Essential role of calcium in the regulation of MAP kinase phosphatase-1 expression

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Mitogen-activated protein (MAP) kinase phosphatase-1 (MKP-1) is a dual-specificity protein phosphatase encoded by an immediate-early gene responsive to growth factors and stress. The MKP-1 protein selectively inactivates MAP kinases *in vitro* by dephosphorylation of the regulatory Thr and Tyr residues. Little is known on the mechanisms that regulate *MKP-1* gene expression. Here, we demonstrate that Ca^{2+} is both necessary and sufficient for the induction of *MKP-1* gene expression. Treatment of Rat1 fibroblasts with the Ca^{2+} chelating agent BAPTA completely suppressed serum-induced MKP-1 expression in a dose- and time-dependent manner. The inhibitory effect of BAPTA was observed at the level of the protein and the mRNA. Importantly, Ca^{2+} chelation blocked the induction of MKP-1 expression in response to all stimuli tested and in different cell types. Increasing the intracellular concentration of Ca^{2+} with the ionophore A23187 was sufficient to induce MKP-1 mRNA and protein expression in rat fibroblasts. We also provide evidence that activation of MAP kinases is not an absolute requirement for induction of the *MKP-1* gene. Exposure of rat fibroblasts to A23187 induced MKP-1 expression without activating the JNK and p38 MAP kinase pathways. Also, inhibition of the ERK pathway with the selective MEK inhibitor PD98059 did not interfere with serum-stimulated *MKP-1* mRNA expression. These results will help define the regulatory mechanisms that govern *MKP-1* gene transcription in target cells.

Keywords: phosphatase; MAP kinase; gene expression; calcium

Introduction

Mitogen-activated protein (MAP) kinases are a family of proline-directed serine/threonine kinases which play a key role in transducing signals from a wide range of extracellular stimuli into intracellular responses. Three distinct subfamilies of MAP kinases are currently known in vertebrates: the extracellular signal-regulated kinases (ERKs), the stress-activated protein (SAP)/Jun N-terminal kinases (JNKs), and p38 (reviewed in (Davis, 1994; Cano and Mahadevan, 1995; Cobb and Goldsmith, 1995)). The ERK isoforms ERK1/ERK2 are activated in response to mitogenic and differentiation factors (Cobb *et al.*, 1991; L'Allemain, 1994), whereas JNKs and p38 respond mainly to environ-

mental stress and proinflammatory cytokines (Kyriakis *et al.*, 1994; Han *et al.*, 1994; Rouse *et al.*, 1994; Raingeaud *et al.*, 1995; Force *et al.*, 1996). The activation of MAP kinases is mediated by a family of dual-specificity enzymes, called MAP kinase kinases, which phosphorylate the proteins on both threonine and tyrosine residues within the sequence T-X-Y (Cobb and Goldsmith, 1995; Waskiewicz and Cooper, 1995). On the other hand, much less is known on the mechanisms leading to inactivation of MAP kinases.

Recently, a novel family of dual-specificity protein phosphatases capable of dephosphorylating the regulatory threonine and tyrosine residues of MAP kinases has been described (reviewed in Keyse, 1995). All these enzymes bear the characteristic active-site motif of protein tyrosine phosphatases (PTPs) and display sequence homology to the vaccinia virus VH1 phosphatase (Guan *et al.*, 1991). The first mammalian gene of this family to be discovered was mouse *3CH134* (also known as *erp*) and its human homologue *CL100* (also known as *hVH-1*) which was isolated as an immediate-early gene inducible by growth factors and stress (Charles *et al.*, 1992; Keyse and Emslie, 1992; Noguchi *et al.*, 1993; Zheng and Guan, 1993). Recombinant 3CH134/CL100 purified from bacteria preferentially dephosphorylates the phosphothreonine and phosphotyrosine residues of MAP kinases *in vitro* resulting in their inactivation (Charles *et al.*, 1993; Alessi *et al.*, 1993; Zheng and Guan, 1993). Overexpression of 3CH134 in COS cells leads to dephosphorylation and inactivation of ERK1/ERK2; and catalytically-inactive 3CH134 physically associates with the phosphorylated form of ERK1/ERK2 in intact cells (Sun *et al.*, 1993). On the basis of these results, the 3CH134/CL100 phosphatase was renamed MAP kinase phosphatase-1 (MKP-1). The expression of MKP-1 was further shown to inhibit ERK-dependent reporter gene activity (Sun *et al.*, 1994b; Brondello *et al.*, 1995), to block cell cycle progression and entry into S phase of fibroblast cells (Noguchi *et al.*, 1993; Sun *et al.*, 1994b; Brondello *et al.*, 1995) and to prevent PC12 cell differentiation (Fukuda *et al.*, 1995). All these observations suggest that MKP-1 might be a physiological MAP kinase phosphatase, but its relative contribution to the inactivation of MAP kinases in specific cellular processes *in vivo* remains to be established.

