ERK1/2 Achieves Sustained Activation by Stimulating MAPK Phosphatase-1 Degradation via the Ubiquitin-Proteasome Pathway*

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Sustained extracellular signal-regulated kinase 1/2 (ERK1/2) activation does not always correlate with its upstream Ras-Raf-mitogen-activated protein kinase kinase 1/2 (MKK1/2) signal cascade in cancer cells, and the mechanism remains elusive. Here we report a novel mechanism by which sustained ERK1/2 activation is established. We demonstrate that Pb(II), a carcinogenic metal, persistently induces ERK1/2 activity in CL3 human lung cancer cells and that Ras-Raf-MKK1/2 signaling cannot fully account for such activation. It is intriguing that Pb(II) treatment reduces mitogen-activated protein kinase phosphatase 1 (MKP-1) protein levels in time- and dose-dependent manners, which correlates with sustained ERK1/2 activation, and that Pb(II) also induces mRNA and de novo protein synthesis of MKP-1. In Pb(II)-treated cells, MKP-1 is polyubiquitinated, and proteasome inhibitors markedly alleviate the ubiquitination and degradation of MKP-1. Inhibiting the Pb(II)-induced ERK1/2 activation by PD98059 greatly suppresses MKP-1 ubiquitination and degradation. It is remarkable that constitutive activation of MKK1/2 triggers endogenous MKP-1 ubiquitination and degradation in various mammalian cell lines. Furthermore, expression of functional MKP-1 decreases ERK1/2 activation and the c-Fos protein level and enhances cytotoxicity under Pb(II) exposure. Taken together, these results demonstrate that activated ERK1/2 can trigger MKP-1 degradation via the ubiquitin-proteasome pathway, thus facilitating long-term activation of ERK1/2 against cytotoxicity.

Members of the family of mitogen-activated protein kinase (MAPK)† proteins are vital intracellular signaling components that become phosphorylated and activated in response to a wide diversity of extracellular stimuli, including growth factors, cytokines, and environmental stresses (reviewed in Refs. 1–5). MAPKs are activated through a three-kinase module composed of a MAPK, a MAPK kinase (MKK), and a MKK kinase (MKKK). These MAPK modules are connected to cell surface receptors and activated via interaction with a family of small GTPases and MKKK kinases. Activated MAPKs phosphorylate many substrates, including cytoskeletal proteins, other kinases, phosphatases, enzymes, and transcription factors, thereby orchestrating several cellular alterations including proliferation, differentiation, survival, and apoptosis. The duration and strength of MAPK activation also affects these biological outcomes. Three major MAPK subfamilies have been extensively studied, i.e. the extracellular signal-regulated kinases (ERK1/2), the c-Jun N-terminal kinases (JNKs), and the p38 kinases. Activation of a particular MAPK signal must be controlled with high specificity and efficiency to achieve precise physiological regulation. The recent discovery of specific docking sites among the members of the MAPK cascades provide a mechanism that explains how specific and efficient signaling is established. For instance, a cluster of positively charged amino acids followed by an LXL motif called the D domain (or the kinase interaction motif, KIM) has been identified in MKKs, MAPK phosphatases (MKPs), and several MAPK substrates (6–8). The D domain binds specifically to an acidic domain (common docking domain) within a docking groove of MAPKs (6–8). Another docking site found in many ERK substrates is called the DEF motif (docking site for ERK, EFXP) (8, 9). These docking interactions facilitate phosphorylation of substrates by MAPKs on specific Ser or Thr residues followed by a Pro residue (S/T/P) sites.

