

Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry

Anne Brunet¹, Danièle Roux,
Philippe Lenormand, Stephen Dowd²,
Stephen Keyse² and Jacques Pouyssegur³

CNRS-UMR 6543 Centre de Biochimie, Université de Nice, Parc Valrose, 06108 Nice, France and ²ICRF Molecular Pharmacology Unit, Biomedical Research Centre, Level 5, Ninewells Hospital, Dundee DD1 9SY, UK

¹Present address: M.E.Greenberg's Laboratory, Children's Hospital, Harvard Medical School, 300 Longwood Avenue, Boston, MA 02115, USA

³Corresponding author
e-mail: pouysseg@unice.fr

Mitogen-activated protein kinase (MAPK) modules, composed of three protein kinases activated by successive phosphorylation, are involved in the signal transduction of a wide range of extracellular agents. In mammalian cells, mitogenic stimulation triggers the translocation of p42/p44MAPK from the cytoplasm to the nucleus, whereas the other protein kinases of the module remain cytosolic. Since MAPK has been shown to phosphorylate and activate nuclear targets, such as the transcription factor Elk1, it has been proposed, but not yet demonstrated, that MAPK nuclear translocation could represent a critical step in signal transduction. In this study, we sequestered p42/p44MAPK in the cytoplasm by the expression of a catalytically inactive form of cytoplasmic MAP kinase phosphatase (MKP-3/Pyst-1). Sequestering MAPK in the cytoplasm did not alter its activation or its ability to phosphorylate cytoplasmic substrates of MAPK (p90RSK1 or an engineered cytoplasmic form of Elk1). In contrast, prevention of MAPK nuclear translocation strongly inhibited Elk1-dependent gene transcription and the ability of cells to reinitiate DNA replication in response to growth factors. Thus the relocalization of MAPK to the nucleus appears to be an important regulatory step for mitogen-induced gene expression and cell cycle re-entry.

Keywords: growth factors/MAP kinase/MAPK phosphatase/nuclear translocation/substrates

Introduction

Mitogen-activated protein kinase (MAPK) modules are involved in the signal transduction of a wide variety of signals in all eukaryotic organisms. In mammalian cells, three well-characterized modules co-exist: p42/p44MAPK, p38MAPK and JNK cascades (for a review see Cano and Mahadevan, 1995). The p42/p44MAPK cascade plays a pivotal role in the re-entry of fibroblasts into the cell cycle (Pagès *et al.*, 1993), and in a variety of other processes, including differentiation of PC12 cells into neurites in

response to nerve growth factor (NGF) (Traverse *et al.*, 1992; Alessi *et al.*, 1995) and long-term potentiation in presynaptic neurons in *Aplysia* in response to serotonin (Martin *et al.*, 1997).

Both p42 and p44MAPK are activated by dual phosphorylation on a threonine and a tyrosine residue, achieved by the dual-specificity kinase MKK1/2. Whereas MKK1/2 remain permanently in the cytoplasm, p42/p44MAPK are relocalized from the cytoplasm to the nucleus upon stimulation (Chen *et al.*, 1992; Gonzalez *et al.*, 1993; Lenormand *et al.*, 1993; Zheng and Guan, 1994). In fibroblasts, a correlation exists between the mitogenic potency of a stimulus and its ability to trigger MAPK translocation. This correlation is also found in PC12 cells (Traverse *et al.*, 1992) and in *Aplysia* (Martin *et al.*, 1997). Comparison of the kinetics of MAPK activation and nuclear translocation suggests that it is the active form of MAPK that translocates into the nucleus (Lenormand *et al.*, 1993; Khokhlatchev *et al.*, 1998).

In mammalian cells, inactivation of MAPK is achieved by a family of dual-specificity MAPK phosphatases which target the two critical phosphorylation sites in the activation loop of MAPK (Keyse, 1998). Several members of this growing family of enzymes, including CL100 (MKP-1), MKP-2 and PAC-1, are tightly localized to the cell nucleus (Rohan *et al.*, 1993; Brondello *et al.*, 1995; Guan and Butch, 1995; King *et al.*, 1995; Kwak and Dixon, 1995). In addition, the genes encoding these proteins are inducible by many of the stimuli which cause MAPK activation, suggesting that they might be involved in feedback control of MAPK activity (Alessi *et al.*, 1993; Sun *et al.*, 1993; Brondello *et al.*, 1997). More recently, a novel subfamily of these proteins with atypical properties has been characterized. The first of these was MKP-3/Pyst-1 which, unlike MKP-1 and -2, is found predominantly in the cytosol and is highly specific for p42/p44MAPK (Groom *et al.*, 1996; Muda *et al.*, 1996). Thus, MAPK dephosphorylation and inactivation may take place in both the nucleus and the cytoplasm, and individual MKPs can be highly selective in targeting different MAPK isoforms.

p42 and p44MAPK are able to phosphorylate multiple substrates, found in various subcellular localizations: membrane-associated, such as the epidermal growth factor (EGF) receptor (Northwood *et al.*, 1991), cytoplasmic, such as c-PLA2 (Lin *et al.*, 1993), or nuclear, such as the Elk1/TCF (Gille *et al.*, 1992; Janknecht *et al.*, 1993; Marais *et al.*, 1993) or c-Myc (Seth *et al.*, 1992). Since many of the nuclear substrates of MAPK are transcription factors, MAPK nuclear translocation is thought to represent a crucial step in the modulation of gene expression. However, it is possible that the cytoplasmic pool of active MAPK could phosphorylate transcription factors in the cytoplasm when they are released from the synthetic

machinery. More importantly, whereas a number of reports have highlighted the role of MAPK activation in triggering different biological responses such as proliferation or differentiation, it has never been addressed whether its nuclear translocation and phosphorylation of nuclear targets was crucial for eliciting such responses or whether the phosphorylation of cytoplasmic substrates might be sufficient.

To address the question of the significance of MAPK nuclear translocation, we forced p42/p44MAPK cytoplasmic retention in fibroblasts by expressing an inactive form of the MKP-3 cytoplasmic phosphatase. These conditions, which abolish MAPK nuclear translocation in response to serum, did not perturb MAPK activation or phosphorylation of cytoplasmic substrates. However, enforced cytoplasmic retention of MAPK inhibited Elk1-dependent gene expression and cell cycle re-entry in response to growth factors. Our results support the notion that regulating the accessibility of MAPK to the nucleus is a key signalling event by which cells may control the intensity and temporal activation of genes during cell growth and differentiation.

Results

Expression of an inactive form of MKP-3 prevents MAPK nuclear translocation

To determine the importance of MAPK nuclear translocation independently of its activation, we attempted to retain MAPK in the cytoplasm without affecting its phosphorylation state. To this end, we used an inactive version of the MKP-3/Pyst1 phosphatase, because it displayed the unique characteristics among the MKP family of being localized in the cytoplasm and being able to form a specific complex with p42/p44MAPK, to the exclusion of the other MAPK family members (Groom *et al.*, 1996; Muda *et al.*, 1998).

