The Dual Specificity Mitogen-activated Protein Kinase Phosphatase-1 and -2 Are Induced by the p42/p44MAPK Cascade*

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Mitogen-activated protein (MAP) kinase phosphatase-1 (MKP-1) and MKP-2 are two members of a recently described family of dual specificity phosphatases that are capable of dephosphorylating p42/p44MAPK. Overexpression of MKP-1 or MKP-2 inhibits MAP kinase-dependent intracellular signaling events and fibroblast proliferation. By using specific antibodies that recognize endogenous MKP-1 and MKP-2 in CCL39 cells, we show that MKP-1 and MKP-2 are not expressed in quiescent cells, but are rapidly induced following serum addition, with protein detectable as early as 30 min (MKP-1) or 60 min (MKP-2). Serum induction of MKP-1 and MKP-2 is sustained, with protein detectable up to 14 h after serum addition. Induction of MKP-1 and, to a lesser extent, MKP-2 temporally correlates with p42/p44MAPK inactivation.

To analyze the contribution of the MAP kinase cascade to MKP-1 and MKP-2 induction, we examined CCL39 cells transformed with either v-raf or a constitutively active direct upstream activator of MAP kinase, mitogen-activated protein kinase kinase 1 (MKK-1; MKK-1(SD/SD) mutant). In both cell models, MKP-1 and MKP-2 are constitutively expressed, with MKP-2 being prevalent. In addition, in CCL39 cells expressing an estradiol-inducible ΔRaf-1::ER chimera, activation of Raf alone is sufficient to induce MKP-1 and MKP-2. The role of the MAP kinase cascade in MKP induction was highlighted by the MKK-1 inhibitor PD 098059, which blunted both the activation of p42/p44MAPK and the induction of MKP-1 and MKP-2. However, the MAP kinase cascade is not absolutely required for the induction of MKP-1, as this phosphatase, but not MKP-2, was induced to detectable levels by agents that stimulate protein kinases A and C. Thus, activation of the p42/p44MAPK cascade promotes the induction of MKP-1 and MKP-2, which may then attenuate p42/p44MAPK-dependent events in an inhibitory feedback loop.

A major characteristic of protein phosphorylation is its reversibility. In the cell, a dynamic balance exists between phosphorylation and dephosphorylation, resulting from interplay between protein phosphatases and protein kinases. As a consequence, a modification of either component is likely to have an equally important impact on signal transduction (1–3).

Protein phosphatases are generally classified into either serine/threonine or tyrosine phosphatases, depending on phosphoamino acid specificity. Dual specificity phosphatases capable of removing both phosphorysine and phosphothreonine from protein targets are a relatively recent discovery. However, an ever increasing number of such phosphatases exist, including Cdc25, which dephosphorylates Thr14 and Tyr15 of Cdc2 (4), and Cdi1 (or MAPK) (5, 6), which dephosphorylates Thr160 of Cdk2. A novel family of dual specificity enzymes harboring the canonical motif (Y/V)HC(X)AGXXR/S/T(G), originally identified in the active site of the VH-1 phosphatase (7), and able to dephosphorylate, at least in vitro, an archetypal substrate, p42/p44MAPK (8, 9), has recently been identified. These phosphatases have been named MAP1 kinase phosphatases (MKPs), with at least eight known members currently identified: MKP-1 (CL100, XCL100, HV1, 3CH134, or epr) (10–14); MKP-2 (HV-2, VTP1) (15, 16); hVH-3 (B23) (17, 18); MKP-3 (rVH-6, Pyst1) (19, 20, 23); hVH-5 (21), PAC1 (22); and two incomplete sequences, MKP-X (19) and Pyst2 (23). p42/p44MAPK are highly homologous ternary members of a group of ubiquitously expressed serine/threonine kinases that, in fibroblasts, are activated in response to all mitogenic stimuli (24). Sustained activation of the MAP kinase cascade is an absolute requirement for fibroblasts to exit from the quiescent G0 state and to pass the restriction point in G1. Thus, blockade of MAP kinase signaling with antisense MAP kinase constructs or dominant-negative MAP kinase molecules (25) or overexpression of MKP-1 (26) all prevent cell cycle reentry. The MAP kinase family is exemplified by three distinct subtypes: p42/p44MAPK (27), p38MAPK (28), and p46/p54JNK (29), which are predominately cytoplasmic proteins in quiescent cells. Upon stimulation, p42/p44MAPK and p46/p54JNK translocate to the nucleus, where they may phosphorylate nuclear transcription factors and thus regulate gene transcription (30–32). Full enzymatic activity of MAP kinase requires a dual phosphorylation on a Thr-X-Tyr motif, which is performed in vivo by specific upstream activators termed MAP kinase kinase (MKK-1/2, MKK-3/6, and MKK-4 for the p42/p44MAPK, p38MAPK, and p46/p54JNK enzymes, respectively) (33–35). Consequently, each MAP kinase family member is a candidate for dual dephosphorylation by MKPs. This raises the question of specificity between cognate kinase-phosphatase partners. However, previous studies have shown that MKP-1, MKP-2, MKP-3, and PAC1 (restricted to hematopoietic cell lines) are all able to inactivate p42/p44MAPK and that specificity is correspondingly low (8, 15, 19, 36).

