Compartment-specific regulation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) mitogen-activated protein kinases (MAPKs) by ERK-dependent and non-ERK-dependent inductions of MAPK phosphatase (MKP)-3 and MKP-1 in differentiating P19 cells

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Activation of mitogen-activated protein kinases (MAPKs), their upstream activators MAPK kinases (MAPKKs or MEKs) and induction of MKP-1 (CL100/3CH134) and MKP-3 (Pyst1/rVH6) dual-specificity MAPK phosphatases (MKPs) were studied in the mouse embryonic stem cell line P19 during the 7 day induction of neuronal differentiation triggered by aggregation and retinoic acid. ERK (extracellular signal-regulated kinase), but not JNK (c-Jun N-terminal kinase), was found activated with biphasic kinetics: a first transient phase on days 1 and 2, followed by a second activation that was sustained until the appearance of a neuronal phenotype. MEK activation appeared coincident with ERK activation. Cytosolic MKP-3 was induced in parallel to ERK activation, the induction being dependent on ERK activation, as was shown using the MEK-1 inhibitor PD98059. In contrast, nuclear MKP-1 was transiently elevated at 48 h, coincident with ERK inactivation and independently of ERK activity. As shown by cell fractionation, activated ERK is translocated to the nucleus. The complementary induction of ERK-specific phosphatases MKP-1 and MKP-3 permits precise and independent control of cytoplasmic and nuclear ERK activity, most probably required to properly induce a complex cellular programme of differentiation.

Key words: MAP kinase, MAP kinase kinase, MAP kinase phosphatase, neuronal differentiation, nuclear translocation.

INTRODUCTION

Mitogen-activated protein kinase (MAPK) modules are used widely to transduce extracellular signals into essential biological responses such as cell-cycle progression, differentiation and programmed cell death [1]. Three protein kinases, activated by a cascade of phosphorylation, compose the MAPK modules: MAPK kinase kinase, MAPK kinase (MAPKKs or MEKs) and MAPK [2,3]. These proteins were identified originally as the downstream mediators of the powerfully transforming oncogene Ras [4], underscoring the importance of MAPK in the control of cell proliferation [5,6]. Genetic studies performed in yeast, *Drosophila* and *Xenopus* [7] have since shown that MAPKs are crucial for differentiation and development.

MAPKs can be inactivated by a family of dual-specificity tyrosine phosphatases, the MAPK phosphatases (MKPs) [8]. The existence of at least ten MKPs in mammals implies a considerable complexity in the regulation of MAPK signalling by these enzymes. MKPs differ by properties such as tissue-specific expression, differential regulation in response to various stimuli, distinct subcellular localization and substrate specificity [9]. Based on these properties MKPs are classified in two families: the MKP-1, CL100/3CH134-like phosphatases (CL100 and 3CH134 are respectively the human and mouse homologues of the mouse MKP-1 gene) [10–12] and the MKP-3, Pyst1/rVH6-like phosphatases (Pyst1 and rVH6 are respectively the human and rat homologues of the rat MKP-3 gene) [13–15]. The transcripts of MKP-1 and its homologues are rapidly induced by growth factors or stress signals and the proteins are located in the nucleus [10–12]. In contrast, MKP-3, MKP-X/Pyst2 (where MKP-X is another rat MKP that is structurally very similar to MKP-3, and Pyst2 is the human homologue) [13,15,16] and MKP-4 (the human gene encoding another MKP) [17] are cytosolic and their transcripts are induced with delayed kinetics by specific stimuli, but not by environmental stress [15–17].

More recently, genetic screening has pointed towards a dynamic role for MKPs in development. In *Drosophila*, the *puckered* gene, closely related to MKP-1, has been described. Mutations in *puckered* lead to failure in dorsal closure associated with the hyperactivation of *basket*, *Drosophila*'s c-Jun N-terminal kinase (JNK) homologue [18–20]. In *Xenopus*, the overexpression of X17C, the homologue of MKP-3, leads to the formation of embryos deficient in posterior mesodermal derivatives, normally dependent upon MAPK activation by fibroblast growth factor [21,22].

To investigate the role of MAPK cascades during development, we have chosen the mouse embryonal carcinoma cell line P19 as a model. These cells derived from murine embryos are developmentally pluripotent and are capable of differentiating *in vitro* into cell types from all the three germinal layers: endoderm, mesoderm and neuroectoderm [23]. P19 cells differentiate along a neuronal pathway when cultured as aggregates and treated with retinoic acid (RA), forming both neuron-like and glia-like cells. We studied MAPKs and ERK (extracellular signal-regulated kinase; also referred to as p42/44 MAPK)-specific MKPs 1 and 3 during this induction of neuronal differentiation. MKP-1 and MKP-3 protein levels increased with different kinetics, in different compartments of the cell and through distinct pathways.

Abbreviations used: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; MEK, MAPK kinase (also referred to as MAPKK); MKP, MAPK phosphatase; RA, retinoic acid; α-MEM, α-modified Eagle's medium; FCS, fetal calf serum; TBS, Tris-buffered saline.

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These findings suggest a complementary regulation of ERK by MKP-3 versus MKP-1, resulting in a timely controlled accumulation of activated ERK in the nucleus.