We expressed in CCL39 fibroblasts Myc-tagged versions of MKP-3, either wild-type (WT) or an inactive mutant in which the crucial cysteine of the catalytic site has been replaced by a serine (C/S), and determined the effect of these constructs on the endogenous MAPK localization by double immunofluorescence. Both WT and inactive forms of MKP-3 were localized cytoplasmically and abrogated endogenous p42MAPK nuclear translocation upon serum stimulation (Figure 1A). Quantification revealed that >95% of the cells expressing MKP-3 (WT or C/S) showed a retention of endogenous MAPK in the cytoplasm. The cytoplasmic retention of MAPK also occurred in cells expressing much lower amounts of MKP-3 (WT or C/S), as judged by immunofluorescence (data not shown).

In agreement with Groom *et al.* (1996), experiments using an antibody directed against the dual-phosphorylated, and thus active, form of MAPK show that WT

MKP-3 completely prevented MAPK activation upon growth factor stimulation, which is consistent with its phosphatase activity. In contrast, MKP-3 C/S did not block the serum-stimulated activation of MAPK (Figure 1B). The fact that MKP-3 inactive mutant did not alter the state of phosphorylation of MAPK was also confirmed by immunoblot (see below, Figure 3C, lower panel). Therefore, the inactive form of MKP-3 specifically blocks MAPK nuclear translocation, without affecting its phosphorylation by upstream MKK1/2.

The inactive form of MKP-3 affects the nuclear translocation of p42/p44MAPK but not of p38MAPK or JNK

To determine whether the effect of the MKP-3 inactive mutant was specific towards p42/p44MAPK family members, we transfected MKP-3 C/S and analysed the localization of the endogenous forms of p42/p44MAPK, p38MAPK and JNK, using polyclonal antibodies directed to the different MAPKs. This experiment showed that MKP-3 C/S retained p42/p44MAPK in the cytoplasm as demonstrated above, but did not affect the localization of the endogenous forms of p38MAPK or JNK (Figure 2A).

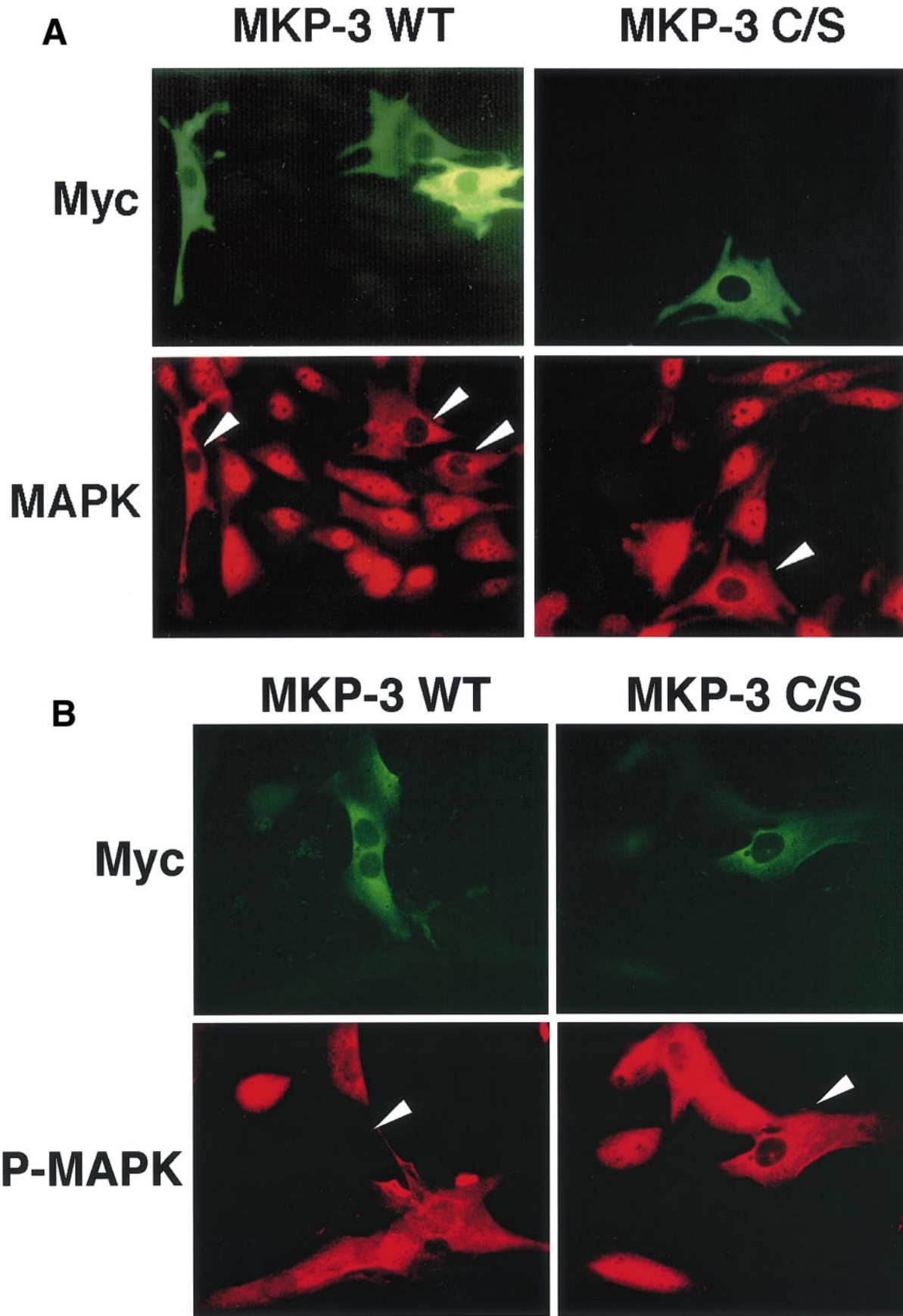
To investigate this point further, we co-transfected MKP-3 C/S together with a hemagglutinin (HA)-tagged form of p44MAPK, p38MAPK and JNK. When overexpressed, all three members of the MAPK family are localized both in the cytoplasm and in the nucleus (Figure 2B). The expression of inactive MKP-3 retained ectopically expressed HA-p44MAPK in the cytoplasm when MKP-3 was overexpressed relative to MAPK (with a 4:1 ratio) (Figure 2B). It has to be noted, however, that in cases where MAPK was overexpressed relative to MKP-3, MAPK bypassed the cytoplasmic retention of MKP-3 and entered the nucleus (data not shown). In contrast to what was seen with p42/p44MAPK, the localization of neither JNK nor p38MAPK was affected by the expression of inactive MKP-3 (Figure 2B). The MKP-3 inactive mutant, therefore, represents a useful and specific tool to assay the role of p42/p44MAPK nuclear localization in the regulation of downstream events.

The inactive form of MKP-3 does not impair cytoplasmic substrate phosphorylation

If MKP-3 inactive mutant was only affecting the ability of MAPK to translocate into the nucleus, then we predicted that it should not affect the phosphorylation of a cytoplasmic substrate of MAPK.