Several MKP family members were identified by virtue of their being encoded by immediate-early genes (10, 37). With the exception of Pyst1 (23), MKPs are not expressed in quiescent cells. This paper is available online at http://www-jbc.stanford.edu/jbc/.

* This work was supported by CNRS Grant UMR134, by l’Association pour la Recherche Contre le Cancer, by La Ligue Nationale contre le Cancer, and by a Roussel Uclaf doctoral fellowship (to J.-M.B.). The abbreviations used are: MAP, mitogen-activated protein; MKP, MAP kinase phosphatase; M KK, MAP kinase kinase; GST, glutathione S-transferase; DMEM, Dulbecco’s modified Eagle’s medium; HA, hemagglutinin; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; FCS, fetal calf serum; IL-1β, interleukin-1β; PMA, phorbol 12-myristate 13-acetate; CRE, CAMP-responsive element.

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cent cells and may be induced upon stimulation with agonists that include mitogens, oxidative stress, heat shock, and UV irradiation (9). Specificity of MKPs for the various MAP kinase members may therefore depend on their presence during a particular cellular event. An understanding of the mechanisms controlling MKP induction may explain how MAP kinase signaling pathways are regulated.

We report that two dual specificity phosphatases (MKP-1 and MKP-2) may be induced in resting CCL39 fibroblasts following serum exposure. We have attempted to correlate their induction with the activity status of signal transduction pathways involving different MAP kinase family members. To this end, we investigated the level of MKP-1 and MKP-2 in cells expressing either constitutively active or inducible forms of each element of the Ras/Raf-1/MKK module. Our results suggest that activation of the p42/p44MAPK cascade is sufficient to promote the expression of MKP-1 and MKP-2. We propose that p42/p44MAPK down-regulation when cells progress through G1 is dependent on a feedback loop that involves MKP family members.

**EXPERIMENTAL PROCEDURES**

**Materials**

| Gene | Vector | Source |
|------|--------|--------|
| p46JNK | pcDNAneo/MKP-1 | Dr. M. Karin |
| p46JNK | pcDNAneo/MKP-2 | Dr. R. Davis |
| cyclin D1 | Anti-cyclin D1 antisera | Dr. Ve´ronique Baldin |

**Cells and Culture Conditions**

The established Chinese hamster lung fibroblast line CCL39 (American Type Culture Collection) and its derivatives (clones Ras5C and MKK-1/SD/SD) were culttivated in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Inc.) containing 7.5% fetal calf serum, 50 mg/ml sodium antibiotic, and 10% in PBS) and counted.

CCL39 cells were seeded at a density of 0.8 \( \times 10^6 \) cells/dish in 10-cm plates and, when confluent, serum-starved for 24 h. Cells were then incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:3000) in blocking solution for 2 h at room temperature. After washing in Tris-buffered saline and 0.1% Triton X-100, blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3000) in blocking solution for 1 h and revealed with ECL. Where indicated, the activity status of p42/p44MAPK was determined by a mobility shift assay in which, following cell lysis, proteins were separated by SDS-PAGE (12.5% acrylamide, 0.0625% bisacrylamide) and Western blotting was performed with antisera E1B, which preferentially recognizes p42MAPK.