The small GTPase, MKKK, and MKK in the ERK pathway are known to be Ras, Raf, and MKK1/2, respectively (1–5). Activation of ERK1/2 requires a dual-phosphorylation by MKK1/2 on the Thr and Tyr residues of TEY sites within the activation loop, whereas dephosphorylation of these residues by MKPs terminates such activation (10–15). Among these MKPs, MKP-1, MKP-2, and MKP-3 are prototypes whose functional associations with MAPK signals have been well characterized in several aspects. The MKP-3 is localized predominantly in the cytosol, whereas the MKP-1 and MKP-2 are localized primarily in the nuclear compartment. mkp-1 and mkp-2 are immediate early genes rapidly induced by many of the stimuli that activate MAPKs, whereas mkp-3 is not an immediate early gene. The phosphatase activity of MKPs is markedly elevated by specific interaction with MAPKs. For example, the association of ERK2 with MKP-3 is highly specific, and the complex formation results in a dramatic enhancement of MKP-3 phosphatase activity to down-regulate ERK2 activity in the cytosol (14). MKP-1 selectively associates with ERK1/2, JNK1, and p38α, which results in the catalytic activation of MKP-1 and the subsequent inactivation of MKAPs in the nucleus (15). Similarly, the catalytic activity of MKP-2 is greatly elevated upon binding with ERK1/2 and JNK1 (16). These findings indicate that MKP's feedback inhibit their activating MAPks precisely. On the other hand, MKP-1 has been
reported to be a labile protein whose stabilization can be enhanced by proteasome inhibitors or glucocorticoids (17–19). Furthermore, ERK1/2 is required for the phosphorylation of MKP-1 on the two extreme C-terminal Ser residues, which enhances MKP-1 stabilization without affecting its phosphatase activity (17). This suggests that ERK1/2 may negatively control its own activity by triggering the stabilization of MKP-1.

Sustained ERK1/2 activity is strongly associated with many types of cancers occurring in pancreas, colon, lung, ovary, prostate, and kidney. This may be attributable to elevated Ras-Raf-MKK1/2 signaling (20–22). Nonetheless, constitutive activation of ERK1/2 has been reported to be independent of MKK1/2 and Raf-1 (23, 24), and the mechanism by which sustained ERK1/2 activation occurs remains elusive. We recently reported that Pb(II) persistently stimulates ERK1/2 without activating JNK, p38, and ERK5 in CL3 human lung cancer cells and normal human fibroblasts, and sustained ERK1/2 signaling is essential for cellular nucleotide excision repair synthesis, anti-cytotoxicity, and anti-mutagenesis (25). In this study, we investigate the roles of activators and regulators in controlling the duration and strength of ERK1/2 activation using Pb(II) as a stimulus. Our results show for the first time that ERK1/2 signaling can trigger MKP-1 degradation via the ubiquitin-proteasome pathway, thereby accomplishing sustained kinase activation. We further demonstrate that Pb(II) triggers not only MKP-1 degradation but also MKP-1 expression at transcriptional and translational levels. The results presented here indicate that ERK1/2 is capable of performing dual and opposing roles in controlling its own activity through up- and down-regulation of MKP-1, thereby determining the duration and strength of kinase activation.

EXPERIMENTAL PROCEDURES

Materials—Lead acetate was purchased from Merck. N-acetyl-Leu-Leu-norleucinal (ALLN), MG132, and PD98059 were obtained from Calbiochem-Novabiochem, cycloheximide came from Sigma, and cell culture media were from Invitrogen. Lead acetate was dissolved in MilliQ-purified water (Millipore, Bedford, MA), and all the other compounds were dissolved in Me2SO. Plasmids containing a full-length wild-type MKP-1 (pSG5-MKP-1-Myc) or a phosphatase-inactive mutant (Cys258 to Ser) of MKP-1 (pSG5-CS-MKP-1-Myc; MKP-1-CS) (26), were kindly provided by Dr. N. K. Tonks (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Plasmids containing a constitutively active form of MKK1 (ΔN3/S218E/S222D; MKK1-CA) (27) or MKK2 (ΔN4/S222E/S226D; MKK2-CA) (28) were gifts from Dr. N. G. Ahn (University of Colorado, Boulder). Dominant negative mutants RasN17 and RasF301 were described previously (29, 30). The antibodies specific against phospho-ERK1/2-Thr202/Tyr204 (catalog number 9101) and phospho-MKK1/2/Ser353/355 (catalog number 9121) were from Cell Signaling (Beverly, MA). The rabbit polyclonal antibodies against ERK2 (C-14 or K-23), MKK1 (12-B), MKP-1 (V-15), ubiquitin (P4D1), c-Fos (sc-52), and α-tubulin (TU-02) were from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal antibody against Myc (MSA-110) was from StressGen Biotechnologies (Victoria, British Columbia, Canada).