To address this issue, we first substituted part of the C-terminal cytoplasmic tail of an integral membrane protein, the Na⁺/H⁺ exchanger (NHE), with the region of Elk1 containing the four MAPK phosphorylation sites (Janknecht *et al.*, 1993; Marais *et al.*, 1993). As expected, this cytoplasmically anchored form of Elk1 was localized at the plasma membrane (Figure 3A). Western blot analysis

Fig. 1. Catalytically inactive mutant of MKP-3 phosphatase prevents endogenous p42MAPK nuclear translocation without affecting its activation. (A) CCL39 cells, transiently transfected with the expression vectors encoding Myc-tagged MKP-3 (WT or the C/S inactive mutant) were rendered quiescent and stimulated with 20% FCS for 3 h. The localization of MKP-3 was revealed by immunofluorescence using the anti-Myc antibody (Myc), whereas the localization of the endogenous p42MAPK was revealed with the MAPK-specific antibody (MAPK). Pictures shown are representative of at least three independent experiments conducted with different methods of fixation (see Materials and methods). (B) CCL39 cells, transiently transfected with the expression vectors encoding Myc-tagged MKP-3 (WT or the C/S inactive mutant), were rendered quiescent and stimulated with 20% FCS for 15 min. The localization of MKP-3 was revealed by immunofluorescence using the anti-Myc antibody (Myc), whereas phosphorylated MAPK was revealed by immunofluorescence using the phospho-MAPK antibody (P-MAPK).



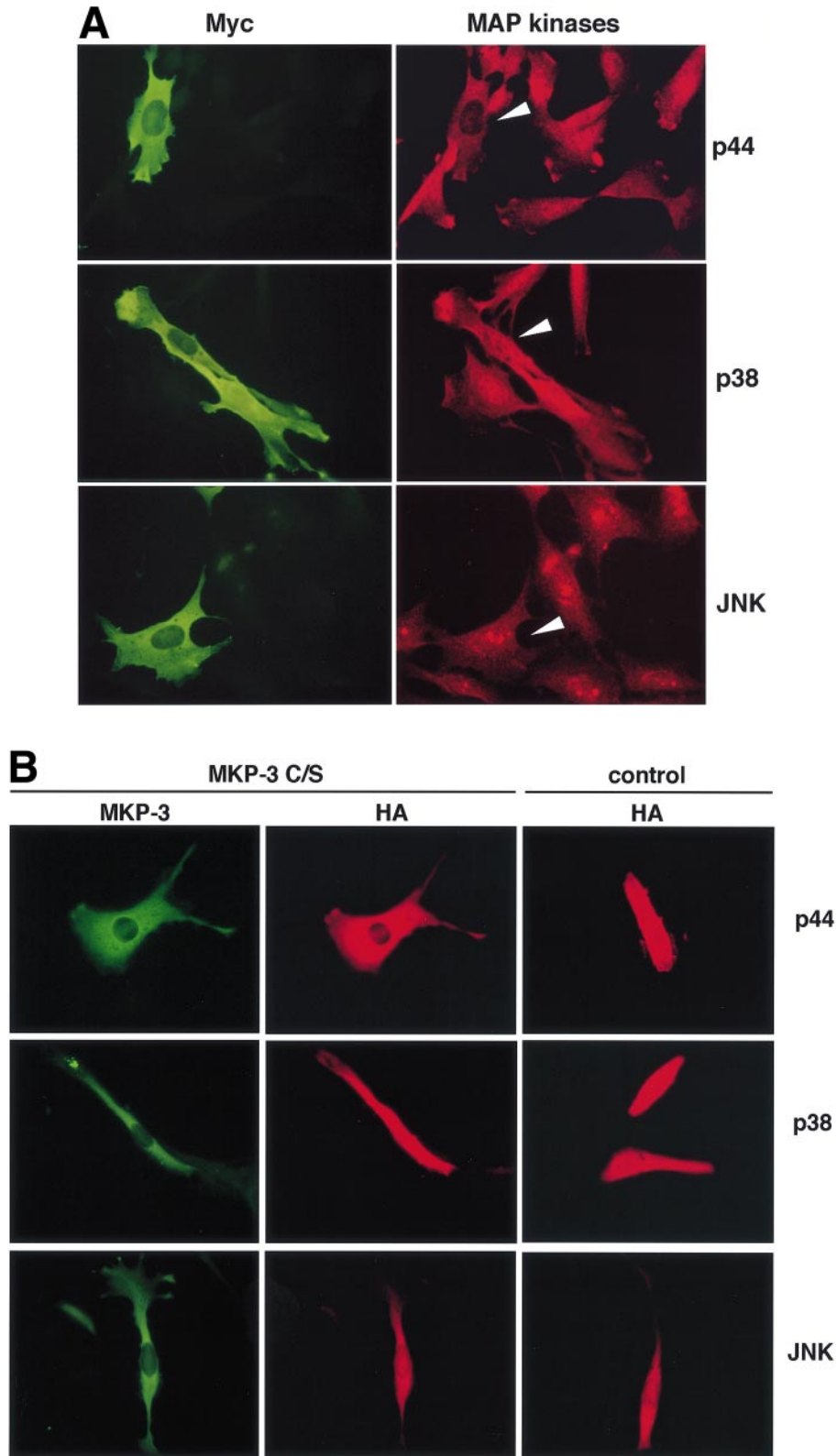


Fig. 2. Inactive form of MKP-3 does not alter the subcellular localization of either p38MAPK or JNK. **(A)** CCL39 cells were transfected with expression vectors encoding Myc-tagged MKP-3 C/S. The localization of the MKP-3 C/S was revealed by immunofluorescence using the anti-Myc antibody (Myc), whereas the localization of the endogenous members of the MAPK family was revealed by specific polyclonal antibodies (see Materials and methods). **(B)** CCL39 cells were co-transfected with expression vectors encoding MKP-3 C/S mutant and vectors encoding HA-p44MAPK, HA-p38MAPK and HA-JNK1 in a 4:1 ratio. The localization of MKP-3 was assessed with the anti-MKP-3 polyclonal antibody, whereas the localization of the different members of the MAPK family was revealed by the anti-HA antibody.