**Immune Complex Kinase Assays**

Cells were washed twice with cold PBS and lysed in Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 40 mM β-glycerophosphate, 200 mM sodium orthovanadate, 10−4 M phenylmethylsulfonyl fluoride, 1 mM/ml leupeptin, 1 mM/ml pepstatin, and 1% Triton X-100) for 15 min at 4 °C. Insoluble material was removed by centrifugation at 12,000 × g for 2 min at 4 °C. Proteins from cell lysates (100 μg) were separated by 10% SDS-PAGE and electrophoretically transferred to Hybond-C Extra membranes (Amersham Corp.) in 25 mM Tris-HCl and 0.19 mM glycine. Membranes were blocked in Tris-buffered saline (25 mM Tris-HCl, pH 7.5, and 137 mM NaCl) containing 5% nonfat dry milk. The blots were then incubated with anti-A装扮1-1 (3:00) in blocking solution for 2 h at room temperature. After washing in Tris-buffered saline and 0.1% Triton X-100, blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3000) in blocking solution for 1 h and revealed with ECL. Where indicated, the activity status of p42/p44MAPK was determined by a mobility shift assay in which, following cell lysis, proteins were separated by SDS-PAGE (12.5% acrylamide, 0.0625% bisacrylamide) and Western blotting was performed with antisera E1B, which preferentially recognizes p42MAPK.

**Northern Blot Analysis**

CCL39 cells were seeded in 10-cm plates and, when confluent, serum-starved for 24 h. Cells were then incubated with the appropriate agonist and, following a suitable time period, washed twice with cold PBS and lysed with Bioprobe RNA preparation (acid phenol and guanidine chloride). RNA was prepared by phenol/chloroform extraction and sodium acetate precipitation. Following RNA separation (30 μM of total RNA), on formaldehyde gels, RNA was transferred to nitrocellulose and probed by the technique of Church and Gilbert (44). The MKP-1 probe was obtained following purification from a StuI digestion (780 base pairs) of pcDNAneo/C100. The MKP-2 probe was purified as a StuIStu1II digest (1055 base pairs) of pcDNAneo/HV-2.
CCL39 cells in 12-well plates were stimulated with FCS for 2 h. After lysis, proteins (75–150 mM Tris, pH 7.5, and 1% Nonidet P-40; once with PBS and 0.5 M by 10% SDS-PAGE. Gels were exposed, and the relevant bands were in the kinase assay for GST-ATF-2 (5). A more detailed analysis revealed that MKP-1 and MKP-2 are induced, with MKP-1 and MKP-2 transiently expressed in HEK 293 cells. When immunoblotting was performed in the presence of the specific peptide used for rabbit immunization, but not with an unrelated peptide sequence, the two bands with apparent molecular masses of 40 and 42 kDa were no longer detectable. Additionally, serum-mediated induction of MKP-1 and MKP-2 was dose-dependent in nature (Fig. 1C). These data strongly suggest that our anti-MKP-1/2 antiserum is capable of specifically detecting the hamster homologues of MKP-1 and MKP-2 in CCL39 cells.

**RESULTS**

Characterization of Rabbit Polyclonal Antiserum Directed against MKP-1 and MKP-2 Proteins—Previous studies that have examined the expression of MKP family members have principally employed Northern blot analysis. In serum-stimulated (2 h), but not quiescent, CCL39 cells, two RNA species that migrate with the size expected of MKP-1 and MKP-2 (2.4 kilobase pairs) were detected using probes that were designed to specifically identify either MKP-1 or MKP-2 mRNA species, respectively (Fig. 1A). A more detailed analysis revealed that the two mRNA species that were identified with MKP-1 and MKP-2 probes were induced by serum with a time course similar to that previously noted for MKP-1 and MKP-2 (data not shown).

To specifically identify MKP-1 and MKP-2 proteins, we raised rabbit polyclonal antiserum directed against a synthetic peptide corresponding to the last 12 C-terminal amino acids of mouse MKP-1 (3CH1/94 protein) (37). This sequence has only one amino acid change from human MKP-1 (Lys to Gln) (10) and one amino acid change in the same position in MKP-2 (Lys to His in both rat and human sequences) (15, 46). In contrast, the C-terminal regions of the other MKPs bear little or no primary structure similarity. Thus, we expected that our anti-MKP antisera would be capable of recognizing both MKP-1 and MKP-2, but not other MKP family members. In Western blots of HEK 293 cells transiently transfected with expression vectors encoding MKP-1 or MKP-2, but not with empty vector, two single major bands were identified by anti-MKP-1/2 antiserum Alb-1 with apparent molecular masses of 40 and 42 kDa, in close agreement with the calculated molecular mass from the primary sequence of MKP-1 and MKP-2, respectively (Fig. 1B).