Cell Culture—The CL3 cell line established from a non-small cell lung carcinoma tumor and the Chinese hamster ovary cell line CHO-K1 was cultured in RPMI 1640 and F12/Dulbecco’s modified Eagle’s medium complete media, respectively. The human diploid fibroblast line HFW and the human embryonic kidney cell line H293 were cultured in Dulbecco’s modified Eagle’s medium complete media. The complete media were supplemented with sodium bicarbonate (2.2%, w/v), t-glutamine (0.03%, w/v), penicillin (100 units/ml), streptomycin (100 μg/ml), and fetal calf serum (10%). CL3 and CHO-K1 cells were maintained at 37 °C in a humidified incubator containing 5% CO2 in air, whereas HFW and H293 cells were cultured in a 10% CO2 incubator at 30 °C.

Treatment—Cells in exponential growth were plated before serum starvation for 16–18 h. Serum-starved cells were then exposed to lead acetate for 15 min to 24 h in serum-free media. In experiments to determine the effect of de novo protein synthesis, cells were exposed to Pb(II) for 12 h, and cycloheximide (10 μg/ml) was added during the final 1–3 h. To determine the effect of MKK1/2 on MKP-1 and ERK1/2 levels, PD98059 (25 μM) was added 1 h before the addition of Pb(II) for 24 h. To determine the effect of proteasome-mediated proteolysis, ALLN (10 μM) and MG132 (25 μM) were added in the final 3 h during treatment of cells with Pb(II) for 24 h.

Transfection—Cells (5 × 105) were plated in a 60-mm Petri dish 1 day before transfection. Plasmids were carried by liposome and transfected into CL3 cells using GenePORTER™ (Gene Therapy Systems, San Diego, CA). After 24 and 48 h, the cells were exposed to Pb(II) for 24 h and 30 min in serum-free media, respectively. The cells were then subjected to whole cell extract preparation and a colony-forming ability assay.

Colony-forming Ability Assay—Immediately after treatment, cells were washed with phosphate-buffered saline (PBS) and trypsinized for determination of cell numbers. The cells were plated at a density of 100–200 cells per 60-mm Petri dish in triplicate for each treatment. The cells were then cultured for 7–14 days, and cell colonies were stained with 1% crystal violet solution (in 30% ethanol).

Western Blot Analysis—After treatment, cells were rinsed twice with cold PBS and lysed in a whole cell extract buffer (20 mM HEPES at pH 7.6, 75 mM NaCl, 2.5 mM MgCl2, 0.1 mM EDTA, 0.1% Triton X-100, 0.1 mM Na3VO4, 50 mM NaF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin and 1 mM 4-2-aminoethylbenzenesulfonyl fluoride). The cell lysate was rotated at 4 °C for 30 min and then centrifuged at 10,000 rpm for 15 min. After that, the precipitates were discarded. The BCA protein assay kit (Pierce) was employed to determine protein concentrations using bovine serum albumin as a standard. Equal amounts of proteins from each set of experiments were subjected to Western blot analysis as described (31). Antibodies were stripped from polyvinylidene difluoride membranes using a solution containing 2% SDS, 62.5 mM Tris-HCl, pH 6.8, and 0.7% (w/v) β-mercaptoethanol at 50 °C for 15 min before re-probing with another primary antibody. Relative protein blot intensities were determined using a computing densitometer equipped with the ImageQuant analysis program (Amersham Biosciences).

Immunoprecipitation—After treatment, cells were washed twice with ice-cold PBS and harvested at 4 °C in an immunoprecipitation lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, 10% glycerol, 1 mM NaF, 1 mM Na3VO4, 1 mM dithiothreitol, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 μg/ml pepstatin). Equal amounts of proteins were immunoprecipitated using anti-ERK2, anti-MKP-1, and anti-ubiquitin antibodies and collected with protein A-Sepharose beads at 4 °C for 1 h. The immunoprecipitate was then washed three times in a cold lysis buffer and subjected to Western blot analysis and an ERK kinase assay.