Other members of the VH1 family of dual-specificity protein phosphatases include VHR (Ishibashi *et al.*, 1992), PAC-1 (Rohan *et al.*, 1993), MKP-2/hVH-2/TYP-1 (Guan and Butch, 1995; Misra-Press *et al.*, 1995; King *et al.*, 1995), B23/hVH-3 (Ishibashi *et al.*, 1994; Kwak *et al.*, 1995), hVH-5 (Martell *et al.*, 1995) and rVH-6/MKP-3 (Mourey *et al.*, 1996; Muda *et al.*,

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1996). These gene products have unique but overlapping tissue distribution patterns. Like MKP-1, all the enzymes exhibit activity towards phosphorylated ERK1/ERK2 *in vitro*. Moreover, *in vivo* expression of PAC-1, MKP-2, and MKP-3 interferes with growth factor-dependent phosphorylation and enzymatic activation of ERK1/ERK2 (Ward *et al.*, 1994; King *et al.*, 1995; Muda *et al.*, 1996; Chu *et al.*, 1996). Interestingly, recent results obtained in transient transfection experiments suggest that individual MAP kinase phosphatases may have distinct specificities for the ERK, JNK and p38 MAP kinases (Chu *et al.*, 1996).

Little is known on the mechanisms by which growth factors and stress activate transcription of the gene encoding MKP-1. The induction of *MKP-1* mRNA expression has been observed in several cell types in response to stimuli as diverse as growth and differentiation factors, cAMP-elevating agents, phorbol esters, heat shock, oxidative stress, and genotoxic agents (Charles *et al.*, 1992; Keyse and Emslie, 1992; Noguchi *et al.*, 1993; Duff *et al.*, 1993; Liu *et al.*, 1995). The aim of this study was to characterize the signalling pathways involved in the regulation of MKP-1 expression. We demonstrate that calcium is both necessary and sufficient for *MKP-1* gene expression in fibroblast and smooth muscle cells. Moreover, we provide strong evidence that the three MAP kinases, ERKs, JNKs and p38, are not obligatory components of the pathways leading to transcription of the *MKP-1* gene in fibroblast cells.

Results

Calcium is necessary for induction of MKP-1 gene expression

To determine whether MKP-1 expression is controlled by reversible protein phosphorylation, we first examined the effect of two non selective protein kinase inhibitors, 2-aminopurine and K-252a (Mahadevan *et al.*, 1990; Kase *et al.*, 1987), on serum-induced expression of MKP-1. Growth-arrested Rat1 fibroblasts were pre-treated for 30 min with increasing concentrations of 2-aminopurine or K-252a and then stimulated for 90 min with 10% calf serum. Cell lysates were prepared and the expression of MKP-1 was analysed by immunoblotting using a specific antibody. Both inhibitors significantly reduced MKP-1 protein expression in a dose-dependent manner (Figure 1). K-252a was more potent than 2-aminopurine with a maximal inhibitory effect observed at 30 nM. In a complementary experiment, we also found that phloretin, an uncoupler and inhibitor of mitochondrial oxidative phosphorylation which depletes cellular stores of ATP (De Jonge *et al.*, 1983), completely abolished the serum-dependent expression of MKP-1 in Rat1 cells (data not shown). These results strongly implicate the action of protein kinase(s) in the regulation of MKP-1 expression.

