectively [10,18]. Cells were treated with 2 $\mu$M actinomycin D for 3 h in the absence of cycloheximide to block synthesis of MKP-1 mRNA and to allow degradation of existing MKP-1 mRNA [10,18]. As a consequence, MKP-1

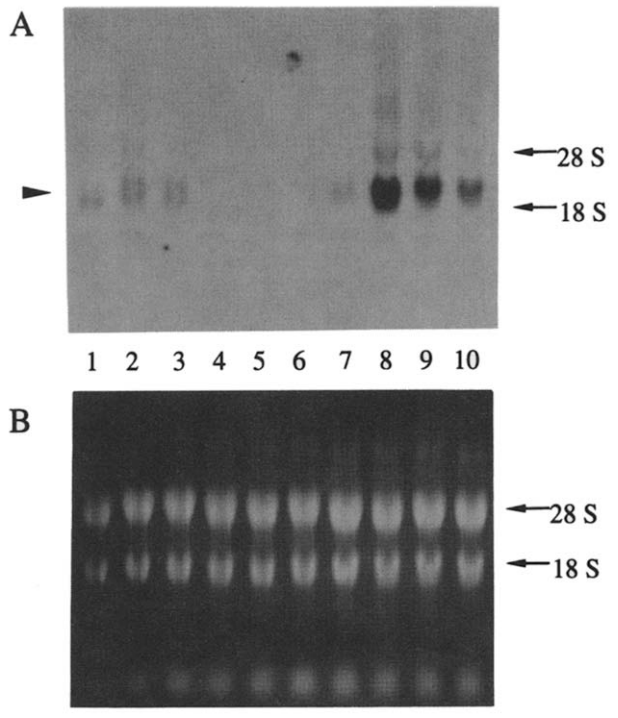


Fig. 2. Northern analysis of MKP-1 mRNA. Total RNA (20 μg/each) of preparations from PC12 cells or NIH 3T3 cells were separated on a formaldehyde–1% agarose gel containing ethidium bromide (0.5 μg/ml). After electrophoresis, the gel was photographed (B), and RNA was transferred to a nitrocellulose filter. The filter was probed with a $^{32}$P-labeled full-length MKP-1 (3CH134) cDNA (A). Lanes 1–3, RNA from serum-starved PC12 cells left unstimulated (lane 1), or stimulated with EGF (150 ng/ml) for 10 min (lane 2) or 60 min (lane 3). Lanes 4–6, RNA from actinomycin D- and cycloheximide-treated, serum-starved PC12 cells left unstimulated (lane 4), or stimulated with EGF for 10 min (lane 5) or 60 min (lane 6). Lanes 7–10, RNA from serum-starved NIH 3T3 cells (lane 7), and serum-starved NIH 3T3 cells stimulated with fetal calf serum (20% v/v, final) for 40 min (lane 8), 60 min (lane 9) or 90 min (lane 10). Arrowhead indicates the MKP-1 mRNA. The rat MKP-1 cDNA has been cloned from a rat brain library and deposited in the GenBank data base (Qian, Z., Gilbert, M.E. and Kandel, E.R, accession number U02553). The nucleotide sequence of the rat MKP-1 cDNA is 93% identical to that of the mouse MKP-1 cDNA used as probe.

protein should also be degraded. Cycloheximide (140 μM) was added to actinomycin D-treated cells during the final 30 min of serum-starvation to inhibit translation of any mRNA that might have remained.

Northern analyses were performed to determine the effects of actinomcyin D and cycloheximide treatments on MKP-1 mRNA in mouse NIH-3T3 and rat PC-12 cells (Fig. 2). Rat and mouse MKP-1 proteins are 98% identical. Low levels of MKP-1 mRNA were detected in serum-starved PC12 cells (lane 1) and NIH 3T3 cells (lane 7). As reported previously [7,10], serum-treatment induced expression of MKP-1 mRNA in NIH 3T3 cells (lanes 8–10), validating our methodologies. No appreciable increase in MKP-1 mRNA was detected in serum-starved PC12 cells treated with EGF (150 ng/ml) for 10 or 60 min (lanes 2, 3). Importantly, no MKP-1 mRNA was detected in PC12 cells treated with actinomycin D and cycloheximide even following treatment with EGF (lanes 4–6).

Immunoprecipitations from $^{35}$S-labeled cells were performed to determine the effects of actinomycin D and cycloheximide treatments on the levels of MKP-1 protein in PC12 cells (Fig. 3). Immunoprecipitations were performed with affinity-purified antibodies prepared against a synthetic MKP-1 peptide [7] (Fig. 3) and against recombinant MKP-1 protein (data not shown). Similar results were obtained with both antibodies. Little MKP-1 protein was detected in serum-starved NIH 3T3 cells (lane 3). Serum-treatment (1 h) of NIH 3T3 cells caused a substantial induction of MKP-1 protein (lane 4 vs. lane 3). The 40 kDa protein is identified as MKP-1 based on: (i) recognition by both previously characterized antibodies; (ii) appropriate mobility; and (iii) especially induction by serum as described by Sun et al. [7]. MKP-1 was detected in PC-12 cells that have been treated with EGF (150 ng/ml, 15 min) (lane 1). Importantly, no MKP-1 protein could be detected in PC12 cells treated with actinomycin D and cycloheximide (lane 2), implying that the protein was depleted in concert with depletion of MKP-1 mRNA, although it is possible that trace amounts of MKP-1 protein below a threshold of detection are still present.