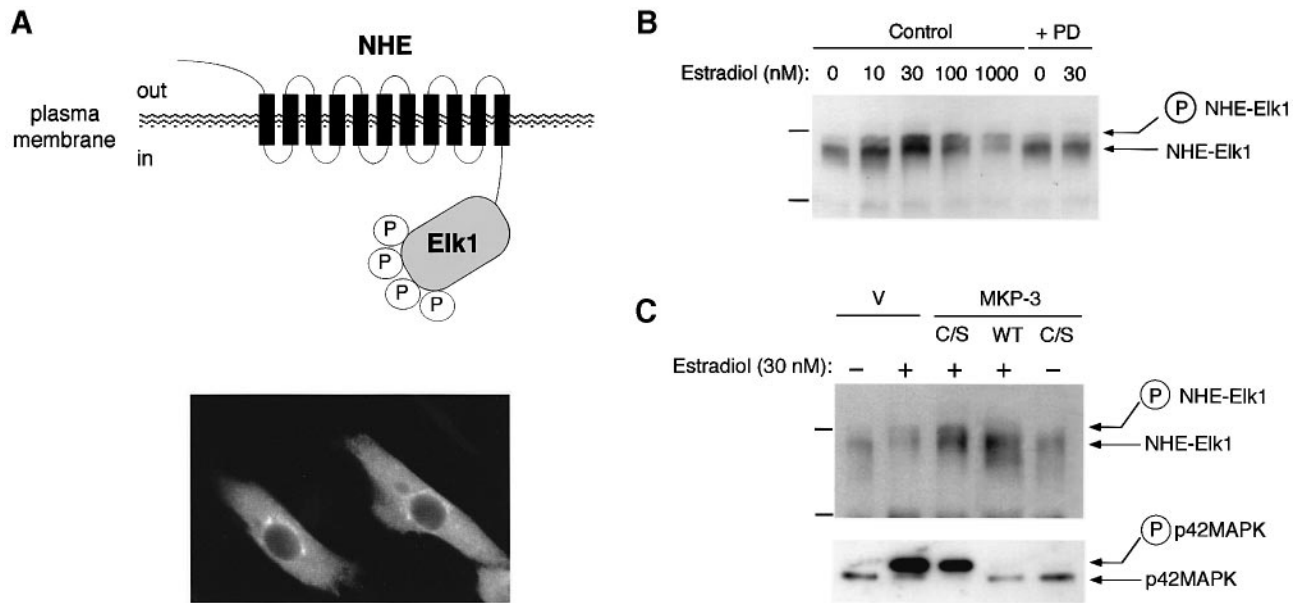


Fig. 3. Inactive form of MKP-3 does not prevent the phosphorylation of a cytoplasmic form of Elk1. **(A)** CCL39 cells were transiently transfected with the construct encoding the fusion between the Na⁺/H⁺ exchanger (NHE1) and Elk1 (NHE-Elk1). The subcellular localization of this construct was assayed by immunofluorescence, using an anti-Elk1 antibody. **(B)** A stable CCL39 cell line expressing both the Raf::ER chimera and the NHE-Elk1 fusion protein was rendered quiescent for 16 h and stimulated with increasing concentrations of estradiol for 30 min, or pre-treated with the MKK1 inhibitor PD 098059 for 30 min and then stimulated with 30 nM estradiol for 30 min. Membrane fractions were resolved on SDS-PAGE and blotted with an anti-Elk1 antibody. **(C)** CCL39 cells expressing the Raf::ER chimeric construct were transiently co-transfected with empty vector (V), MKP-3 WT, MKP-3 C/S and NHE-Elk1. Cells resistant to the acid load selection were rendered quiescent and stimulated with 30 nM estradiol for 30 min. Membrane fractions were resolved on SDS-PAGE and blotted with an anti-Elk1 antibody (upper panel). Cytoplasmic fractions were resolved on SDS-PAGE and blotted with an anti-p42MAPK antibody (lower panel). The immunoblots shown are representative of two independent experiments.

conducted with an anti-Elk1 antibody shows that in fibroblasts expressing the Raf::ER chimeric protein, estradiol stimulation, which specifically activates the Raf-MAPK cascade, led to a retardation of the electrophoretic mobility of NHE-Elk1. This mobility shift is MAPK-dependent as it is abolished by the MKK1-specific inhibitor PD 098059 (Dudley *et al.*, 1995) (Figure 3B). Altogether, these results indicate that we have engineered a cytoplasmic form of Elk1 which, like WT Elk1, is dependent on MAPK activation for phosphorylation.

We next asked whether the estradiol-stimulated phosphorylation of NHE-Elk1 was affected by the expression of MKP-3. As expected, the WT form of MKP-3 was able to suppress the phosphorylation shift of NHE-Elk1 induced by estradiol (Figure 3C, upper panel, compare lanes 2 and 4). In contrast, this mobility shift was not affected by the inactive form of MKP-3 (Figure 3C, upper panel, compare lanes 2 and 3).

To extend this finding to other substrates of MAPK and obtain more quantitative analysis on the phosphorylation of cytoplasmic substrate in cells expressing the inactive form of MKP-3, we assayed the activity of the kinase p90RSK1, which is dependent upon its phosphorylation by p42/p44MAPK (Grove *et al.*, 1993). p90RSK1 has been shown to be cytoplasmic under resting conditions but can translocate to the nucleus upon growth factor stimulation (Chen *et al.*, 1992). We therefore prepared cytoplasmic fractions of 293 cells transfected with MKP-3 and assayed the kinase activity of p90RSK1 in such fractions. The inactive MKP-3 C/S mutant, in contrast to the WT form, did not alter p90RSK1 activation (Figure 4).

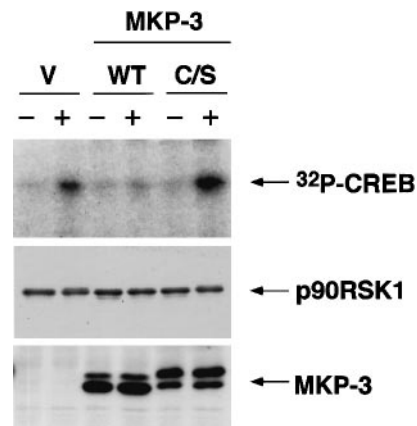


Fig. 4. Inactive form of MKP-3 does not prevent the phosphorylation of the cytoplasmic fraction of p90RSK1. 293 cells were transiently co-transfected with empty vector (V), or vectors encoding MKP-3 (WT or the C/S inactive mutant) and the construct encoding HA-p90RSK1. Cells were starved for 20 h (-) and stimulated for 10 min with 20% FCS (+). The cytoplasmic fraction of HA-p90RSK1 was immunoprecipitated with the anti-HA antibody, and its kinase activity was assayed using CREB as a substrate in the presence of [γ -³²P]ATP (upper panel). The expression of HA-p90RSK1 was determined by immunoblot using the anti-p90RSK1 antibody (middle panel). The expression of MKP-3 was monitored by immunoblot using the anti-Myc antibody (lower panel).

Taken together, these experiments indicate that the inactive form of MKP-3, although binding tightly to MAPK and preventing its nuclear translocation, did not impair MAPK's ability to phosphorylate cytoplasmic substrates.

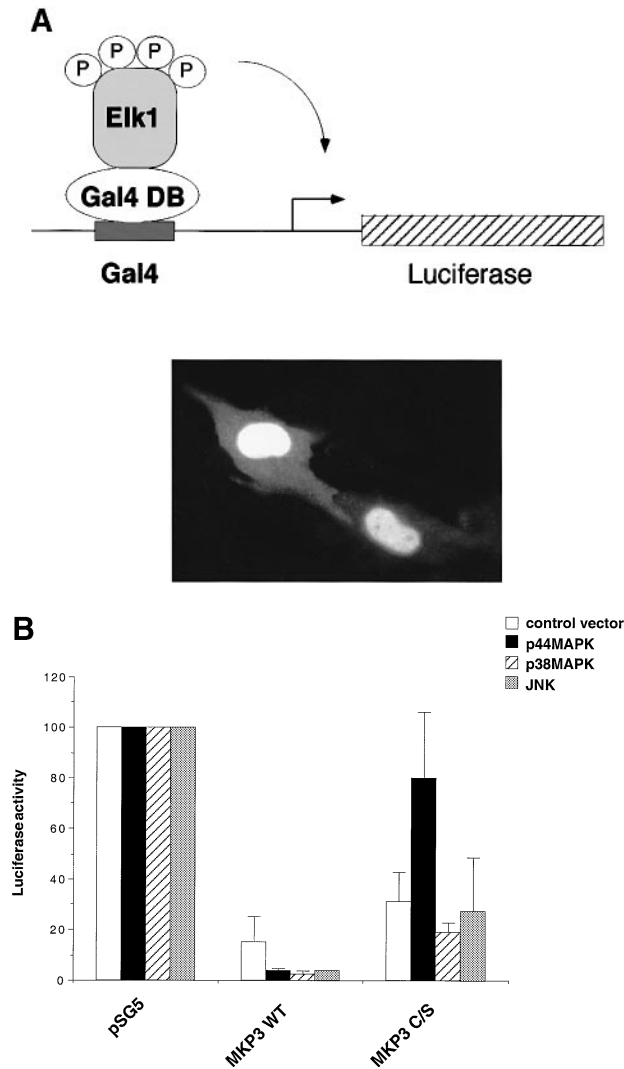


Fig. 5. Inactive form of MKP-3 inhibits nuclear Elk1 activity. (A) CCL39 cells were transiently transfected with the construct encoding the Gal4–Elk1 fusion protein. The subcellular localization of this construct was assayed by immunofluorescence, using an anti-Elk1 antibody. (B) CCL39 cells were transiently co-transfected with the empty vector (pSG5), or vectors encoding MKP-3 (WT or the C/S inactive mutant), Gal4–Elk1 fusion protein and the Gal4–luciferase reporter constructs. Vectors encoding HA-p44MAPK, HA-p38MAPK or HA-JNK were added at a 2:1 ratio compared with the MKP-3 vectors. Luciferase activity was assayed 2 days after transfection. The values represent the mean \pm SEM of four independent experiments conducted in triplicate.