We next performed Western blotting on cell lysates derived from serum-deprived or serum-stimulated CCL39 cells. Following serum stimulation (3.5 h) of quiescent CCL39 cells, Alb-1 antisera recognized two major bands with the expected molecular mass of MKP-1 and MKP-2 (Fig. 1B). In addition, these two bands comigrated with MKP-1 and MKP-2 transiently expressed in HEK 293 cells. When immunoblotting was performed in the presence of the specific peptide used for rabbit immunization, but not with an unrelated peptide sequence, the two bands with apparent molecular masses of 40 and 42 kDa were no longer detectable. Additionally, serum-mediated induction of MKP-1 and MKP-2 was dose-dependent in nature (Fig. 1C). These data strongly suggest that our anti-MKP-1/2 antiserum is capable of specifically detecting the hamster homologues of MKP-1 and MKP-2 in CCL39 cells.

**MKP-1 and MKP-2 Are Rapidly but Differentially Induced in Response to Serum:** Correlation with p42/p44 MAPK Inactivation Time Course—To analyze the induction of MKP-1 and MKP-2 in CCL39 cells, we performed Western blotting on cell lysates from serum-stimulated cells (Fig. 2A). In quiescent CCL39 cells, both MKP-1 and MKP-2 are undetectable. Upon addition of serum, MKP-1 and MKP-2 are induced, with MKP-1 present as early as 1 h after addition of serum. Comparison of a range of experiments shows that MKP-1 is often detectable as early as 30 min after addition of serum (data not shown). In contrast, MKP-2 is induced later, with detectable protein present at 2–3 h after serum addition. Each phosphatase is present for at least 14 h after serum addition. After 10–12 h of serum addition, the CCL39 cell populations had started to enter S phase, correlating with the expression of cyclin D1. Thus, MKP-1 and MKP-2 are induced and present during G_1 phase, transition and S phase entry in CCL39 cells. As we have previously reported (38), serum stimulation of p44 MAPK is rapid, being maximal after 5 min. p44 MAPK then declines slowly, until 1–2 h following serum addition, where there is a significant loss of activity (Fig. 2B, lower panel) (47). This loss of activity correlates temporally with the induction and presence of MKP-1 protein and, to a lesser extent, with the production of MKP-2 (Fig. 2, upper panel). As most members of the MKP family are encoded by immediate-early genes, addition of protein synthesis inhibitors to quiescent CCL39 cells should block their induction. To examine the contribution of MKP-1 and
MKP-2 to the inactivation of p42/p44MAPK, we examined the time course of MAP kinase activity in the presence or absence of cycloheximide (Fig. 2C). As detailed in Fig. 2B, serum-stimulated p42/p44MAPK activity is maximal at 5 min and then declines such that ~30% of the maximal activity elicited by serum (5-min time point) is present 4 h after addition of serum. However, in the presence of cycloheximide, the long-term inactivation of p42/p44MAPK is reduced such that 54% of the maximal activity elicited by serum (5-min time point) is present 4 h after addition of serum. Additional experiments revealed that cycloheximide inhibited the serum-mediated induction of both MKP-1 and MKP-2 in a dose-dependent manner that correlated with the extent of protein synthesis inhibition (data not shown). Thus, although a considerable fraction of the p42/p44MAPK inhibitory activity is independent of cycloheximide treatment, immediate-early genes and probably MKP-1 and MKP-2 play a role in setting the level of p42/p44MAPK activity.