EXPERIMENTAL

Cell culture

P19 mouse embryonal carcinoma cells (ATCC, Rockville, MD, U.S.A.) were cultured at 37 °C in α-modified Eagle's medium (α-MEM) supplemented with 10 % (v/v) fetal calf serum (FCS), in an air atmosphere with 5 % CO_2 (v/v). To induce neuronal differentiation, the cells were cultured for 4 days in 100 mm bacterial Petri dishes containing 10 ml of α-MEM/10 % FCS, in the presence of 0.3 μM all-*trans* RA (Sigma, Buchs, Switzerland). Aggregates were collected and transferred to tissue-culture plates, where they were cultured in α-MEM/10 % FCS without RA for 3 more days [23]. Appearance of the neuronal phenotype after day 7 was detected by microscopy.

Northern blots

Total RNA was isolated by the acid guanidium isothiocyanate/phenol/chloroform method [24]. Following RNA separation (15 μg of total RNA) on 1.2 % agarose gels, RNA was transferred to nylon membranes by capillarity. The blots were then hybridized with two specific [α-^{32}P]dCTP-labelled (3000 Ci/mmol) cDNA probes obtained by random priming (Prime-a-gene labeling system, Promega, Madison, WI, U.S.A.). The MKP-3 probe corresponded to the *Pvu*II 300–1250 bp fragment from rat MKP-3 cDNA. The MKP-1 probe corresponded to the *Pst*I 160–1100 bp fragment from rat MKP-1 cDNA. Signal intensity was analysed with a Molecular Dynamics PhosphorImager.

Western blots

P19 cells were washed twice with cold PBS and lysed in Triton X-100 lysis buffer (20 mM Tris/HCl, pH 8/137 mM NaCl/1.5 mM $MgCl_2$/1 mM EGTA/50 mM NaF/1 mM sodium orthovanadate/0.1 μM PMSF/10 μg/ml leupeptin/10 μg/ml aprotinin/1 % (v/v) Triton X-100/8 % (v/v) glycerol). Homogenates (100 μg of total protein per lane) were resuspended in 3 × sample buffer [125 mM Tris/HCl, pH 6.8/4 % (w/v) SDS/20 % glycerol (v/v)/0.2 % (w/v) Bromophenol Blue/10 % (v/v) mercaptoethanol], boiled for 5 min and resolved by SDS/PAGE (8 % gel). Semi-dry electrotransfer on to PVDF membranes (Millipore, Volketswil, Switzerland) was performed at 25 V for 40 min in 48 mM Tris solution containing 39 mM glycine, 0.037 % (w/v) SDS and 20 % (v/v) methanol. Membranes were blocked with Tris-buffered saline (TBS; 20 mM Tris/HCl, pH 7.5/500 mM NaCl) containing 0.1 % Tween-20 and 5 % non-fat dry milk. The blots were then incubated overnight at 4 °C with one of the following polyclonal primary antibodies: anti-phospho-specific p44/42 MAPK (1/1000; New England Biolabs, Allschwill, Switzerland), anti-phospho-JNK (1/200; Santa Cruz Biotechnology, Basel, Switzerland), anti-phospho-MEK1/2 (1/1000; New England Biolabs), anti-MKP-1 (1/500; Santa Cruz Biotechnology) and anti-MKP-3 (1/1000; a gift from Dr J. Pouysségur, CNRS, Nice, France). Primary antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit IgG (1/20000; Transduction Laboratories, Basel, Switzerland) and the enhanced chemoluminescence (ECL) kit (Amersham, Zurich, Switzerland).

Biochemical fractionation for subcellular localization and mini-immunoblotting

P19 cells were washed with PBS, pelleted and resuspended in lysis buffer [10 mM Tris, pH 7.4/10 mM NaCl/3 mM $MgCl_2$/0.5 % (v/v) Nonidet P40/0.5 mM PMSF/50 mM NaF/1 mM sodium orthovanadate/0.1 μM PMSF/10 μg/ml leupeptin/10 μg/ml aprotinin]. After 20 min on ice the lysate was loaded on to a 1 ml cushion of 1 M sucrose in hypotonic lysis buffer (sucrose-hypotonic buffer) and centrifuged for 10 min at 4 °C and 1600 ***g***. The supernatant was collected and centrifuged for 10 min at 4 °C and 13000 ***g*** to get rid of the membranes. This supernatant was used as the cytoplasmic extract. The pelleted nuclei from the first centrifugation were washed once with 1 ml of sucrose-hypotonic buffer and centrifuged for 5 min at 4 °C and 1600 ***g***. The nuclear pellet was then resuspended in 50 μl of nuclear extract buffer [200 mM NaCl/10 mM Hepes, pH 7.9/1.5 mM $MgCl_2$/0.1 mM EDTA/5 % (v/v) glycerol/0.5 mM PMSF/50 mM NaF/1 mM sodium orthovanadate/0.1 μM PMSF/10 μg/ml leupeptin/10 μg/ml aprotinin]. After a 30 min incubation at 4 °C with constant stirring, the nuclei were centrifuged for 10 min at 4 °C and 13000 ***g***. The supernatant was then removed and used as the nuclear extract.

Contamination of the nuclear fraction by the cytosol was determined by measuring the activity of lactate dehydrogenase in the nuclear extract. Lactate dehydrogenase is a cytosolic enzyme and its activity was measured following the change in absorbance at 340 nM in the presence of NADH and pyruvate (Sigma). The specific activity of lactate dehydrogenase (ΔA/min per μg of protein) measured in the nuclear extract was 50 times less than in the cytosolic extract.