To gain insights into the nature of the protein kinase(s) involved, we evaluated the effect of selective inhibitors of protein kinase C (PKC) isoforms, tyrosine kinase inhibitors and a membrane-permeable Ca^{2+} chelating agent on serum-induced expression of MKP-1. CGP41251 is a compound which inhibits

Ca^{2+} -dependent and independent isoforms of PKC (Meyer *et al.*, 1989; Marte *et al.*, 1994), while Go6976 is more selective for the classical Ca^{2+} -dependent PKC isoforms (Martiny-Baron *et al.*, 1993). As shown in Figure 2a, the two PKC inhibitors, and the biologically inactive analog CGP42700, had no effect on the phosphatase expression level. Similarly, genistein and herbimycin A, two broad spectrum tyrosine kinase inhibitors (Akiyama *et al.*, 1987; Uehara *et al.*, 1989), did not modify the effect of serum on MKP-1 expression (Figure 2b). In contrast, treatment of Rat1 cells with the Ca^{2+} chelating agent BAPTA resulted in

Figure 1 Inhibition of serum-induced MKP-1 expression by non selective protein kinase inhibitors. Quiescent Rat1 fibroblasts were pre-treated for 30 min with the indicated concentrations of 2-aminopurine (2-AP) or K-252a. The cells were then stimulated or not (cont) with 10% calf serum for 90 min. Equal amounts of lysate proteins were resolved by SDS-gel electrophoresis on 10% acrylamide gel and transferred to nitrocellulose membrane. The membrane was probed with anti-MKP-1 antibody and the proteins visualized by chemiluminescence detection. The position of MKP-1 is indicated by an arrow

Figure 2 Effect of signalling inhibitors on serum-induced MKP-1 expression in Rat1 fibroblasts. Quiescent Rat1 fibroblasts were pre-treated for 30 min with the indicated signalling inhibitors, with the exception of herbimycin A which was present during 20 h. The cells were then stimulated or not (cont) with 10% calf serum (CS) for 90 min. The expression of MKP-1 protein was analysed by immunoblotting as described in Figure 1. The concentrations of drug inhibitors were: 10 μM BAPTA, 10 μM CGP41251, 10 μM CGP42700, 10 μM Go6976, 30 μM genistein (Gen) and 0.5 μM herbimycin A (HA)

a complete inhibition of the serum-dependent expression of MKP-1.

These results prompted us to investigate the effect of BAPTA in more detail. Pharmacological studies revealed that BAPTA inhibits MKP-1 expression in a dose-dependent manner with a half-maximal inhibition observed at a concentration of approximately $1 \mu\text{M}$ (Figure 3a). A complete inhibition was observed at $10 \mu\text{M}$ BAPTA. Time-course experiments indicated that addition of BAPTA 60 or 30 min before serum induced a total inhibition of MKP-1 expression (Figure 3b). When the chelating agent was added together with serum, only a partial inhibition was observed. The inhibitory effect was further reduced when BAPTA was added 30 min after serum stimulation, and completely disappeared at 60 min. BAPTA also reduced the rate of protein synthesis at this concentration, which may partly account for the effect seen on MKP-1 protein levels (data not shown). In order to determine if the involvement of Ca^{2+} was specific to the stimulus and/or the cellular context, we examined the effect of BAPTA on two different cell types, Rat1 fibroblasts and rat aortic smooth muscle cells (SMC), treated with various extracellular stimuli. As shown in Figure 4a, MKP-1 protein expression was strongly induced by serum in Rat1 fibroblasts, but also in response to epidermal growth factor, thrombin, phorbol esters, and heat shock. Pre-incubation of the cells with BAPTA completely abolished MKP-1 expression, whatever the stimulus used. The same experiment was performed in rat aortic SMC using the same stimuli, except for thrombin which was substituted by angiotensin II. As observed for Rat1 cells, MKP-1 expression was induced by all treatments and the response was blocked by BAPTA (Figure 4b).

Figure 3 The inhibition of MKP-1 expression by BAPTA is dose- and time-dependent. (a) Quiescent Rat1 fibroblasts were pre-treated for 30 min with different concentrations of BAPTA prior to stimulation with medium (cont) or 10% calf serum for 90 min. (b) BAPTA ($10 \mu\text{M}$) or vehicle was added at various times before and after the addition of 10% calf serum to quiescent Rat1 cells. Time 0 corresponds to the beginning of the 90 min incubation period with serum. The cells were treated with medium (cont) or 10% calf serum (CS) for 90 min as control. The expression of MKP-1 protein was analysed by immunoblotting as described in Figure 1

Since MKP-1 is encoded by an immediate-early gene, it was of interest to determine if the effect of BAPTA is exerted at the transcriptional level. Rat1 fibroblasts were pretreated in the absence or presence of BAPTA before serum stimulation and the level of *MKP-1* mRNA was analysed by Northern blotting. As expected, serum rapidly and strongly increased the expression of *MKP-1* mRNA, which reached a maximal level at 30 min and declined thereafter (Figure 5). In the presence of BAPTA, the induction of *MKP-1* mRNA expression was almost completely abolished.