ERK Kinase Assay—The anti-ERK2 immunoprecipitate was washed in a kinase reaction buffer (20 mM HEPES, pH 7.6, 20 mM MgCl2, 2 mM dithiothreitol, 0.1 mM Na3VO4, and 1 mM NaF). The kinase assay was carried out in a total volume of 30 μl of a kinase reaction buffer containing 20 μM ATP, 1 μCi of [γ-32P]ATP (6000 Ci/mmol), and 0.5 μg of myelin basic protein (MBP) at 30 °C for 30 min. Phosphorylated MBP was resolved on 12% SDS-polyacrylamide gels followed by autoradiography.

Northern Blot Analysis—Total RNA (30 μg), isolated via a guanidium isothiocyanate/phenol/chloroform extraction procedure, was subjected to electrophoresis in a 1% agarose-2.2% formaldehyde gel, transferred to nylon membranes, and hybridized to 32P-labeled DNA probes that were prepared using T4 kinase end-labeling. The synthetic human mkp-1 probe sequence used was 5′-AGGGCGCGAGCAAAAAGAAACC-3′. Hybridization was performed at 42 °C for 3 h in a solution containing 50% formamide, 6× SSC (1× SSC contains 180 mM NaCl and 10 mM sodium citrate, pH 8.0), 5× Denhardt’s reagent, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA. Membranes were washed with 2× SSC/0.1% SDS for 5 min at room temperature and 0.1× SSC/0.1% SDS for 5 min at 42 °C for 5 min and then exposed to x-ray film.

RESULTS

Ras-Raf-MKK1/2 Signaling Is More Important for the Early than the Persistent ERK1/2 Activation by Pb(II)—CL3 cells were exposed to lead acetate (30 μM) in serum free medium for 15 min to 24 h, and the activation of MKK1/2 and ERK1/2 was determined by Western blot analysis using antibodies specific to phospho-MKK1/2 and phospho-ERK1/2. Fig. 1A shows that Pb(II) increased the phospho-ERK1/2 protein levels in CL3 cells in a time-dependent manner, e.g. exposure for 15 min and
24 h yielded increases of 1.7- and 4.4-fold, respectively, over untreated controls. Pb(II) also increased phospho-MKK1/2 protein levels in CL3 cells with the maximum level of induced phospho-MKK1/2 (2.0-fold) observed at an exposure time of 15 min (Fig. 1A). Reprobing these blots with anti-ERK2 and anti-MKK1 antibodies showed similar endogenous ERK2 and MKK1 protein levels in each cell extract, respectively (Fig. 1A). These results indicate that Pb(II) elicits different kinetics for the activation of ERK1/2 and their upstream MKK1/2 signals.

To investigate whether Pb(II) elicits MKK1/2-ERK1/2 activation via the Ras-Raf pathway, CL3 cells were transfected with plasmids containing dominant negative forms of RasN17 or Raf301. The cells were then allowed expression for 48 and 24 h before exposure to 100 μM Pb(II) for 30 min and 24 h, respectively. ERK activity was determined by immunoprecipitation and kinase assay using MBP as a substrate. As shown in Fig. 1B, the expression of RasN17 or Raf301 completely blocked the induction of ERK kinase activity in cells exposed to Pb(II) for 30 min. However, only partial suppression of the Pb(II)-elicited ERK kinase activity was observed when these RasN17- or Raf301-transfected cells were exposed to Pb(II) for 24 h (Fig. 1C). These results imply that Ras-Raf-MKK1/2 signaling is essential for early activation of ERK1/2 but cannot fully account for the persistent activation of ERK1/2 elicited by Pb(II).