Cytosolic and nuclear extracts were resuspended in 3 × sample buffer and boiled for 5 min. Samples (25 μg of total protein/lane) were loaded on to a SDS/PAGE mini-gel (8 % gel), electrophoresed and transferred to PVDF membranes. Membranes were assembled with the multiscreen apparatus (Mini-Protean II, Bio-Rad, Glattbrugg, Switzerland) in such a way that each sample-blot lane was covered by two channels, allowing incubation with two different antibodies at the same time. Thus after blocking the membrane with TBS containing 0.1 % Tween-20 and 5 % non-fat dry milk, a different antibody was added to each of the two channels for incubation with the samples. The primary polyclonal antibodies used were: anti-ERK (1/6000, Santa Cruz Biotechnology), anti-phospho-specific p44/42 MAPK (1/1000), anti-MKP-1 (1/500) and anti-MKP-3 (1/1500). After washing the membranes with TBS three times for 5 min the primary antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit IgG (1/20000) and the ECL kit.

RESULTS

Activation of ERK-2 but not JNK during neuronal differentiation of P19 cells

It is well established that MAPK cascades play a crucial role in differentiation and development of various organisms [7]. In order to identify which MAPK is important for the neuronal differentiation of P19 cells, we analysed the activation of the ERK and JNK MAPKs during several stages of differentiation using specific antibodies against phospho-ERK and phospho-JNK, an approach that has been validated in numerous previous studies [5,25–27]. P19 cells lysed during normal exponential growth served as controls.

The induction of P19 cells by aggregation and RA leads to a strong biphasic activation of ERK-2 (Figure 1A). During the first transient phase, a significant activation over the control was

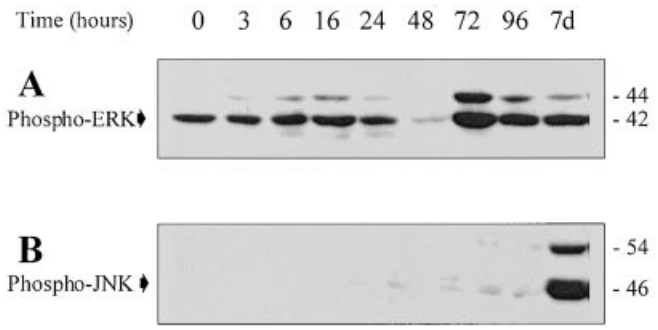

Figure 1 ERK, but not JNK, is activated during the neuronal differentiation of P19 cells

P19 cell homogenates (100 μg of total protein/lane) were analysed by SDS/PAGE (8% gel) followed by Western blotting using polyclonal antibodies against the phosphorylated (active) forms of two major MAPKs, ERK and JNK. (**A**) Anti-phospho-specific p44/42 MAPK antibodies detected two bands corresponding to phospho-ERK-1 (44 kDa) and phospho-ERK-2 (42 kDa). (**B**) Anti-phospho-JNK antibodies detected two specific bands corresponding to two isoforms of phospho-JNK of 46 and 54 kDa. Blots were revealed with ECL as described in the Experimental section and are representative of three separate experiments that showed the same pattern.

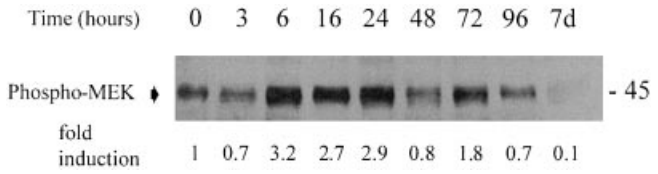

Figure 2 MEKs are activated during the neuronal differentiation of P19 cells

P19 cell homogenates (100 μg of total protein/lane) were analysed by SDS/PAGE (8% gel) followed by Western blotting using polyclonal antibodies against the phosphorylated (active) forms of MEK1 and MEK2. Anti-phospho-specific MEK1/2 antibodies detected one band corresponding to phospho-MEKs (45 kDa). Blots were revealed with ECL as described in the Experimental section and are representative of three separate experiments showing the same pattern.

