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

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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 MAPK 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 MKP-3 inhibited MAPK phosphorylation and showed a retention of endogenous MAPK in the cytoplasm. The cytoplasmic retention of MAPK also occurred in cells expressing much lower amounts of cytoplasmic substrate phosphorylation.

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.

### 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 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 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. 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 p21/44MAPK (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).

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.
Role of p42/p44MAPK nuclear translocation

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 ± 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 potently 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 G1/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 G0 phase (Figure 6A and B). This suggested that the blockage of MAPK nuclear translocation prevented G1/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 G1/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 G1/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 relocation 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 (Kokhatchev et al., 1998). Mutants of the C-terminal region of MAPK which are unable to dimerize remain cytoplasmic even upon growth factor stimulation (Kokhatchev 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-κB pathways (Karin and Hunter, 1995). Since MAPK nuclear

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**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 rendered quiescent 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 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 et al., 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 (MPK-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 p42MAP 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 (Rcp28) 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 (CIS), were described previously (Groom et al., 1996). The expression vectors encoding HA-tagged forms of p44MAPK, p38MAPK (Brunet and Pouysségur, 1996), JNK1 (Déjardin 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 Apal restriction site at the level of amino acid 317 of Elk1 in-frame with the Apal restriction site corresponding to amino acid 698 of NHE1. The construct encoding a fusion protein between NHE1 and MKP-3/C5/MKP-3 mutant was generated by PCR, by inserting an Apal site at the level of the first amino acid of MKP-3/C5 in-frame with the Apal 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; Biowhittaker) 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/aceton (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 phospo-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 secondary 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 (Biowhittaker), 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-NHE, 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 (PBS-T). 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 µg of the 5× Gal4–Luciferase reporter gene, 0.5 µg of the Gal4–Elk1 fusion construct, 0.5 µg of the hCMV promoter driving expression of a control p53 protein over the entire duration of the experiment.

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