Increased calcium concentration is sufficient to induce MKP-1 gene expression

Together, the preceding results indicated that Ca^{2+} is required for MKP-1 gene expression. We then wanted to determine if an increase in the intracellular concentration of Ca^{2+} alone was sufficient to activate the *MKP-1* gene. Rat1 fibroblasts were treated with the Ca^{2+} ionophore A23187 and the level of MKP-1 expression was monitored as above. As shown in Figure 6a, addition of A23187 increased the expression of MKP-1 protein in a dose-dependent manner with a maximal effect observed at $3 \mu\text{M}$. Time-course experiments revealed that A23187-induced MKP-1 expression reaches a maximum at approximately 2 h and slowly declines thereafter (Figure 6b). The effect of A23187 was most likely exerted at the level of gene transcription since the ionophore induced a strong and transient accumulation of *MKP-1* mRNA (eightfold stimulation over basal level) in these cells (Figure 6c). It is interesting to note that the kinetics of MKP-1 induction is slightly slower and the extent of MKP-1 expression is lower in cells treated with A23187 as

Figure 4 Effect of BAPTA on the induction of MKP-1 expression by various stimuli. Quiescent Rat1 cells (a) or rat aortic SMC (b) were pre-treated for 30 min in the absence (vehicle) or presence of $10 \mu\text{M}$ BAPTA. The cells were then exposed to the following stimuli: medium (cont), 10% calf serum (CS) for 90 min, 100 ng/ml epidermal growth factor (EGF) for 90 min, 3 U/ml thrombin (Thr) for 90 min, 100 nM PMA for 90 min, 100 nM angiotensin II (AII) for 90 min and heat shock at 42°C for 30 min followed by 60 min at 37°C (HS). The expression of MKP-1 protein was analysed by immunoblotting as described in Figure 1

Figure 5 Inhibition of *MKP-1* mRNA expression by BAPTA in Rat1 fibroblasts. Quiescent Rat1 cells were pre-treated for 30 min with vehicle or with 10 μ M BAPTA. The cells were then stimulated for the indicated times with 10% calf serum. Total RNA was extracted from the cells and analysed by Northern hybridization using a ^{32} P-labeled *MKP-1* probe. The results were normalized by rehybridization of the blot with a *GAPDH* probe. The extent of hybridization was visualized by phosphorimaging analysis

Figure 6 The Ca^{2+} ionophore A23187 induces *MKP-1* gene expression in Rat1 fibroblasts. Quiescent Rat1 fibroblasts were treated with different concentrations of A23187 for 4 h (a) or with 3 μ M A23187 for different period of times (b). The expression of MKP-1 protein was analysed by immunoblotting as described in Figure 1. The cells were stimulated with medium (cont) or with 10% calf serum (CS) for 90 min as control. (c) Quiescent Rat1 cells were stimulated with 3 μ M A23187 for the indicated times. Total RNA was extracted from the cells and analysed by Northern hybridization using a ^{32} P-labeled *MKP-1* probe. Results were normalized by rehybridization of the membrane with a *GAPDH* probe

compared to serum. This suggests that additional signalling pathways might be required for full transcriptional activation of the *MKP-1* gene.

Induction of *MKP-1* gene expression in the absence of MAP kinases activation

We also performed a series of experiments to investigate if MAP kinases, which are putative substrates of the phosphatase, are required for the induction of MKP-1 expression in response to extracellular signals. Indeed, it has been recently proposed that activation of the JNK pathway induces *MKP-1* gene transcription in NIH3T3 cells (Bokemeyer *et al.*, 1996). We first measured the enzymatic activity of JNK2, ERK1, and p38 in Rat1 cells following stimulation with A23187. Ca^{2+} influx caused a strong activation of ERK1, which reached a maximum at approximately 15 min (eightfold stimulation) and remained elevated for at least 2 h (Figure 7b). In contrast, addition of A23187 did not activate JNK2