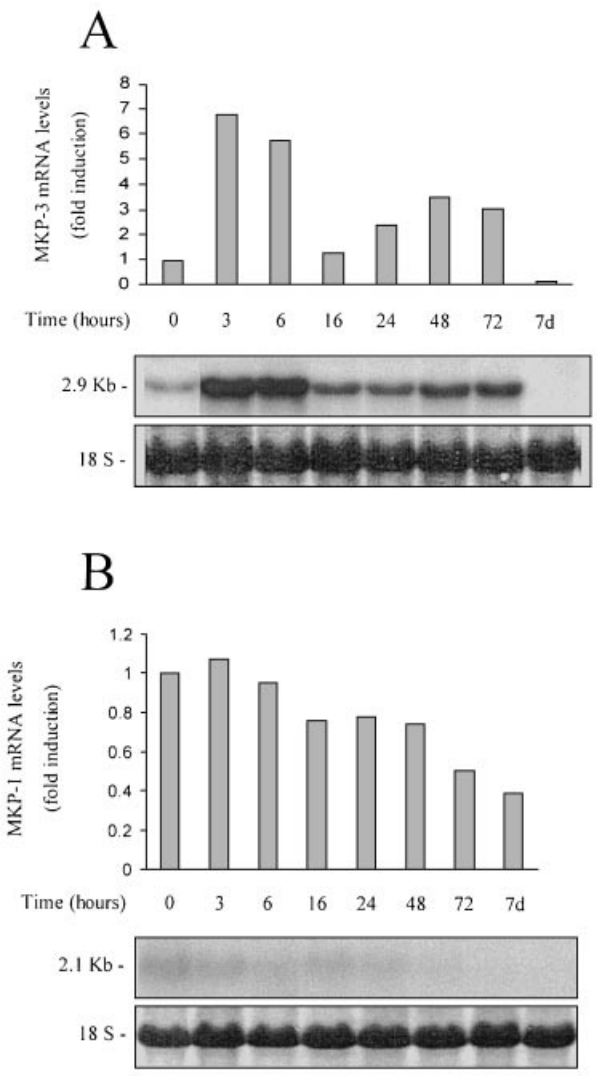

Figure 3 Strong and selective induction of MKP-3 mRNA upon aggregation and RA treatment of P19 cells

Total RNA (15 μg/lane) extracted from P19 cells at various times following induction was separated on a 1.2% agarose gel, transferred to a nylon membrane and analysed by Northern blotting. Hybridization was performed with random-primed ^{32}P-labelled probes derived from the *Pvu*II 300–1250 bp fragment of the MKP-3 cDNA (**A**) and from the *Pst*I 160–1100 bp fragment of the MKP-1 cDNA (**B**). Shown below each graph is the Methylene Blue-stained 18 S rRNA used as a loading control for equal RNA loading; shown is a single blot of which a sector between 0 and 3 h has been cut out. Blots were quantified by PhosphorImager and results are plotted as the average ratio relative to levels detected in exponential growth. Values are the means from three independent experiments (S.D. $\pm$ 5–15%).

detectable as early as 6 h after the start of the induction procedure; active ERK-2 reached a maximal level at 16 h and decreased dramatically at 48 h. During the second phase, ERK-2 activation peaked at 72 h and remained substantially elevated all through to fully differentiated neurons found after 6 days. Activation of ERK-1 in parallel to ERK-2 was also discernible.

In contrast to ERK, JNK remains inactive at all the stages of the neuronal differentiation of P19 cells. However, when the neurons appear by day 7 JNK activity is switched on (Figure 1B). This shows that the initial stimuli, aggregation and RA, are triggering selectively the ERK MAPK cascade.

Activation of MEK during neuronal differentiation of P19 cells

It is well known that ERKs are phosphorylated and activated by upstream regulators MEK1 and MEK2 [28,29]. MEK during P19 cell differentiation was analysed by Western blotting, using a polyclonal antibody against phosphorylated MEK1 and MEK2 (active forms). P19 cells lysed during normal exponential growth served as controls. The induction of differentiation by aggregation and RA leads to a biphasic activation of MEK (Figure 2). We observed during the first transient phase a 3.2-fold activation over the control, detectable as early as 6 h after the start of the induction procedure; active MEK maintained high levels until 24 h and decreased at 48 h. MEK was found to be significantly activated again at 72 h. MEK activation (Figure 2) and ERK activation (Figure 1) show qualitatively a very similar time course, consistent with MEK causing ERK activation.

Induction of the dual-specificity MKP-3 and MKP-1

Mammalian cells express many phosphatases that can inactivate ERK, including the MKP family, which selectively dephosphorylate and inactivate different MAPKs [8,9]. Northern-blot analysis (Figure 3A) showed that MKP-3 mRNA was very strongly

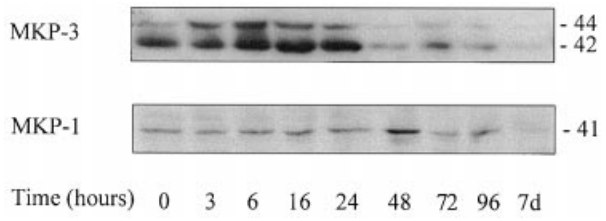

Figure 4 MKP-3 and MKP-1 protein levels during the neuronal differentiation of P19 cells

Western-blot analysis of P19 cell lysates (100 μg of total protein/lane) was performed as in Figure 1. Upper panel: a specific polyclonal antibody against MKP-3 protein revealed two MKP-3 isoforms of 42 and 44 kDa. Lower panel: a commercial anti-MKP-1 antibody revealed a specific band of 41 kDa. The blots shown are representative of three separate experiments yielding the same pattern.

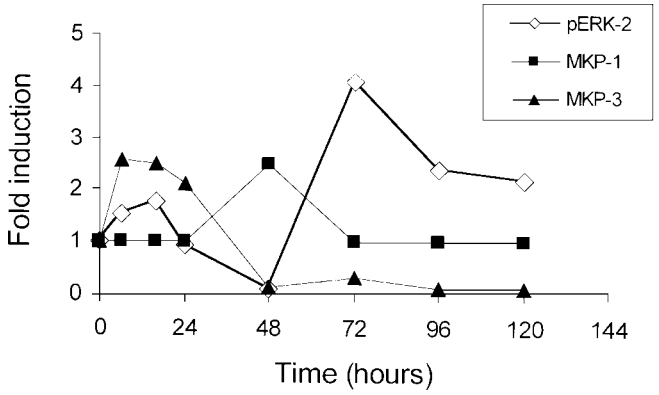

Figure 5 During the early stages of neuronal differentiation of P19 cells, ERK activation coincides with MKP-3 induction and ERK inactivation with MKP-1 induction

Western-blot analysis of P19 cell lysates collected at various times during neuronal differentiation were performed using specific polyclonal antibodies against phospho-ERK (pERK-2), MKP-3 and MKP-1, as in Figures 1 and 4, and quantified by densitometry. The data are presented as fold induction relative to control levels seen in P19 cells during exponential growth. The values shown are for ERK-2 (◇), for the sum of the two isoforms of MKP-3 (▲) and for MKP-1 (■). Shown are the mean values from three separate experiments (S.D. ± 5–15%).