MKP-1 and MKP-2 Block the Activation of p44MAPK, p46JNK, and p38MAPK and Inhibit CCL39 Cell Proliferation—To explore the substrate specificity of the MKP-1 and MKP-2 expressed by CCL39 cells, we performed cotransfection assays with each phosphatase together with epitope-tagged p44MAPK, p38MAPK, or p46JNK (Fig. 3A). In response to serum, HA-p44MAPK may be activated and can phosphorylate its substrate, myelin basic protein. However, following cotransfection with either MKP-1 or MKP-2, p44MAPK is no longer activable. In a similar manner, in response to specific stress stimulus, such as anisomycin, both HA-p38MAPK and Flag-p46JNK are able to phosphorylate their respective substrates in the absence, but not in the presence, of either MKP-1 or MKP-2. Thus, the transient expression of both MKP-1 and MKP-2 attenuates the activation in vivo of each of the three kinases examined: p44MAPK, p38MAPK, and p46JNK.

We have previously shown that deregulated expression of MKP-1 in CCL39 fibroblasts prevents cell cycle reentry (26). As MKP-2 is also able to inhibit p42/p44MAPK in vivo (Fig. 3A), we expected that MKP-2 would also exert a strong antiproliferative effect. To examine this hypothesis, we transfected CCL39 cells with either MKP-1 or MKP-2 and determined the colony-forming ability of transfected cells (Fig. 3B). In the presence of both MKP-1 and MKP-2, colony formation and hence cell division are blocked by up to 80%, demonstrating that both MKP-1 and MKP-2 inhibit the proliferation of CCL39 cells.

Inactivation of Stress Kinases Does Not Require MKP-1 or MKP-2—The transient expression studies described above (Fig. 3) show that overexpression of MKP-1 or MKP-2 can inhibit p38MAPK and p46JNK activity. However, this does not unequivocally demonstrate that endogenous MKP-1 and MKP-2 are required for inactivation of either kinase. Hence, we examined the time course of activation of p46/p54JNK in response to a range of agonists, together with the possible induction of MKP-1 or MKP-2 (Fig. 4). Activation of p46/p54JNK by osmotic shock (sorbitol), sodium arsenite (equivalent to heat shock (48)), or IL-1β was slow in comparison with agonist-mediated activation of p42/p44MAPK (Fig. 2), peaking at ~30 min after addition of either sorbitol or IL-1β. In the case of sodium arsenite and sorbitol, this activity was sustained, lasting for at least 3 h in the continual presence of agonist. In contrast, stimulation of p46/p54JNK by IL-1β was transient, having returned to basal values within 2–3 h after addition of agonist. None of the agonists tested were able to induce MKP-1 or MKP-2 to detectable levels, in contrast to control cells, which were stimulated with serum. Thus, inactivation of p46/p54JNK
proceeds in the absence of detectable MKP-1 and MKP-2.

**Serum Induction of MKP-1 and MKP-2 Does Not Require p38MAPK Activity—** p38MAPK is weakly, although significantly, activated by serum in CCL39 cells (data not shown) and could therefore play a role in MKP induction. Pretreatment of CCL39 cells with the specific p38MAPK inhibitor SB 203580 (49, 50) completely blocks the activation of p38MAPK (51). However, following inhibitor pretreatment, the ability of FCS both to stimulate p42/p44MAPK and to induce MKP-1 and MKP-2 was unimpaired (Fig. 5). Hence, activation of the p38MAPK signaling pathway is not a requirement for the serum-mediated induction of MKP-1 and MKP-2.

**MKP-1 and MKP-2 Are Constitutively Expressed in CCL39 Cells Expressing v-ras or MKK-1 (SD/SD)—** As both MKP-1 and MKP-2 are able to inactivate p42/p44MAPK and agents that activate MAP kinase also provoke the induction of MKP-1 and MKP-2, we hypothesized that p42/p44MAPK might regulate the induction of its own inhibitor. Activation of p42/p44MAPK requires the sequential activation of Ras, Raf-1, and MKK-1/2, with p42/p44MAPK being the last step in the kinase cascade. To examine the contribution of the MAP kinase cascade to the induction of MKP-1 and MKP-2, we analyzed CCL39 cells expressing constitutively active members of this pathway. In CCL39 cells expressing v-ras, p42/p44MAPK is constitutively active (Ref. 52 and data not shown), and MKP-2 is constitutively expressed, with MKP-1 present at a much lower level, but still detectable. Addition of serum results in the induction of MKP-1, with no appreciable change in MKP-2 (Fig. 6). In addition to the p42/p44MAPK cascade, Ras is also known to control signaling pathways such as those linked to phosphati-
Induction of MKP-1 and MKP-2 by MAP Kinase