The inactive form of MKP-3 prevents nuclear Elk1-dependent transcription

We asked whether the blockage of MAPK nuclear translocation affected the activation of MAPK-dependent nuclear targets, such as transcription factors. To this end, we took advantage of the Gal4–Elk1 fusion protein which is localized predominantly in the nucleus (Figure 5A) and whose phosphorylation and subsequent activation by MAPK can be assayed using a Gal4–luciferase reporter gene. As expected, expression of MKP-3 WT which potentially inactivates p42/p44MAPK (see Figure 1B) inhibited Gal4–Elk1 activation (Figure 5B). Interestingly, expression of the inactive form of MKP-3 inhibited Gal4–Elk1 activation by 60–70% (Figure 5B). The effect of MKP-3 C/S, but not that of MKP-3 WT, was reverted by

the exogenous expression of high levels of p44MAPK (Figure 5B), but not by lower levels (data not shown). This is consistent with the fact that overexpression of MAPK bypasses the cytoplasmic trapping achieved by MKP-3 C/S.

In contrast, the overexpression of neither p38MAPK nor JNK reversed the MKP-3 C/S negative effect on Gal4–Elk1 activity (Figure 5B). These results indicate that MAPK nuclear translocation is essential to maximally phosphorylate and activate one of its nuclear targets, Elk1.

The inactive form of MKP-3 reduces the rate of S phase entry in response to mitogenic stimulation

We then asked more generally whether the nuclear translocation of MAPK was necessary for the mitogenic response of the cells. To examine whether the prevention of MAPK nuclear translocation interferes with G_1/S phase progression, we monitored DNA replication by bromodeoxyuridine (BrdU) incorporation. In contrast to cells expressing the PTP1C phosphatase, which apparently plays no role in S phase entry (Rivard *et al.*, 1995), cells expressing WT MKP-3 were unable to enter S phase (Figure 6A and B). More importantly, cells expressing the inactive form of MKP-3 were also blocked in the G_0 phase (Figure 6A and B). This suggested that the blockage of MAPK nuclear translocation prevented G_1/S phase progression.

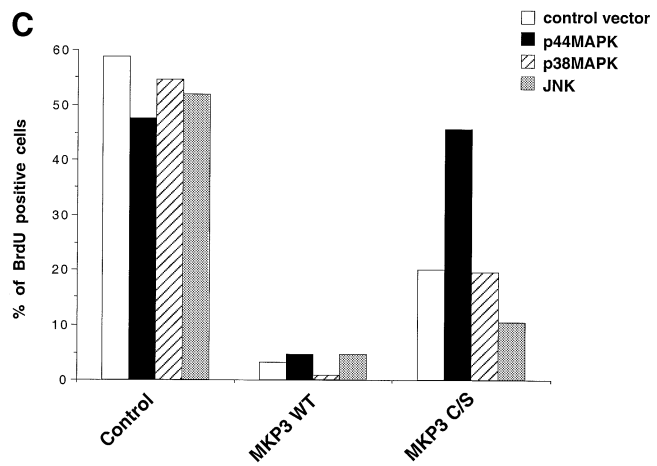
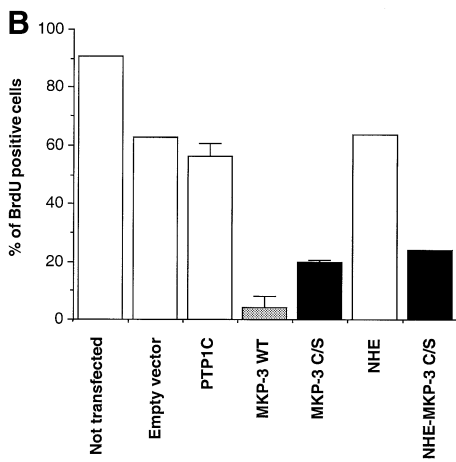
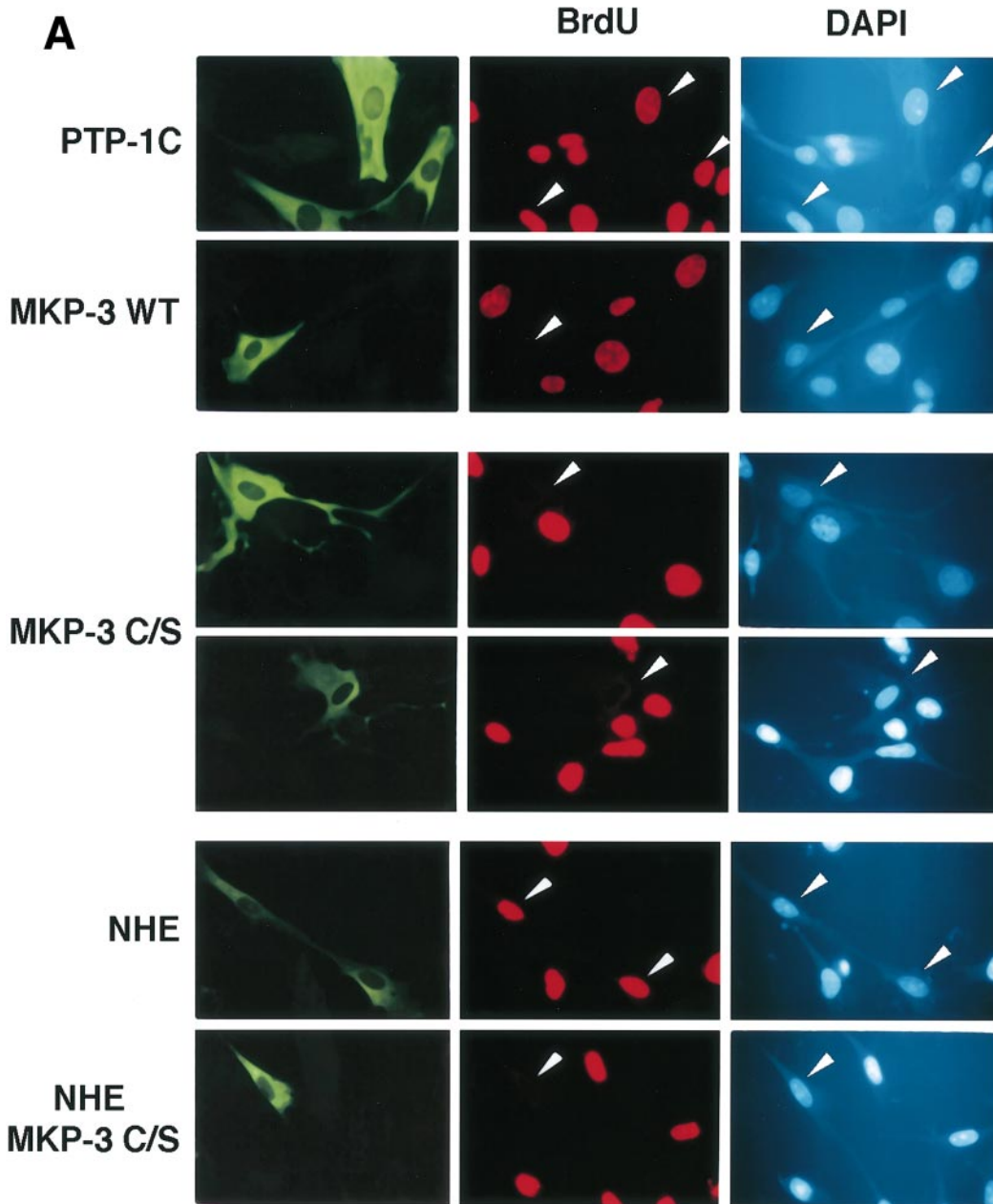
To exclude the possibility that MKP-3 could enter the nucleus transiently, as a shuttling molecule, we targeted MKP-3 C/S to the plasma membrane by generating a fusion protein with the sodium/proton exchanger (NHE–MKP-3 C/S). Ectopic expression of membrane-targeted MKP-3 C/S prevented MAPK nuclear translocation (data not shown) and also prevented DNA synthesis, whereas NHE alone did not (Figure 6A and B). Interestingly, similar results were obtained by expressing a version of NHE–MKP-3 in which we deleted the phosphatase catalytic domain, leaving intact the N-terminal MAPK-interacting domain (Muda *et al.*, 1998) (see Materials and methods). This construct, when overexpressed, prevented MAPK nuclear translocation and G_1/S progression up to 70% (data not shown).