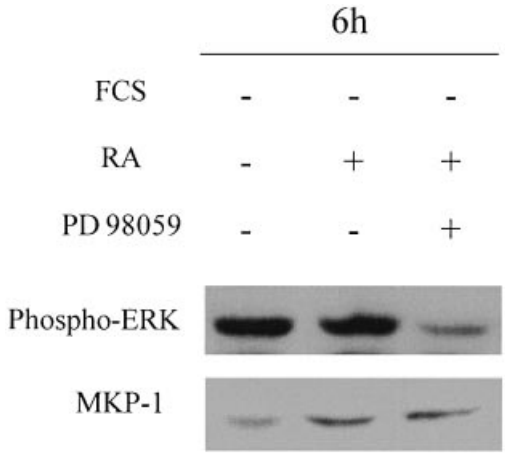

Figure 7 MKP-1 protein induction by RA is independent of ERK activation

P19 cells were seeded in six-well plates and rendered quiescent when confluent by withdrawal of the serum from the medium during a 16 h period. Cells were then stimulated with RA (0.3 μM) in the presence or absence of the MEK-1 inhibitor PD98059 (50 μM), which was added 1 h prior to the addition of RA. Cells were recovered after 6 h, extracted and analysed by Western blotting with polyclonal antibodies against phospho-ERK as in Figure 1, and against MKP-1 as in Figure 4. The blots shown represent three independent experiments that resulted in the same pattern.

induced after 3 h, reaching maximal levels 5–8-fold over the control at 3 and 6 h; thereafter MKP-3 mRNA decreased at 16 and 24 h. MKP-3 mRNA increased again slightly at 48 and 72 h. In contrast, MKP-1 mRNA levels (Figure 3B) were low and only declined slightly before the end of the differentiation.

During the first phase of differentiation, the induction of MKP-3 protein (as monitored by Western blotting using a polyclonal antibody; Figure 4, upper panel) was evident as a marked increase over the levels seen in exponentially growing cells (control, time 0). MKP-3 immunoreactivity was found in two specific bands representing two isoforms (42 and 44 kDa), probably due to the presence of two in-frame 5′-methionines, resulting in an alternative translation-initiation site, already described in [15]. A time lag of about 3 h between the kinetics of

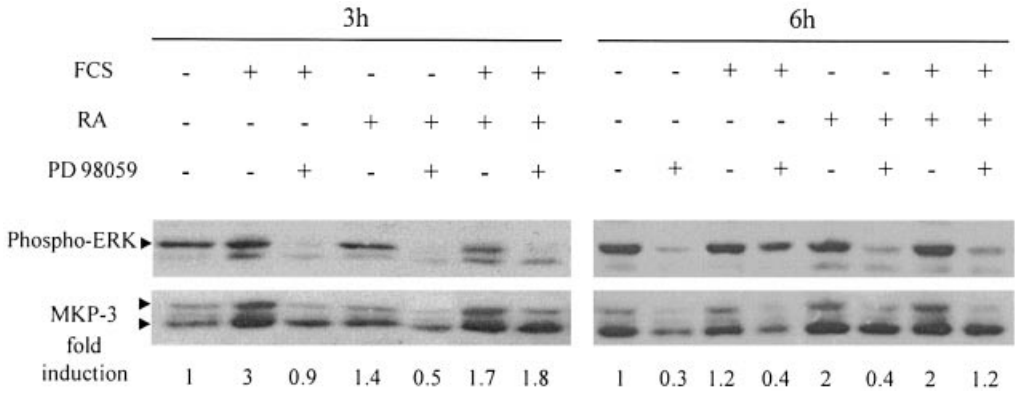

Figure 6 MKP-3 protein induction by serum and RA requires ERK activation

P19 cells were seeded in six-well plates and rendered quiescent when confluent by withdrawal of the serum from the medium during 16 h. Cells were then stimulated with FCS (10%) or RA (0.3 μM) in the presence or absence of the MEK-1 inhibitor PD98059 (50 μM), which was added 1 h prior to the addition of serum or RA. Cells were lysed after 3 h (left-hand panels) or 6 h (right-hand panels) and Western blots were performed with polyclonal antibodies against phospho-ERK and MKP-3 as in Figures 1 and 4, respectively. The blots shown represent three independent experiments that resulted in the same pattern.