**Figure 5.** Induction of MKP-1 and MKP-2 does not require p38MAPK activity. CCL39 cells were seeded in 12-well plates and rendered quiescent when confluent. Cells were then pretreated or not with the specific p38MAPK inhibitor SB 203580 (10 μM) for 18 h prior to addition of FCS (10%) for a further 3 h. Cells were then lysed; and p42/p44MAPK activity was determined following immunoprecipitation as described under “Experimental Procedures” (upper panel), or proteins (100 μg) were separated by 10% SDS-PAGE and Western-blotted with anti-MKP-1/2 antisemur as described in the legend to Fig. 1 (lower panel).

**Figure 6.** MKP-1 and MKP-2 are constitutively expressed in CCL39 cells expressing v-ras or MKK-1(SD/SD). Parental CCL39 cells or CCL39 cells expressing and transformed by either v-ras or a constitutively active MKK-1 mutant (MKK-1(SD/SD)) were seeded in 12-well plates and serum-deprived when quiescent. Cells were then stimulated with FCS (20%) for the times indicated or not (NS) prior to cell lysis. Proteins (100 μg) were separated by 10% SDS-PAGE and Western-blotted with anti-MKP-1/2 antisemur as described in the legend to Fig. 1. A Western blot is shown that is representative of four such blots performed.

**Figure 7.** Activation of the Raf-1/MKK/MAP kinase module is sufficient to induce MKP-1 and MKP-2. CCL39 cells transfected with and expressing an estrogen-inducible Raf-1 construct (ΔRaf1::ER cells) were seeded in 12-well plates and rendered quiescent when confluent. Cells were then stimulated with 5% FCS, 1 μM estradiol (Est), or the two together for 3 or 5 h. Cells were lysed, and proteins (100 μg) were separated by 10% SDS-PAGE and Western-blotted with anti-MKP-1/2 antisemur as described in the legend to Fig. 1. Data are from a single experiment that was performed two times.

FCS - + - +
SB 203580 - - + +
MKP-1 - + + +
MKP-2 - + - +
Est - + + +

**Table 1.** Interaction of MAP Kinase Activation and Protein Kinase C Activity

| v-ras | MKK-1(SD/SD) | CCL39 |
|-------|-------------|-------|
| 15    | NS 180      | NS 180|

**Figure 5.** The transformation of fibroblasts by the ectopic expression of oncogenic proteins may result in the secretion of growth factors or hormones, which may then stimulate the cell population in an autocrine/paracrine manner. Indeed, conditioned serum-free medium from v-ras-transformed, but not wild-type, CCL39 cells can modify signal transduction pathways when added back to wild-type CCL39 cells (data not shown). Hence, the constitutive expression of MKP-1/2 in v-ras- or MKK-1(SD/SD)-transformed CCL39 cells may not be due solely to the constitutive activation of the p42/p44MAPK cascade, but may involve additional signaling pathways. To rigorously assess the role of the p42/p44MAPK cascade alone in the regulation of MKP induction, we used CCL39 cells expressing an inducible member of the MAP kinase signaling cascade, ΔRaf1::ER (42, 55). This chimeraic protein has been shown to be conditionally activable by exposure to the estrogen analogue estradiol and to be able to specifically activate p42MKK1 and p42/p44MAPK with no interference with stress MAP kinase pathways (55). In ΔRaf1::ER cells, estradiol addition potently activates p42/p44MAPK (Ref. 42 and data not shown) and promotes MKP-1 and MKP-2 expression (Fig. 7), whereas in parental cells, estradiol alone has no effect on p42/p44MAPK activities and MKP-1/2 expression (data not shown). The ability of the ΔRaf1::ER construct to induce MKP-1 and more markedly MKP-2 is augmented in the presence of serum. Hence, activation of Raf-1, MKK-1, and, by consequence, p42/p44MAPK is sufficient to induce expression of MKP-1 and MKP-2.