As was the case for the luciferase activity (see Figure 5B), the overexpression of p44MAPK reversed the blockage of G_1/S phase progression due to the expression of MKP-3 inactive mutant but not that of the WT form (Figure 6C). This reversion was specific to p44MAPK and was not observed with either JNK or p38MAPK (Figure 6C).

Altogether, these experiments strongly suggest that MAPK nuclear translocation is a crucial step in signal transduction leading to gene expression and promoting cell cycle re-entry upon mitogenic stimulation.

Discussion

In this study, we have reported a means to specifically prevent p42/p44MAPK nuclear translocation without affecting its activation. Different methods can theoretically be used to achieve the blockage of MAPK nuclear translocation. The one we employed here was to create an artificial anchor for MAPK based on two criteria: a specific interaction with MAPK and a cytoplasmic localization. Several proteins could possibly fulfil the criteria to create



a cytoplasmic anchor for MAPK. For instance, the activator of MAPK, MKK1, is a cytoplasmic protein that also binds specifically to MAPK (Bardwell *et al.*, 1996; Fukuda *et al.*, 1997) and has therefore been proposed to play the role of an MAPK anchor *in vivo* (Fukuda *et al.*, 1997). Moreover expression of MKK1 in *Xenopus* has been shown to impair MAPK nuclear translocation (Fukuda *et al.*, 1997). However, in our fibroblast CCL39 cell line, the ability of MKK1 expression to prevent MAPK nuclear translocation was much weaker than that of inactive MKP-3. This can be explained by the fact that MKK1, when overexpressed, is less excluded from the nucleus than is MKP-3, which is in agreement with the observation that MKK1 can enter the nucleus under certain circumstances (Jaaro *et al.*, 1997). More importantly, MKK1 interacts more weakly with MAPK than does MKP-3 (P.Lenormand, unpublished observation).

Another method to prevent MAPK nuclear translocation would be to affect its transport into the nucleus. The mechanism by which MAPK is transported into the nucleus is not yet known, but it has been hypothesized that transcription factors could co-transport with it, via a 'piggy-back' system. Recent experiments conducted with Spc1, the p38MAPK homologue in fission yeast, show that the deletion of the transcription factor Atf1, which is a major substrate of this kinase, prevented Spc1 relocalization into the nucleus (Gaits *et al.*, 1998). Previous work from our laboratory has also supported the notion that nuclear proteins could play a role in MAPK nuclear translocation and retention (Lenormand *et al.*, 1998). However, interfering with transcription factors, which are MAPK major targets, cannot be used to assay the role of MAPK translocation in biological responses since these responses may themselves require transcription.

A third way to prevent MAPK nuclear translocation is to act on MAPK itself. In a recent report, M.Cobb's group showed that MAPK could form dimers when one of the monomers is phosphorylated and that this dimerization could lead to MAPK nuclear translocation (Khokhlatchev *et al.*, 1998). Mutants of the C-terminal region of MAPK which are unable to dimerize remain cytoplasmic even upon growth factor stimulation (Khokhlatchev *et al.*, 1998). Since those mutants still retain their kinase activity, they could be used to assay the role of MAPK nuclear translocation. Using a similar approach, we fused an NES to the N- or C-terminal ends of p44MAPK in order to enforce its nuclear exit. However, these latter approaches rely both on the fact that MAPK dimer formation is efficient *in vivo* and that the introduction of a mutant form of MAPK will also be able to prevent the endogenous form of MAPK from translocating into the nucleus, in a dominant-negative fashion. Preliminary results obtained with the NES-MAPK indicate that these forms are not able to function efficiently as dominant-negatives with

the endogenous form of MAPK (A.Brunet, unpublished observation).

Therefore, preventing MAPK nuclear translocation by anchoring it in the cytoplasm with an inactive phosphatase represents an attractive method to assay the role of MAPK translocation. However, one potential drawback to this approach was that an efficient trapping of MAPK might also prevent the accessibility to the upstream MKK1/2 or to all the substrates. Our experiments, either using antibodies directed against the phosphorylated form of MAPK or analysing the retardation in the electrophoretic mobility of MAPK clearly showed that MAPK, in a complex with MKP-3 C/S, was phosphorylated. This complexed MAPK was also active towards a substrate *in vitro*, as demonstrated with *in vitro* kinase assays (P.Lenormand, unpublished observation). As far as the accessibility to substrates is concerned, we were able to show that when complexed to MKP-3, MAPK was still able to phosphorylate cytoplasmic substrates *in vivo*. Therefore, the complex between MKP-3 inactive mutant and MAPK appears to be tight enough to prevent MAPK nuclear translocation, but does not affect MAPK general activation and substrate recognition. The domains of MKP-3 involved in MAPK specificity of recognition recently have been mapped in the N-terminal region of the MKP-3 (Camps *et al.*, 1998; Muda *et al.*, 1998). This domain alone, when targeted to the plasma membrane as a fusion with the NHE appears to be sufficient to prevent MAPK nuclear translocation. This result indicates that the cytoplasmic retention of MAPK is mediated via the N-terminal MAPK-interacting site and is not as a result of 'substrate trapping' by the C/S mutant of MKP-3. It would be interesting to determine the corresponding region of MAPK that interacts with MKP-3. Since a mutant of MAPK resistant to MKP action previously had been mapped to the extreme C-terminal domain of the MAPK (Bott *et al.*, 1994; Camps *et al.*, 1998), this region could be the one interacting with MKP-3. The fact that the domains involved in the recognition of substrates were mapped in a more upstream domain of MAPK (Kallunki *et al.*, 1994; Brunet and Pouyssegur, 1996) reinforces the observation that MAPK accessibility to substrate would not be impaired by its interaction with MKP-3.