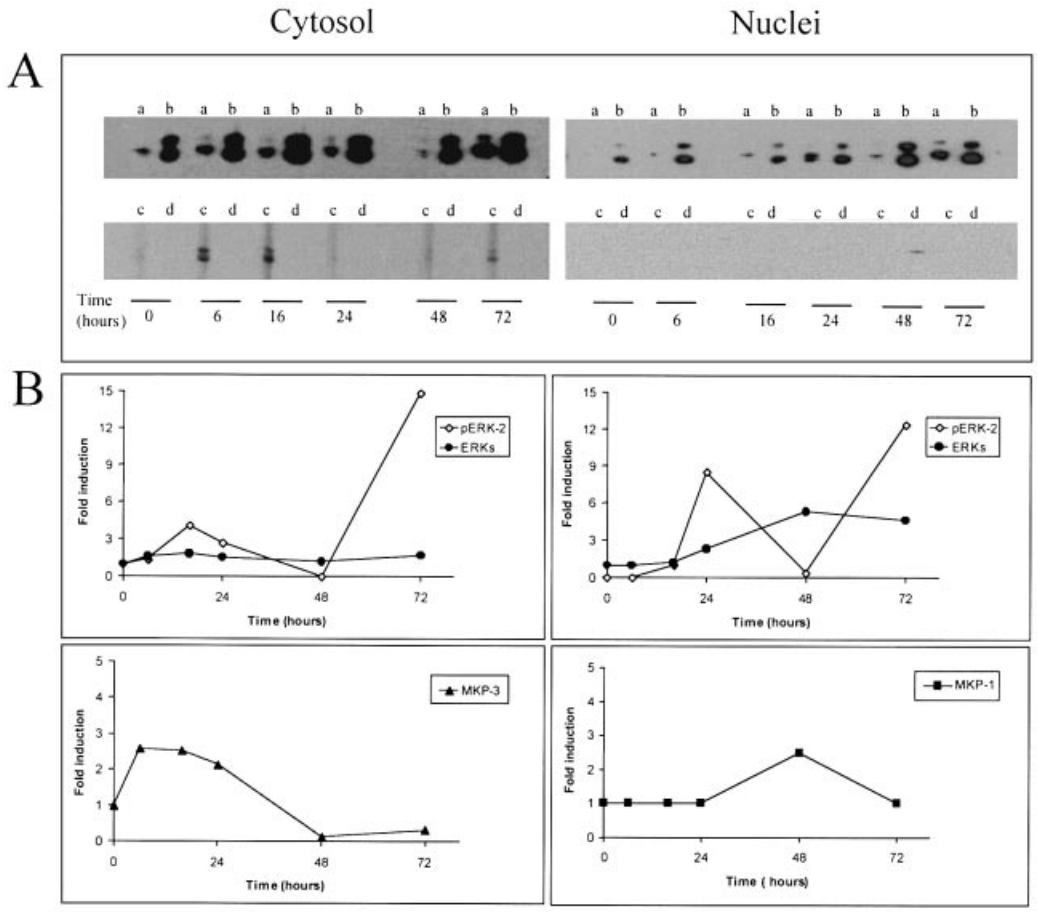

Figure 8 Timed accumulation and inactivation of ERK in the nuclei of P19 cells by complementary induction of MKP-3 and MKP-1 during neuronal differentiation

P19 cells collected at the indicated times during the early stages of neuronal differentiation were fractioned into nuclei and cytosol as described in the Experimental section. To minimize inter-blot variability, Western-blot analysis was performed using a multi-channel blotting device allowing the incubation of each sample lane on the same blot with two different antibodies at the same time. Lanes a and b were blotted with anti-phospho-specific p44/42 MAPK (pERK) and anti-ERK, lanes c and d with anti-MKP-3 and anti-MKP-1, respectively. Antibodies and revelation procedures were as in Figures 1 and 4. Shown are the Western blots (**A**) and values of their densitometric quantification (**B**), the latter plotted as the average ratio relative to levels in exponential growth. Values are the means from three independent experiments (S.D. $\pm$ 5–15%).

MKP-3 mRNA and those of the MKP-3 protein (maximal induction of MKP-3 mRNA was at 3 h compared with 6 h for the protein) was consistent with the induced mRNA being the cause of a rise in the levels of the MKP-3 protein. In contrast to the first phase, the modest rise in MKP-3 mRNA occurring at late times (Figure 3, 48 and 72 h) was not accompanied by the reappearance of MKP-3 protein, which in fact dropped below the control levels seen in exponentially growing P19 cells.

To show the specificity of MKP-3 induction, we tested the presence of another dual-specificity phosphatase, MKP-1 [10–12] (Figure 4, lower panel). Surprisingly, MKP-1 protein was found strongly induced at the single time point of 48 h. This induction was not preceded by a change in the levels of MKP-1 mRNA (Figure 3B).

Comparing the kinetics of MKP-3 protein induction with those of ERK-2 activation (Figure 5), one may notice that MKP-3 protein induction paralleled the first transient ERK-activation phase. In contrast, the strong induction of MKP-1 at 48 h coincided with ERK inactivation. The pattern of induction of the two potential inactivators of ERK, MKP-3 and MKP-1 (the former parallel, the latter anti-parallel to ERK activation), suggests different induction mechanisms and a distinct role in ERK inactivation for these two dual-specificity protein phosphatases.

MKP-3 induction due to ERK activation

MKP-3 could be induced in response to ERK activation to constitute a negative-feedback regulatory loop [30]. To test the involvement of the ERK pathway in MKP-3 induction, we blocked ERK activation with a specific MEK-1 inhibitor (PD98059) [31,32]. P19 cells were rendered quiescent when confluent, by 16 h of serum deprivation. Cells were then stimulated with FCS (10%) or RA (0.3 μM) in the presence or absence of the MEK-1 inhibitor PD98059 (50 μM), which was added 1 h prior to stimulation. MKP-3 induction and ERK activation were then analysed by Western blotting at 3 and 6 h (Figure 6).