Serum-mediated Induction of MKP-1 and MKP-2 Requires MKK-1/2 Activity—The experiments above (Fig. 7) show that activation of Raf-1 is sufficient to induce MKP-1 and MKP-2. To determine whether activation of p42/p44MAPK is necessary for the serum-mediated induction of MKP-1 and MKP-2, we employed a specific inhibitor of MMK-1/2, PD 098059 (56, 57). In the presence of PD 098059 (10 μM), the ability of serum to stimulate p42MAPK is inhibited by 70%, and the induction of MKP-1 and MKP-2 is significantly, although not completely attenuated (Fig. 8). Higher concentrations of PD 098059 are able to completely block the activation of p42/p44MAPK and the induction of MKP-1 and MKP-2, but also inhibit protein synthesis. However, this inhibitor, when used at a concentration of 10 μM, allows us to conclude that p42/p44MAPK are required for full serum-mediated induction of MKP proteins in CCL39 cells.

MKP-1 and MKP-2 Are Differentially Induced by Protein Kinase C- and cAMP-elevating Agents—Addition of the tumor-promoting agent PMA to fibroblasts leads to the stimulation of protein kinase C and p42/p44MAPK, but not p38MAPK and p46/p44JNK. When CCL39 cells are treated with PMA, MKP-1, but not MKP-2, is transiently induced, with maximal induction evident 1 h after addition of agonist (Fig. 9A, upper panel). To determine whether protein kinase C pathways are also associated with the serum-dependent induction of MKP-1/2, we pretreated quiescent cells with a specific inhibitor of protein kinase C enzymes, GF 109203X (58), and followed the serum-mediated induction of MKP-1 and MKP-2. In addition to attenuating the ability of PMA to induce MKP-1 (Fig. 9B), the serum-mediated induction of MKP-1 and MKP-2 is also reduced by pretreatment with GF 109203X. In addition to an AP-1 element present in the MKP-1 promoter, two cyclic AMP-responsive elements (CRE) are evident. Sustained activation of protein kinase A in CCL39 cells leads to the induction of MKP-1, with MKP-2 undetectable over a 3-h time course. Maximal induction of MKP-1 is evident after 1 h of agonist addition. The level of induction of MKP-1 is significantly lower than that elicited by serum (Fig. 9A, lower panel). As activation of protein kinase A does not stimulate the p42/p44MAPK pathway in CCL39 cells, this suggests that although full induction of MKP-1 and MKP-2 requires p42/p44MAPK activation, p42/p44MAPK activity is not an absolute requirement.

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FIG. 8. Serum-mediated induction of MKP-1 and MKP-2 requires p42/p44MAPK activity. CCL39 cells were seeded in 12-well plates and rendered quiescent when confluent. Cells were then stimulated or not with FCS (10%) in the presence or absence of the MKP-1 inhibitor PD 098059 (10 µM), which was added 30 min prior to FCS addition. Cells were lysed; and p42MAPK activity was determined by a mobility shift assay (upper panel), or proteins (100 µg) were separated by 10% SDS-PAGE and Western-blotted with anti-MKP-1/2 antisera as described in the legend to Fig. 1 (lower panel). Western blots representative of three performed that gave qualitatively identical results are shown.

DISCUSSION

Dual phosphorylated p42/p44MAPK is an excellent substrate for the MKP family of dual specificity phosphatases in vitro (9), and all MKP family members tested have been shown to inactivate p42/p44MAPK in vivo. At least eight MKPs are known to exist in mammalian systems (see the Introduction), which, together with the identification of additional MAP kinase family members, reflects signaling complexity (59).

MKPs, with the exception of Pyst1 (23), are the product of immediate-early genes and, based on Northern analysis, share overlapping tissue distributions (17, 46). Purified MKPs are constitutively active (15, 60, 61). Although this does not rule out the possibility that post-translational regulation of MKP activity occurs, it suggests that the principal point of MKP regulation is at the level of transcription. Hence, the specificity of interaction between MAP kinase and MKP family members may depend on the specific induction of one or more MKP. A detailed analysis of the factors required for induction of MKPs would increase our understanding of their physiological role.