Figure 7 Effect of A23187 on JNK, ERK and p38 MAP kinase pathways in Rat1 fibroblasts. Quiescent Rat1 cells were stimulated with 3 μ M A23187 for the indicated period of times. Cell lysates were prepared and p38 (upper panel), ERK1 (middle panel) or JNK2 (lower panel) were immunoprecipitated using specific antibodies preadsorbed to protein A-Sepharose beads. The phosphotransferase activity of each MAP kinase was assayed directly using GST-ATF2 (p38), MBP (ERK1), or GST-c-Jun (JNK2) as substrate (see Materials and methods)

and only had a weak (twofold) and transient stimulatory effect on p38 activity (Figure 7c and 7a). Exposure of the cells to heat shock or anisomycin confirmed that JNK2 and p38 are fully active. We next

used a pharmacological approach to address the role of the ERK pathway in the induction of *MKP-1* gene expression. PD98059 is a synthetic drug inhibitor which selectively inhibits the activity of ERK1/ERK2 by

Figure 8 Effect of the MEK inhibitor PD98059 on *MKP-1* gene expression in Rat1 fibroblasts. Quiescent Rat1 fibroblasts were pre-treated for 30 min with vehicle alone (open symbols) or with 50 μ M PD98059 (closed symbols). The cells were then stimulated with 10% calf serum (Δ , ∇) or 1% calf serum ($\circ$, $\bullet$) for the indicated period of times. (a) Enzymatic activity of ERK1. Cell lysates were prepared and subjected to immunoprecipitation with a specific ERK1 antiserum. The phosphotransferase activity of ERK1 was assayed directly using MBP as substrate. The enzymatic activities are expressed as pmoles of phosphate incorporated into the substrate per min per mg of lysate protein. (b) expression of MKP-1 protein analysed by immunoblotting as described in Figure 1. (c) Expression of *MKP-1* mRNA analysed by Northern hybridization using a ^{32} P-labeled *MKP-1* cDNA as probe. Rehybridization of the membrane with a *GAPDH* probe confirmed that similar amounts of RNA were loaded in each lane (data not shown)

interfering with the upstream activation of the MAP kinase kinases MEK1/MEK2 (Dudley *et al.*, 1995; Alessi *et al.*, 1995; Servant *et al.*, 1996). Incubation of Rat1 cells with 50 μ M PD98059 resulted in little inhibition of MKP-1 protein expression in response to 10% serum (Figure 8b). However, in the presence of high serum concentration, PD98059 was found to only partially block the activation of ERK1 (Figure 8a). Other workers also reported that PD98059 does not inhibit the activation of ERK1/ERK2 completely when cells are stimulated with high concentrations of agonists (Alessi *et al.*, 1995; Flynn and Proud, 1996). Thus, to further evaluate the involvement of ERK1/ERK2 in the regulation of MKP-1 expression, we tested the effect of PD98059 at sub-optimal serum concentration. When Rat1 cells were stimulated with 1% serum, PD98059 completely suppressed the activation of ERK1 and, under these conditions, partially inhibited the expression of MKP-1 protein (Figure 8a and b). However, PD98059 had no significant effect on the accumulation of *MKP-1* mRNA in these cells (Figure 8c). It is important to underline that PD98059 inhibits growth factor-induced protein synthesis at this concentration (Servant *et al.*, 1996), which may explain the decrease in MKP-1 protein. These results argue that activation of MAP kinases is not an absolute requirement for the induction of *MKP-1* gene transcription by extracellular stimuli.

Discussion

MKP-1 is an immediate-early gene that is rapidly and transiently induced in response to growth factors and cellular stress. The *MKP-1* gene encodes a VH1-like dual-specificity phosphatase which exhibits catalytic activity towards MAP kinases *in vitro*. Little is known about the mechanisms that regulate *MKP-1* gene expression and about the physiological roles of the enzyme. In this study, we show that in fibroblast cells: (1) Ca^{2+} is both necessary and sufficient for the induction of *MKP-1* gene expression; and (2) activation of MAP kinases is not an absolute requirement for induction of the *MKP-1* gene.

Pharmacological experiments with various signalling inhibitors have revealed that chelation of intracellular Ca^{2+} with BAPTA completely blocks serum-induced MKP-1 expression in a time- and dose-dependent manner. The inhibitory effect of BAPTA was observed at the level of the protein and the mRNA, thereby suggesting that Ca^{2+} may be required for transcriptional activation of the *MKP-1* gene. The following observations illustrate the central role of Ca^{2+} in the regulation of MKP-1 expression. First, Ca^{2+} chelation blocks the induction of MKP-1 expression in response to all stimuli tested, including G protein-coupled receptor agonists, receptor tyrosine kinase agonists, or physical stress. Second, the Ca^{2+} requirement of MKP-1 expression is not restricted to fibroblasts but is also observed in other cell types such as vascular SMC. Third, increasing the intracellular concentration of Ca^{2+} with the ionophore A23187 is sufficient to induce the expression of MKP-1 mRNA and protein. Similar observations were made in the lymphoma cell line 2PK-3 (Mittelstadt and Defranco, 1993) and in rat aortic SMC (Duff *et al.*, 1993) using the calcium