Persistent ERK1/2 Activation by Pb(II) Is Accompanied by Decreased MKP-1 Protein Levels—The activation of ERK1/2 kinase activity is mainly a kinetic control between their activating kinases and inactivating phosphatases. The dual specific phosphatase MKP-1 is known to down-regulate ERK activity in the nucleus (10–13). To determine whether constitutive activation of ERK1/2 by Pb(II) is associated with mechanisms involved in down-regulation of MKP-1, we examined MKP-1 protein levels in CL3 cells exposed to 100 μM Pb(II) for 1–24 h. As shown in Fig. 2A, MKP-1 protein levels were slightly elevated during Pb(II) exposure for 1–6 h, whereas MKP-1

![Fig. 1](image1.png)

**Fig. 1.** Ras-Raf-MKK1/2 signaling is more important for early activation rather than persistent activation of ERK1/2 induced by Pb(II). A, CL3 cells were treated with 30 μM Pb(II) in a serum-free medium for the indicated times or left untreated. ERK1/2 and MKK1/2 activations in whole cell extracts were examined using phospho-specific antibodies. The relative activities shown under the blots were determined by densitometric analyses calculated by averaging the results of four independent experiments normalized by arbitrarily setting control cell densitometrics to 1. Western blot analyses of ERK2 and MKK1 protein levels in the same extracts are shown in the bottom panels. B and C, CL3 cells were transfected with RasN17 or Raf301 vectors. The cells were then allowed expression for 48 and 24 h before exposure to 100 μM Pb(II) for 30 min (B) and 24 h (C), respectively. ERK activity was determined by immunoprecipitation and kinase assay using MBP as a substrate. Relative intensities were calculated by averaging the results of four transfection experiments.

![Fig. 2](image2.png)

**Fig. 2.** Persistent activation of ERK1/2 by Pb(II) is accompanied by decreased MKP-1 protein levels. A, CL3 cells were treated with 100 μM Pb(II) in serum-free media for the indicated times. B, cells were exposed to various concentrations of Pb(II) for 24 h. C, after exposure to 30 μM Pb(II) for 24 h, the cells were washed with PBS and kept cultured in serum-free medium for 0–8 h. The cells were then extracted to determine MKP-1 protein levels using Western blot analysis or subjected to ERK kinase assay using MBP as a substrate. After resolving MKP-1, the anti-MKP-1 antibody was striped, and the α-tubulin in the same blots was detected using a specific antibody to serve as an internal control. The relative MKP-1 protein levels and ERK kinase activity were calculated by averaging the results of at least four independent experiments.
levels substantially decreased after longer exposure times (12–24 h). This time-dependent increase and decrease in MKP-1 protein levels was also observed in HFW human diploid fibroblasts (data not shown). Exposing CL3 cells to various doses of Pb(II) for 24 h also led to down-regulation of MKP-1 in a dose-dependent manner (Fig. 2B). Moreover, the reduced MKP-1 levels had not recovered 6 h after removal of the Pb(II) (Fig. 2C). Fig. 2, B and C also show that lowered MKP-1 levels were accompanied by increased ERK1/2 activities under Pb(II) exposure. These results indicate that down-regulation of MKP-1 protein levels by Pb(II) is strongly associated with the persistent activation of ERK1/2.

**MKP-1 Lowering by Pb(II) Is Not Associated with Down-regulation of Its mRNA or de Novo Protein Synthesis**—The long-term effect of Pb(II) in down-regulating MKP-1 suggests a decrease in mRNA expression/protein synthesis or protein stability. To assess whether Pb(II) affects the expression of mkp-1, mRNA was isolated and identified by Northern blotting. Fig. 3A shows that mkp-1 mRNA levels constantly increased during exposure to 100 μM Pb(II), indicating that reduced MKP-1 protein levels are not due to suppression at the transcriptional level. To explore whether Pb(II) exposure affects MKP-1 at the translational level, cycloheximide (10 μg/ml), an inhibitor of de novo protein synthesis, was added for the final 3 h of cell exposure to Pb(II) (100 μM) for 12 h. Cycloheximide did not lower MKP-1 protein levels in untreated cells (Fig. 3B), indicating that the endogenous MKP-1 protein is rather stable. However, cycloheximide co-treatment further decreased MKP-1 levels in cells treated with Pb(II) (Fig. 3B), indicating that new synthesis of MKP-1 does occur with Pb(II) exposure. These results suggest that down-regulation of MKP-1 protein levels is not due to decreases in its mRNA or protein synthesis.