Using MKP-3 inactive mutant as a tool, we were able first to show that the blockage of MAPK nuclear translocation impaired MAPK-dependent activation of the transcription factor Elk1. Although this result was expected, it had not yet been formally excluded that MAPK nuclear substrates could have been phosphorylated in the cytoplasm, where they undergo obligatory transit during their biosynthesis. In this alternative model, the relay between the cytoplasm and the nucleus would have been established by transcription factors, as is the case for the JAK-STAT or the NF- κ B pathways (Karin and Hunter, 1995). Since MAPK nuclear

Fig. 6. Inactive MKP-3 mutant prevents S phase entry upon mitogenic stimulation. (A) CCL39 cells, either not transfected or transiently transfected with the expression vectors encoding PTP1C, MKP-3 (WT or C/S mutant), NHE or an NHE-MKP-3 C/S fusion construct were rendered quiescent for 24 h and stimulated for 24 h with 20% FCS, the BrdU being added for the last 4 h. Cells expressing the different constructs were revealed by immunofluorescence using polyclonal primary antibodies directed against each protein (left panel). Reinitiation of DNA synthesis was followed by BrdU incorporation, detected by an anti-BrdU monoclonal antibody (middle panel). The DNA was stained using DAPI (right panel). (B) Quantification of the experiment presented in (A). Experiments were performed in duplicate, and 100 cells were counted for each condition. For PTP1C, MKP-3 WT and C/S, the values represent the mean and SEM of three independent experiments. (C) Expression vectors encoding HA-p44MAPK, HA-p38MAPK or HA-JNK were transfected in a 2:1 ratio compared with MKP-3. Experiments were conducted in the same way as in (A). Experiments were performed in duplicate and 100 cells were counted for each condition.

translocation is required for Elk1-dependent activation, our results suggest that the phosphorylation of Elk1 by MAPK occurs in the nucleus. Moreover, the blockage of MAPK nuclear translocation inhibited the activity of the total *c-fos* promoter, confirming that MAPK entry into the nucleus is crucial for immediate early gene expression (A. Brunet, unpublished observation). Therefore, in the MAPK signaling pathway, the relay between the cytoplasm and the nucleus appears to be constituted by MAPK itself.

The approach of blocking the nuclear relocalization of MAPK could also be used to assay in which subcellular compartment different MAPK substrates are phosphorylated. Another question that remains to be answered in terms of compartmentalization is where the dephosphorylation and consecutive inactivation of MAPK by the MKPs occurs *in vivo*. Observations made in *Aplysia* (Martin *et al.*, 1997) and in fibroblasts (P. Lenormand, in preparation) show that after long-term stimulation, active MAPK is only found in the cytoplasm. Therefore, one important role for MAPK nuclear translocation could be to enter in contact with nuclear phosphatases (MKP-1 and -2 for instance) in order to shut down the signal. Endogenous MKP-3 could also participate in signal extinction by dephosphorylating the cytoplasmic pool of MAPK.

Finally, as far as biological responses are involved, our results show that MAPK nuclear translocation appears to be required for progression into S phase in response to mitogenic stimulation. It would be interesting to test other biological responses that have been shown to be under the control of MAPK, such as differentiation of PC12 cells (Traverse *et al.*, 1992) or thymocytes (Alberola-Ila *et al.*, 1995; Crompton *et al.*, 1996), survival of PC12 cells (Xia *et al.*, 1995) or long-term potentiation in *Aplysia* (Martin *et al.*, 1997). Since most of them are dependent upon gene expression and protein synthesis, it seems likely that they will also be affected by MAPK retention in the cytoplasm. However, other MAPK-controlled responses, such as the initiation of protein synthesis (Pause *et al.*, 1994) or the suppression of integrin activation (Hughes *et al.*, 1997) may be independent of MAPK nuclear translocation.

In conclusion, our study provides a way to specifically block MAPK nuclear translocation without affecting its activation. In addition to demonstrating the importance of MAPK nuclear translocation for cellular processes such as mitogen-induced gene expression and cell cycle progression, this system should be useful for specifically blocking subsets of MAPK responses, an effect which is not possible when one interferes more generally with MAPK activation.

Materials and methods

Material

Monoclonal antibodies directed against the HA and Myc epitopes were purchased respectively from Boehringer Mannheim and Santa Cruz. We used commercially available polyclonal antibodies directed against p42MAPK kinase (Upstate Biotechnology), the dual-phosphorylated p42MAPK (Promega), p38MAPK and JNK1/2 (Sigma), and p90RSK1 (Santa Cruz). Polyclonal antibodies directed against MKP-3/Pyst1 were generated against the last 20 amino acids of the human isoform (Groom *et al.*, 1996). The polyclonal antibodies against the PTP1C phosphatase were kindly provided by Dr E.R. Stanley (Rivard *et al.*, 1995). The polyclonal antibodies (RpC28) directed against the C-terminal region of the human NHE1 isoform were described previously (Sardet *et al.*, 1990). The purified CREB protein was kindly provided by Dr A.J. Shaywitz.

Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse or anti-

rabbit antibodies were purchased from Sigma. Texas Red X-conjugated goat anti-mouse or anti-rabbit antibodies were from Molecular Probe. The BrdU proliferation kit was purchased from Amersham. The MKK1 inhibitor PD 098059 was obtained from New England Biolabs.

Constructs

The expression vectors encoding the Myc-tagged phosphatase Pyst1/MKP-3, either WT or an inactive mutant in which Cys293 of the catalytic site has been replaced by a serine (C/S), were described previously (Groom *et al.*, 1996). The expression vectors encoding HA-tagged forms of p44MAPK, p38MAPK (Brunet and Pouyssegur, 1996), JNK1 (Dérjard *et al.*, 1994) and p90RSK1 (Grove *et al.*, 1993), the PTP1C phosphatase (Rivard *et al.*, 1995) and the human NHE1 (Sardet *et al.*, 1989) have all been described. The expression vectors encoding the Gal4-Elk1 fusion protein and the Gal4-luciferase reporter gene have been described previously (Janknecht *et al.*, 1994) and were kindly provided by Dr K. Kelly. The vector encoding human Elk1 was kindly provided by Dr R.A. Hipskind.

The construct encoding a fusion protein between the NHE1 and the C-terminal domain of Elk1 was generated by PCR, by inserting an *ApaI* restriction site at the level of amino acid 317 of Elk1 in-frame with the *ApaI* restriction site corresponding to amino acid 698 of NHE1. The construct encoding a fusion protein between NHE1 and MKP-3 C/S mutant was generated by PCR, by inserting an *ApaI* site at the level of the first amino acid of MKP-3 C/S in-frame with the *ApaI* site corresponding to amino acid 698 of NHE1. The construct encoding a fusion protein between NHE1 and the N-terminal domain of MKP-3 was generated by PCR, using the *EcoRI* site of MKP-3 and deleting the region encoding amino acids 230–359 of MKP-3, so that the last amino acid and the Myc epitope tag were in-frame with the N-terminal domain of MKP-3. The constructs were verified by sequencing.