ionophore ionomycin. It is noteworthy that the stimulatory effect of A23187 on MKP-1 expression is slightly delayed in comparison to serum and of lower magnitude. This suggests that an additional Ca^{2+} -independent pathway(s) is recruited by serum to stimulate full transcription of the *MKP-1* gene.

The mechanism by which Ca^{2+} regulates *MKP-1* gene expression is currently unknown. Ca^{2+} is a widespread second messenger which participates to a host of cellular changes including gene expression (Clapham, 1995). Elevation of intracellular Ca^{2+} levels has been shown to activate transcription of a number of eukaryotic genes through phosphorylation of transcription factors that bind to the CRE (cAMP-responsive element) (Sheng *et al.*, 1990; 1991; Dash *et al.*, 1991; Liu *et al.*, 1993). This effect of Ca^{2+} may be mediated by the multifunctional Ca^{2+} /calmodulin-dependent protein kinases (Schulman, 1993) which can phosphorylate and activate different members of the CRE-binding protein (CREB)/activating transcription factor (ATF) family, such as CREB, CREM τ and ATF-1 (Dash *et al.*, 1991; Sheng *et al.*, 1991; Shimomura *et al.*, 1996; de Groot *et al.*, 1993; Sun *et al.*, 1994c). In T lymphocytes, the activation of nuclear factor of activated T cells (NF-AT) is quantitatively correlated with the intracellular concentration of Ca^{2+} (Negulescu *et al.*, 1994). The influx of Ca^{2+} through voltage-sensitive Ca^{2+} channels also stimulates serum response factor (SRF)-dependent transcription in the pheochromocytoma cell line PC12 (Miranti *et al.*, 1995). Sequencing of the 5' flanking region of the mouse *MKP-1* gene revealed the presence of several consensus sequences that include one CRE, two SP1 sites, two CTF/NF1-like sites, and one E-box motif (Noguchi *et al.*, 1993). The human *MKP-1* gene is also flanked by two CREs (Kwak *et al.*, 1994). In contrast to other immediate-early genes, no serum response element (SRE) has been found. Although the role of these different elements in the transcriptional activation of *MKP-1* remains to be determined, it is tempting to speculate that the CRE may mediate the response to Ca^{2+} . In support of this hypothesis, cAMP was found to strongly induce *MKP-1* mRNA expression in NIH3T3 cells (Noguchi *et al.*, 1993).

The expression of *MKP-1* mRNA is induced by a range of stimuli that also trigger the activation of MAP kinase pathways in mammalian cells. This suggests that MAP kinase activity may be required for *MKP-1* transcription, thereby providing a feedback loop to inactivate the signalling pathway (Sun and Tonks, 1994a). In fission yeast, activation of the stress-activated MAP kinase Spc1/Sty1 leads to expression of the *pyp2⁺* gene in a manner dependent upon the Atf1 transcription factor (Millar *et al.*, 1995; Shiozaki and Russel, 1996; Wilkinson *et al.*, 1996). The *pyp2⁺* gene encodes a tyrosine-specific protein phosphatase which dephosphorylates Spc1/Sty1 in a feedback inhibition loop. Recently, Grumont *et al.* (1996) have provided evidence suggesting that ERK2 activation is necessary for phorbol esters and v-Ras-dependent transcription of the *Pac-1* gene in T lymphocytes. In another study, Bokemeyer *et al.* (1996) reported that expression of an active mutant of MEK kinase-1 in NIH3T3 cells leads to *MKP-1* gene induction. However, the findings reported in this study together with observations by other groups suggest that MAP

kinases are not obligatory components of the signalling pathways that regulate *MKP-1* gene expression. We have found that exposure of rat fibroblasts to the Ca^{2+} ionophore A23187 induces *MKP-1* mRNA expression without significantly activating the JNK and p38 MAP kinase pathways. Moreover, treatment with SB203580, a highly selective inhibitor of p38 MAP kinase (Lee *et al.*, 1994), had little or no effect on the induction of *MKP-1* expression by serum (data not shown). We also show that incubation of rat fibroblasts with a concentration of PD98059 that blocks ERK1/ERK2 activation does not interfere with serum-stimulated *MKP-1* mRNA expression. The observation that PD98059 partly inhibits *MKP-1* protein expression most likely reflects the inhibitory effect of the compound on protein synthesis. Indeed, we have recently demonstrated that addition of PD98059 (Servant *et al.*, 1996) or expression of a dominant-negative mutant of ERK1 (Gopalbhai K and Meloche S, unpublished data) block growth factor-stimulated protein synthesis in target cells.