We show that MKP-1 and MKP-2 are expressed in the well-established CCL39 fibroblast cell line (Fig. 1). Both phosphatases are induced by serum, albeit with a different time course of induction, suggesting that the mechanisms involved in their induction may not be identical (Fig. 2). Induction of MKP-1 and, to a lesser extent, MKP-2 correlates with an attenuation of p44MAPK activity. It is therefore possible that p44MAPK is specifically targeted by MKP-1 in CCL39 cells. However, it should be noted that in contrast to NIH3T3 fibroblasts (8) and Rat-1 cells (data not shown), where there is very little inactivation of p42/p44MAPK in the presence of cycloheximide, in CCL39 cells, ~50% of the p42/p44MAPK inactivating activity is insensitive to cycloheximide and hence does not involve the majority of MKP family members. Overexpression of either MKP-1 or MKP-2 completely blocks the activity of p44MAPK and two members of the stress kinase family, p38MAPK and p46JNK. Thus, both MKP-1 and MKP-2 are potent inhibitors of CCL39 cell cycle reentry (Fig. 3). We cannot conclude that this block is entirely due to p42/p44MAPK inhibition or is a result of inhibition of p42/p44MAPK stress kinases, and some of the more recently identified MAP kinase family members. However, it is important to note that overexpression of MKP-1 does not result in a complete loss of substrate specificity as we have previously shown that activation of p70S6K occurs normally in cells that overexpress MKP-1 (42). Our results are not in total agreement with those reported by Chu et al. (62), who have demonstrated by a similar transient transfection technique of several cell types that while MKP-1 dephosphorylates p42MAPK, p38MAPK, and p54JNK, MKP-2 is more discriminating and will dephosphorylate p42MAPK and p54JNK, but not p38MAPK. This discrepancy may arise from differences in the expression levels of each phosphatase.

To define specificity between endogenous MAP kinases and MKPs, we examined the ability of a range of agonists known to stimulate either the mitogenic p42/p44MAPK pathway or the stress kinase pathways to induce MKP-1 or MKP-2. In apparent contradiction to previous reports based on Northern blot analysis (63), we found that none of the stress agents tested, IL-1β, osmotic shock, or sodium arsenite, were capable of inducing MKP-1 or MKP-2 to detectable levels. However, with the exception of IL-1β, all of these agents are potent inhibitors of protein synthesis in CCL39 cells. Hence, it is not surprising that we were unable to detect protein expression of two immediate-early genes. In addition, protein synthesis inhibition has been shown to up-regulate immediate-early gene mRNA induction, most probably through an increase in mRNA stability (64). Thus, an analysis of MKP mRNA induction is responsive to stress agents may be difficult to interpret. Stimulation of CCL39 stress pathways with the above agonists, in particular IL-1β, resulted in only a transient activation of both p46/p44JNK and p38MAPK (data not shown). As we were unable to detect induction of MKP-1 or MKP-2 in response to any stress agonists tested, we conclude that neither phosphatase is involved in the inactivation of stress kinases following addition of these agonists alone (Fig. 4). Unfortunately, it is not possible to block production of MKP family members with protein synthesis inhibitors and then follow a time course of stress kinase activity in response to agonist stimulation, as an inhibition of protein synthesis by itself activates stress kinases (Ref. 65 and data not shown). The inhibition of p38MAPK failed to modify serum-stimulated induction of MKP-1 and MKP-2, demonstrating that activation of this stress kinase is not a require-
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Finally, we have shown that MKP-1 and MKP-2 are principally induced in CCL39 cells by the MAP kinase cascade, but maximal induction involves the interplay of at least one additional signaling pathway. However, we have been unable to address whether the endogenous level of expression of MKP-1 or MKP-2 is sufficient to dephosphorylate p42/p44MAPK and whether p42/p44MAPK is preferentially dephosphorylated by endogenous MKP-1 or MKP-2. In an attempt to address the latter question, we are adopting an antisense strategy to specifically target and prevent the induction of endogenous MKP-1 and MKP-2. Current research in this laboratory is aimed at developing cell models in which MKPs are maximally inducible in the absence of activation of the MAP kinase pathway, which may allow us to answer the former question.

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In yeast Schizosaccharomyces pombe, the MAP kinase homologue Spc1p is activated in response to osmotic stress and is inactivated by its cognate phosphatase, Pyp2 (74). Interestingly, Pyp2 is also transcriptionally induced by osmotic stress, indicating that a Spc1-Pyp2 negative feedback loop exists (41). In agreement with this model, we suggest that p42/p44MAPK down-regulation may depend on a similar negative feedback loop involving MAPK family members.
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