MKP-3 protein was strongly induced by serum at 3 h and by RA at 6 h, as well as by the two combined stimuli at both times. PD98059 blocked completely the induction of MKP-3 by serum and RA alone and attenuated strongly the induction by the

combined action of the two stimuli. Also, the MEK-1 inhibitor completely blocked ERK-2 activation by serum (3 h) and by RA (6 h) and markedly inhibited ERK-2 activation by the combined stimulation, both at 3 and 6 h (Figure 6).

The strikingly parallel action of PD98059 on ERK activation and MKP-3 induction suggests strongly that MKP-3 is induced via the activation of the ERK pathway in P19 cells. We extrapolate these findings to the induction of differentiation in which ERK is activated by combined aggregation, serum and RA. Note that PD98059 could not be applied during the normal induction protocol, which requires the continuous presence of serum both for the survival of P19 cells in suspension culture and for their aggregation.

In parallel experiments it was found that the ERK pathway is most probably not involved in MKP-1 protein induction, as neither serum nor RA seemed to induce MKP-1 protein after 3 h of stimulation (results not shown). However, as is shown in Figure 7, RA at 6 h increased MKP-1 protein, irrespective of the presence or absence of the MEK-1 inhibitor.

Distinct compartmentalization of ERK and its specific phosphatases MKP-3 and MKP-1

An apparently paradoxical aspect of the data presented so far was that the activation of the ERK pathway persisted in spite of the strong induction of MKP-3, a phosphatase which inactivates ERK preferentially both *in vivo* and *in vitro* [13,15,30,33]. MKP-3, in contrast to many other MKPs, including MKP-1, is excluded from the nucleus and thus appears to be a regulator of cytoplasmic ERK-1 and ERK-2 [13–15]. Cytoplasmic versus nuclear regulation of ERK during the neuronal differentiation of P19 cells was thus investigated by following the state of activation of ERK separately in cytosolic and nuclear extracts (Figure 8). We performed Western-blot analysis after cellular fractionation using a multiscreen apparatus to minimize inter-blot variability; this system allows the simultaneous incubation of each sample lane on the same blot with two different antibodies. Membranes were blotted with the polyclonal antibodies anti-phospho-specific ERK (Figure 8A, lanes a), anti-ERK (lanes b) or with anti-MKP-3 (lanes c) and anti-MKP-1 (lanes d).

Western bands were quantified by densitometry. Comparison between the signal obtained with the anti-phospho-specific ERK versus the anti-ERK antibody gave us the kinetics of activation of ERK (Figure 8B). As can be seen, ERK activation in the cytosol (maximal at 16 h, decreased at 24 h) preceded ERK activation in the nucleus (not active before 24 h). At 48 h, ERK was completely inactivated in both the cytoplasmic and nuclear fractions. Furthermore, the total amount of ERK in the nuclear fraction increased at 24 and 48 h of neuronal differentiation, in contrast to cytosolic ERK, which remained stable, indicating a major translocation of activated ERK from the cytosol to the nucleus between 16 and 24 h. This result was confirmed by immunocytochemical staining using an anti-ERK antibody and confocal microscopy analysis (results not shown).

In parallel, we verified the presence of the induced MKP-1 and MKP-3 in these cytosolic and nuclear fractions (Figure 8). MKP-3 was detected only in the cytosolic fraction and paralleled ERK activation during the first 48 h (transient phase). MKP-1 was only transiently induced at 48 h, coincident with completely inactivated ERK, and was restricted to the nuclear fraction.

The whole compartmentalization pattern was consistent with the following scenario: in the cytoplasm, the activation of ERK is followed rapidly both by an induction of MKP-3 and the nuclear translocation of active ERK, the former inactivating cytoplasmic ERK and the latter securing nuclear activity of ERK. At the end of the initiation of differentiation, ERK is inactivated rapidly by MKP-1, and possibly by other nuclear phosphatases, to permit further progression towards the differentiated state.

Taken together, these data demonstrate that during the neuronal differentiation of P19 cells, two dual-specificity phosphatases that are highly selective for ERK, MKP-3 and MKP-1, are induced with different kinetics, in different compartments of the cell and by distinct pathways. This strongly suggests that their physiological roles are different.

DISCUSSION

We show here that the early phase of neuronal differentiation of mouse P19 cells triggered by aggregation and treatment with RA is accompanied by the activation of the MEK/ERK MAPK cascade and by the induction of MKP-3 and MKP-1. We also show the complementary nature of induction of MKP-3 in the cytoplasm and MKP-1 in the nucleus, which results in distinct control of cytoplasmic and nuclear ERK activities, probably a crucial element in the proper induction of differentiation.

The ERK MAPK cascade and its homologues are largely implicated in differentiation and development. This is evident from a vast body of genetic studies in *Drosophila* [34], *Xenopus* [21,35] and *Caenorhabditis elegans* [36], and also from many studies in mammalian cells and cell lines. ERK activity is important, e.g. for the differentiation of C2 muscle cells [37], thymocytes [38] and megakaryocytes [39]. In PC12 pheochromocytoma cells, epidermal growth factor transiently activates ERK, whereas sustained activation of ERK following treatment with nerve growth factor induces cell differentiation [35,40].