Cell culture

The Chinese hamster lung fibroblast cell line CCL39 (ATCC) or CCL39 expressing the chimeric Raf::ER chimeric construct (Lenormand *et al.*, 1996) was cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) supplemented with 7.5% fetal calf serum (FCS; Biowhitaker) and antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin) at 37°C in an atmosphere of 95% air, 5% CO₂.

Immunofluorescence

CCL39 cells were plated onto glass coverslips at a density of 10⁵ cells/35 mm dish. They were transfected by the calcium phosphate technique with 7.5 µg of the different constructs. The day after transfection, cells were rendered quiescent by incubation in serum-free medium for 16 h, and stimulated with various agonists. They were then fixed for 15 min at –20°C with methanol/acetone (70:30 v/v) or methanol/formaldehyde (99:1 v/v) or for 15 min in 10% formaldehyde at room temperature, followed by a methanol permeabilization for 10 min at –20°C for phospho-MAPK antibody. Coverslips were washed with phosphate-buffered saline (PBS) and the non-specific sites were blocked by incubation with PBS + 3% bovine serum albumin (BSA). Coverslips were incubated with the first antibody diluted in PBS/BSA (anti-HA, 1/500; anti-Myc, 1/100; anti-MAPK, 1/2000; anti-phosphoMAPK, 1/150; anti-p38MAPK, 1/300; anti-JNK, 1/300; anti-Elk1, 1/500; anti-MKP-3, 1/100) for 1 h, then washed five times with PBS. Cells were incubated with the second antibody (goat anti-mouse FITC-conjugated, 1/100; goat anti-rabbit Texas Red-conjugated, 1/500) diluted in PBS/BSA for 1 h. After extensive washes in PBS and in distilled water, coverslips were mounted in Mowiol (Calbiochem) and examined under epifluorescent illumination.

BrdU incorporation

CCL39 cells were transfected with the relevant constructs as described above. The day after transfection, cells were starved for 24 h and stimulated for 24 h with 20% FCS (Biowhitaker), with a pulse of BrdU (1/500) for the last 4 h. Cells were fixed for 15 min at –20°C with methanol/formaldehyde (99:1 v/v) and incubated with primary antibody (anti-MKP-3, 1/100; anti-PTP1C, 1/250; anti-NHE1, 1/500) prior to incubation with the secondary antibody (FITC anti-rabbit: 1/100) as described above. The cells were then fixed in 3.7% formaldehyde for 10 min at room temperature, rinsed in PBS and the chromatin was rendered accessible by a 10 min treatment with HCl (2 M). Cells were washed extensively with PBS and blocked in PBS + 10% FCS + 0.1% Tween-20. The coverslips were incubated with the anti-BrdU monoclonal antibody (1/2) for 1 h, then washed five times with PBS + 0.1% Tween-20 (PBST). The secondary antibody (Texas Red anti-mouse, 1/500) was incubated for 45 min. DAPI (4',6 diamidine dihydrochloride; Boehringer Mannheim) was added for the last 15 min at a final concentration of 0.2 µg/ml. Cells were washed

extensively in PBST then in distilled water and mounted in Mowiol (Calbiochem).

Luciferase assays

CCL39 cells were seeded in 24-well plates, at a density of 10^5 cells/well, and were co-transfected with 0.25 μ g of the 5 \times Gal4-luciferase reporter gene, 0.5 μ g of the Gal4-Elk1 fusion construct, 0.5 μ g of the constructs corresponding to MKP-3 and 1 μ g of the constructs corresponding to the different MAPK family members. Two days after transfection, cells were lysed in 100 μ l of lysis buffer and the luciferase activity of one-tenth of the samples was assayed according to the Promega protocol.

Western blotting

CCL39 cells expressing the Raf:ER chimera (Lenormand *et al.*, 1996) were seeded in 10 cm dishes at a density of 2×10^6 cells/dish. They were transfected by the SuperFect reagent (Qiagen) with 5 μ g of the vector encoding the NHE-Elk1 fusion protein and 10 μ g of the relevant constructs. The day after transfection, cells were submitted to an acid load selection (Pouyssegur *et al.*, 1984), in order to kill cells that did not express the NHE-Elk1 protein. Resistant cells were rendered quiescent in serum-free medium for 5 h and stimulated with 30 nM estradiol for 30 min.

Membrane fractions were obtained by incubating the cells in a hypotonic buffer [10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM phenanthroline, 1 mM iodoacetamide] for 10 min at 4°C. Cells were then scrapped and the membranes were pelleted by centrifugation at 40 000 r.p.m. for 20 min at 4°C and directly resuspended in 1 \times Laemmli sample buffer. Proteins were resolved on SDS-PAGE (7.5%; 19:1 acrylamide/bisacrylamide) and transferred onto PVDF membranes (Immobilon). The membranes were incubated with the anti-Elk1 antibody (1/1000) overnight at 4°C. Signal was detected using horseradish peroxidase (HRP)-conjugated anti-rabbit antibodies and the enhanced chemiluminescence method. Cytoplasmic fractions were resolved on SDS-PAGE (12.5%; 30:0.2 acrylamide/bisacrylamide) and immunoblotted with the anti-p42MAPK antibody.

Kinase assay

293 cells were seeded in 35 mm dishes at a density of 750 000 cells per dish. They were transfected by the calcium phosphate technique with 5 μ g of the vector encoding HA-p90RSK1 and 1 μ g of the vector encoding the relevant constructs.

At 24 h after transfection, cells were starved in serum-free medium for 20 h, then stimulated with 20% FCS for 10 min. Cytoplasmic fractions were obtained by lysing the cells in a hypotonic buffer [5 mM Tris-HCl, pH 7.4, 1 mM MgCl₂, 2 mM EGTA, 2 mM dithiothreitol (DTT), 5 mM β -glycerophosphate, 1 mM PMSF, 1 mM aprotinin]. Extracts were Dounce homogenized 40 times and centrifuged at 10 000 r.p.m. for 5 min. The supernatant was centrifuged at 15 000 r.p.m. for 30 min. Cytoplasmic extracts were immunoprecipitated with the anti-HA antibody coupled to protein A-Sepharose beads for 2 h. The immunoprecipitate was washed twice in buffer A (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1% NP-40, 2 mM DTT, 40 mM β -glycerophosphate, 50 mM NaF, 1 mM PMSF, 1 mM aprotinin), twice in buffer B (10 mM Tris-HCl pH 7.4, 1 M NaCl, 0.1% NP-40, 2 mM DTT, 40 mM β -glycerophosphate, 50 mM NaF, 1 mM PMSF, 1 mM aprotinin), once in buffer C (50 mM Tris-HCl pH 7.4, 150 mM NaCl) and once in kinase buffer (20 mM HEPES pH 7.2, 10 mM MgCl₂, 2 mM DTT, 0.1 mg/ml BSA). Kinase assay was performed using 5 μ g of purified CREB as a substrate and 5 μ Ci of 50 μ M [γ -³²P]ATP for 15 min at 30°C. The reaction was stopped by addition of Laemmli sample buffer, resolved on SDS-PAGE (12%; 29:1 acrylamide/bisacrylamide) and autoradiographed.

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