Other studies also reported a lack of correlation between the activation of MAP kinases and the induction of *MKP-1* gene transcription in various cell types. For example, treatment of B lymphocytes (Mittelstadt and DeFranco, 1993) or NIH3T3 cells (Bokemeyer *et al.*, 1996) with phorbol esters was found to stimulate the phosphorylation of ERK isoforms without inducing *MKP-1* gene expression. Also, expression of a constitutively-active MEK1 mutant failed to increase *MKP-1* mRNA expression in NIH3T3 cells, whereas a dominant-negative form of the kinase potentiated serum or anisomycin-induced *MKP-1* gene expression (Bokemeyer *et al.*, 1996). In HeLa cells, treatment with the genotoxic agent methyl methanesulfonate strongly induced *MKP-1* mRNA expression but had little effect on ERK1/ERK2 activity (Liu *et al.*, 1995). In murine macrophages expressing an oncogenic $\Delta\text{Raf-1}$: estrogen receptor ($\Delta\text{Raf-1-ER}$) fusion protein, the *MKP-1* gene was shown to be induced in response to lipopolysaccharide stimulation but not in response to activation of $\Delta\text{Raf-1-ER}$ with estradiol, despite the rapid and prolonged activation of ERK1/ERK2 (Hambleton *et al.*, 1995). Finally, treatment of Rat1 cells with the PKC inhibitor Ro-31-8220 was recently reported to activate the JNK pathway, but to inhibit growth factor-stimulated expression of *MKP-1* (Beltman *et al.*, 1996). Taken together, these observations indicate that activation of signalling pathways other than the three known MAP kinase modules is required for full transcription of the *MKP-1* gene.

Materials and methods

Materials and antibodies

Tissue culture reagents and epidermal growth factor were purchased from Life Technologies. Thrombin, 2-aminopurine, bovine myelin basic protein (MBP), anisomycin, and protease inhibitors were from Sigma. Angiotensin II was obtained from Hukabel Scientific. Phorbol 12-myristate 13-acetate (PMA), K-252a, and genistein were from LC Services. BAPTA (1,2-bis(o-aminophenoxy) ethane- N,N,N',N' -tetraacetic acid tetra (acetoxymethyl) ester) and A23187 were from Calbiochem. Go6976 and herbimycin A

were from Biomol. CGP41251 and the biologically inactive analogue CGP42700 were gifts from Ciba-Geigy. [γ - ^{32}P]ATP was from Amersham Corp. PD98059 was a gift from Parke-Davis. The bacterial expression plasmids encoding GST-ATF2 and GST-c-Jun were kindly provided by Dr Roger Davis (University of Massachusetts) and Dr Jim Woodgett (Ontario Cancer Institute), respectively. The recombinant fusion proteins were expressed in *E. coli* and purified as described (Guan and Dixon, 1991b).

The anti-MKP-1 polyclonal antibody was obtained from Santa-Cruz Biotechnology. Antiserum SM1 has been described and specifically immunoprecipitates ERK1 isoform (Meloche, 1995). The polyclonal antibodies to JNK2 and p38 were generously provided by Dr Jacques Landry (Université Laval).

Cell culture

Rat1 cells were cultured in minimum essential medium (MEM) supplemented with 10% calf serum, 2 mM glutamine and antibiotics (Meloche, 1995). They were made quiescent by incubation in serum-free Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) containing 15 mM HEPES (pH 7.4) and 1 mg/ml BSA for 24 h. Rat aortic SMC were cultured and synchronized as described previously (Giasson and Meloche, 1995).