To investigate the contribution of inducible phosphatases to the rapid deactivation of MAP kinase, PC12 cells were treated with actinomycin D and cycloheximide as above prior to stimulation with EGF and assay of MAP kinase kinase (Fig. 1B) and MAP kinase (Fig. 1A). Actinomycin D and cycloheximide pretreatment had no effect on the activation of MAP kinase kinase or MAP kinase (Fig. 1B). Under conditions in which no MKP-1 mRNA and protein were detectable (Fig. 2), deactivation of MAP kinase was not affected. The time courses for MAP kinase activity with and without treatment with actinomycin D and cycloheximide were nearly superimposable.

We conclude from these experiments that the induction of the dual specificity protein-tyrosine/threonine phosphatase MKP-1 is not the principal mechanism accounting for the rapid deactivation of MAP kinase in PC12 cells. This conclusion seems unequivocal. Furthermore, the conditions used would be likely to block induction of any unidentified inducible protein phosphatase(s) in PC12 cells, should any exist, provided they have similar turn-over rates. Our experiments were not de-

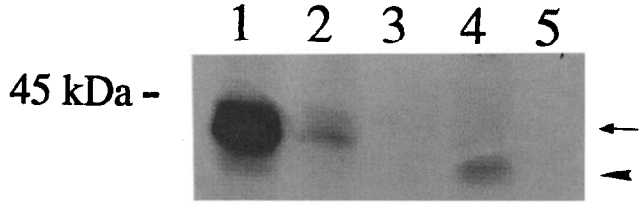


Fig. 3. Analysis of MKP-1 protein. PC12 cells or NIH 3T3 cells were metabolically labeled with [$^{35}$S]methionine/cysteine and stimulated with growth factors as described in section 2. MKP-1 was immunoprecipitated with an anti-MKP-1-peptide antibody [7]. The antibody–antigen complexes were isolated by protein A-agarose and washed eight times with the specified lysis buffer [7]. Lanes 1, serum-starved PC12 cells stimulated with EGF (150 ng/ml, 15 min); lanes 2, serum-starved PC12 cells that were treated with actinomycin D/cycloheximide and stimulated with EGF; lanes 3, serum-starved NIH 3T3 cells; lanes 4, serum-starved NIH 3T3 cells that were stimulated with dialyzed calf-serum (20% v/v, 60 min). Lane 5, NIH 3T3 cell lysate were prepared as in lane 4 and incubated with protein A-agarose without antibody. Arrowheads indicate MKP-1 protein bands. Arrow indicates a prominent $^{35}$S-labeled protein in PC12 cells which binds nonspecifically to protein A-agarose. The intensity of this $^{35}$S-labeled band is reduced in lane 2 due to the inhibition of protein synthesis by actinomycin D/cycloheximide. This nonspecific, $^{35}$S-labeled protein has been observed previously in immunoprecipitates from Balb/c 3T3 cells and rat vascular smooth muscle cells [10,22].

signed to identify the responsible MAP kinase phosphatase(s), only to test the importance of induction of MKP-1 to the rapid deactivation of MAP kinase in PC12 cells. While induction of MKP-1 is not required for the rapid deactivation, it is possible that MKP-1 basally expressed in PC12 cells is sufficient for rapid deactivation. However, the amount of MKP-1 expressed basally would have to be in considerable excess because significant depletion of MKP-1 protein by actinomycin and cycloheximide did not affect the kinetics of deactivation. It seems more likely that other protein phosphatases than MKP-1 contribute to the rapid deactivation of MAP kinase in PC12 cells. Phosphatase 2A catalytic subunit can inactivate MAP kinase in vitro [8] and Ser/Thr phosphatases are still tenable candidates for MAP kinase phosphatases in vivo. Phosphatase 2A has been strongly implicated in regulation of the MAP kinase pathway in vivo by the finding that SV40 small T antigen causes up-regulation of both MAP kinase kinase and MAP kinase activities in CV-1 cells (19). Evidence for a tyrosine specific MAP kinase phosphatase in interphase extracts prepared from *Xenopus* eggs has been obtained by Sarevic et al. [20]. However, the *Xenopus* protein-tyrosine phosphatase failed to bind to a column of thio-phosphorylated MAP kinase whereas an okadaic-sensitive MAP kinase phosphatase was retained by the column.

MAP kinase activity in vivo is determined by the relative activities of the immediate upstream activators, the MAP kinase kinases, and the MAP kinase phosphatase(s). While differences in phosphatase regulation are a possible explanation for the differences in MAP kinase activation observed with NGF- versus EGF-stimulation, the data of Muroya et al. [21] on Ras activation in PC12 cells is consistent with the steady-state level of Ras-GTP being the major determinant of MAP kinase activity. The temporal profiles of EGF- and NGF-stimulated MAP kinase activation (Fig. 1A) and MAP kinase kinase (Fig. 1B) for PC12 cells are remarkably similar to temporal profiles of EGF- and NGF-stimulated p21 Ras activation in PC12 cells (see Fig. 4 in [20]). Thus, there may be no need to invoke differences in downstream regulation to explain differences in the kinetics of activation of MAP kinase by NGF and EGF in PC12 cells.

*Acknowledgements:* We thank Leigh Ann Vincent for excellent technical assistance, John W. Haycock for advice on culture of PC12 cells, Michael Cox for PC12 cells, Nick Tonks and Hong Sun for anti-MKP-1 antibody. This research was supported by a grant (to T.W.S.) from the National Institutes of Health (DK 41077). Postdoctoral fellowship support for J.W. was provided by the Juvenile Diabetes Foundation International.

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