A signalling cascade involved in the triggering of differentiation should be controlled very precisely and should act finally in the nuclear compartment. Indeed, it has been demonstrated recently that nuclear translocation of active ERK is crucial to induce neurite outgrowth, fibroblast transformation and also cell-cycle entry [41,42]. We show here that following activation via MEK, activated ERK is translocated to the nucleus during the differentiation of P19 cells into neurons and that cytoplasmic and nuclear ERK activation have distinct kinetics. This supports the notion that the functional versatility of the ERK cascade resides not only in the multiple ways by which ERK can be activated, but also in the precise definition of the kinetics and of the cellular compartment(s) in which ERK is active. ERK activation is controlled not only by the activating cascade, but also by the multiple ERK inactivation processes.

Dual-specificity phosphatases, also termed MKPs, constitute the largest family of tyrosine phosphatases capable of regulating MAPKs in mammalian systems [8]. Different MKPs (MKP-1, MKP-X, MKP-3 and B23) analysed by *in situ* hybridization show a well-defined and selective distribution in the brain during development or following seizure [43], underlining the potential of MKPs to play distinct specific roles during development and for differentiated cell functions. MKP-1, MKP-2 and MKP-3 are able to inactivate ERK both *in vitro* and *in vivo* [9]. MKP-3 appears to be most specific for ERK as, in contrast to MKP-1 and MKP-2, it normally does not inactivate the stress-activated protein kinases [JNK/SAPK (stress-activated protein kinase) or p38] [13,15,33]. In addition, binding of the N-terminal domain of MKP-3 to ERK, but not to JNK/SAPK or p38, activates its phosphatase activity up to 30-fold [30].

Our results show that MKP-3 is induced at the onset of the differentiation of P19 cells into neurons by a mechanism dependent upon activation of the ERK pathway, and we confirm in P19 cells that endogenous MKP-3 is restricted to the cytoplasm.

The time course of cell activation in the P19 model of neuronal differentiation is dependent upon the progression of aggregation: the major growth of aggregates occurs between 6 and 24 h after induction, a time during which we observe the most pronounced activation of MEK and ERK, and induction of MKP-3. Note that the kinetics of ERK activation in individual cells may be faster than the kinetics shown for the cell population in Figure 1. Furthermore, the cell population is not absolutely homogeneous. Besides cells entering the G_1 phase and progressing towards neuronal phenotype [44,45], there is a small fraction of cells that re-enter the S phase and later undergo apoptosis [46]. In any case, we propose that MKP-3 is induced as a rapid response to ERK activation, in order to tightly regulate cytosolic ERK. In PC12 cells, nerve growth factor causes an up-regulation of MKP-3 mRNA [15] and protein [47] with concomitant neuronal differentiation. In contrast, withdrawal of basic fibroblast growth factor from MM14 muscle cells decreases MKP-3 expression, resulting in the subsequent increase in ERK activity concomitant with muscular differentiation [14].

MKP-3 can only inactivate cytosolic ERK, and other phosphatases must be involved in the inactivation of ERK translocated to the nucleus; MKP-1 is a good candidate. MKP-1 was identified originally as an immediate early gene responsive to oxidative stress and heat shock, as well as to serum stimulation [10,48], and the only substrate of MKP-1 identified at that time was phospho-ERK [11,12]. However, MKP-1 is not a phosphatase specific for ERK, since in UV-stimulated PC12 cells MKP-1 inactivates JNK/SAPK rather than ERK [49]. Several further studies suggest that among the three MAPKs (ERK, JNK/SAPK and p38) inactivated by MKP-1, the target will differ according to the cell type and the kind of stimulation. In P19 cells we find the induction of MKP-1 protein only at the time point of 48 h, at which ERK is fully inactivated. Together with the exclusive nuclear localization of MKP-1, this is a strong indication that MKP-1 inactivates nuclear ERK.

Multiple pathways are involved in MKP-1 expression. MKP-1 mRNA and protein levels appear to be regulated by ERK1/2, JNK/SAPK or Ca^{2+}-dependent pathways in a cell-type-specific fashion. Recently, Lee et al. [50] have shown that RA acting through RA receptors increases the expression of MKP-1. As we show in Figure 7, RA strongly induces MKP-1 protein at 6 h. The parallel activation of ERK appears of no direct relevance for MKP-1 induction, since ERK activation can be completely prevented without any change in MKP-1 expression at 6 h. Recent data [51] show that MKP-1 phosphorylation by ERK stabilizes MKP-1 protein by reducing its proteolytic degradation. The induction of MKP-1 at 48 h of neuronal differentiation observed without concomitant change in mRNA levels (Figures 3 and 4) would be consistent with such a mechanism. MKP-1 phosphorylation by ERK would be dependent upon the nuclear accumulation of active ERK (Figure 8). Note that MKP-1 phosphorylation does not modify its intrinsic ability to dephosphorylate ERK [51].

We conclude that during the neuronal differentiation of P19 cells, two dual-specificity phosphatases, MKP-3 and MKP-1, are induced with different kinetics, in different compartments of the cell and involving distinct pathways; this results in a timely controlled accumulation of activated ERK in the cytosol and in the nucleus. Our data illustrate how timed and localized control by MKPs makes it possible for a single MAPK cascade module to control a variety of complex cellular processes.

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