Immunoblot analysis of MKP-1

Following stimulation with the indicated agents, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 50 mM sodium fluoride, 5 mM EDTA, 40 mM β -glycerophosphate, 1 mM sodium orthovanadate, 10^{-4} M phenylmethylsulfonyl fluoride, 10^{-6} M leupeptin, 10^{-6} M pepstatin A, 1% Triton X-100). After 25 min at 4°C, the lysates were clarified by centrifugation at $13\,000 \times g$ for 10 min at 4°C. Equal amounts of lysate proteins (100 μg) were subjected to SDS-gel electrophoresis on 10% acrylamide gels and transferred to Hybond-C nitrocellulose membranes (Amersham) in 25 mM Tris, 192 mM glycine. Membranes were blocked in Tris-buffered saline (TBS) containing 5% non-fat dry milk and 0.05% Tween-20 for 45 min at 37°C prior to incubation for 60 min at room temperature with anti-MKP-1 antibody (1 $\mu\text{g}/\text{ml}$) in blocking solution. The membranes were washed three times in TBS containing 0.05% Tween-20 (TBST) prior to incubation for 30 min at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG diluted 1:10 000 in blocking solution. After washing as above, the immunoreactive bands were visualized by enhanced chemiluminescence (Amersham).

Protein kinase assays

The enzymatic activity of JNK2, ERK1 and p38 was measured by specific immune complex kinase assays using GST-c-Jun, MBP and GST-ATF2 as substrates, respectively. The assay of ERK1/ERK2 has been described previously (Meloche, 1995; Giasson and Meloche, 1995). For JNK2 assays, cell lysates were prepared as described above and 500 μg of proteins were incubated for 2 h at 4°C with 2 μl of anti-JNK2 antibody preadsorbed to protein A-Sepharose (Pharmacia) beads. The immune complexes were washed three times with lysis buffer and once with JNK assay buffer (20 mM HEPES, pH 7.4, 20 mM MgCl_2 , 2 mM dithiothreitol (DTT), 20 mM β -glycerophosphate, 100 μM sodium orthovanadate). The beads were then resuspended in 40 μl of JNK assay buffer containing 1 μg GST-c-Jun, 50 μM ATP and 5 μCi [γ - ^{32}P]ATP. The reaction was initiated with ATP, incubated at 30°C for 15 min, and stopped by addition of 2 $\times$ Laemmli's sample buffer. The

samples were analysed by SDS-gel electrophoresis and autoradiography.

For p38 assays, 150 µg of lysate proteins were incubated for 2 h at 4°C with 5 µl of anti-p38 antibody coupled to protein A-Sepharose. The immune complexes were washed as above. The beads were then resuspended in 20 µl of p38 kinase assay buffer (25 mM HEPES, pH 7.4, 25 mM MgCl₂, 2 mM DTT, 25 mM β-glycerophosphate, 100 µM sodium orthovanadate) containing 1 µg GST-ATF2, 50 µM ATP, and 3 µCi [γ -³²P]ATP. The enzymatic reaction was carried out at 30°C for 30 min and analysed as described for JNK2.

Northern blot analysis

Total RNA was extracted by a modified version of the guanidinium thiocyanate procedure as described (Chomczynski and Sacchi, 1987; Chomczynski, 1993). Equal amounts of total RNA (15–25 µg) were denatured by heating for 15 min at 65°C in 2.2 M formaldehyde and 50% formamide and resolved by electrophoresis in a 1% agarose gel containing 1.8% formaldehyde. The RNA were transferred to Hybond-N membranes (Amersham), fixed, and hybridized with ³²P-labeled probes. Hybridization was carried out in hybridization medium [5 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate), 0.1% SDS, 5 × Denhardt's solution (1 × Denhardt's = 0.02% Ficoll 400, 0.02% polyvinyl pyrrolidone and 0.02% bovine serum albumin), 50% formamide, and 100 µg/ml herring sperm DNA] containing the labeled probe (1–2 × 10⁶ c.p.m./ml) for 16 h at 42°C. The membranes were

washed twice at 25°C for 15 min in 2 × SSC, 0.1% SDS and twice at 60°C for 30 min in 0.5 × SSC, 0.1% SDS. The extent of hybridization was analysed with a phosphorimager apparatus (Molecular Dynamics). The results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

The probes used were: a 1.2 kb PvuII–NotI fragment of human *MKP-1* cDNA (provided by Dr Stephen Keyse, ICRF), and a 1.2 kb XbaI–PstI fragment of rat *GAPDH* cDNA. All the probes were labeled by random priming.

Other methods

Protein concentrations were measured using the BCA protein assay kit (Pierce) with bovine serum albumin as standard.

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