**MKP-1 Lowering by Pb(II) Is Mediated through Ubiquitin-directed Proteolysis**—The 26 S proteasome machinery is known to be the major regulator of protein turnover in eukaryotic cells by which many labile proteins are targeted for degradation mediated through the covalent addition of polyubiquitin chains to lysine residues (32–34). To investigate the involvement of proteasome in the Pb(II) lowering of MKP-1 proteins, ALLN, a common 26 S proteasome inhibitor, was added at a concentration of 10 μM for the final 3 h of the treatment of CL3 cells with 100 μM Pb(II) for 24 h. As shown in Fig. 4A, ALLN restored the Pb(II)-decreased MKP-1 proteins to their untreated level. MG132, another proteasome inhibitor, also prevented the degradation of the MKP-1 protein caused by Pb(II) treatment (Fig. 4A). To determine whether MKP-1 proteins are modified by ubiquitination before degradation, cells were co-exposed to Pb(II) and ALLN as described above, and the MKP-1 protein in whole cell extract was immunoprecipitated and then subjected to immunoblot analysis using an anti-ubiquitin antibody. As shown in Fig. 4B, the endogenous MKP-1 was recognized by the antibody against ubiquitin, indicating that MKP-1 was modified by ubiquitin. Similar results were observed in immunoblot analysis using an anti-MKP-1 antibody to detect ubiquitinated proteins derived from anti-ubiquitin immunoprecipitates in Pb(II)-exposed cells (Fig. 4B). Taken together, these results indicate that Pb(II) triggers MKP-1 protein degradation via the ubiquitin-directed 26 S proteasome pathway in CL3 cells.

**Ubiquitin-directed Proteolysis of MKP-1 Is Dependent on ERK1/2 Signaling**—To determine whether Pb(II)-elicited ERK1/2 signaling is involved in controlling MKP-1 degradation, cells were pretreated with the MKK1/2 inhibitor PD98059 (25 μM) for 1 h before exposure to 100 μM Pb(II) for 24 h. The whole cell extract was isolated for determination of MKP-1 levels by Western blot analysis. As shown in Fig. 5A, PD98059 completely blocked Pb(II)-elicited ERK1/2 activation. Interestingly, PD98059 co-treatment restored the MKP-1 protein low-
The role of ERK1/2 signaling in triggering proteolysis of the MKP-1 protein was further examined by transient transfection of CL3 cells with a plasmid carrying MKK1-CA, a constitutively active form of MKK1. As shown in Fig. 6, the expression of MKK1-CA significantly increased MKP-1 ubiquitination and decreased the levels of this protein in CL3 cells. Ubiquitination of the endogenous MKP-1 protein by forced expression of MKK1-CA also occurred in H293 and CHO-K1 cells (Fig. 6B), and forced expression of MKK2-CA also induced MKP-1 ubiquitination and degradation in CL3, H293, and CHO-K1 cells (Fig. 6B, and data not shown). These results indicate that activation of MKK1/2/ERK1/2 signaling that elicits MKP-1 protein ubiquitination and degradation may be a widespread phenomenon.

Exogenous MKP-1 Phosphatase Activity Decreases ERK Phosphorylation and c-Fos Protein Level and Enhanced Cytotoxicity upon Pb(II) Exposure—To explore whether the phosphatase activity of MKP-1 is essential for dephosphorylation of ERK1/2 in CL3 cells, plasmids containing either wild-type MKP-1 or the catalytically inactive mutant MKP-1-CS were transfected into cells, allowed expression for 24 h, and then exposed to 100 μM Pb(II) for another 24 h. As Fig. 7 shows, forced expression of wild-type MKP-1 decreased the phospho-ERK1/2 levels induced by Pb(II), whereas the MKP-1-CS did not have such an effect. On the other hand, phospho-ERK1/2 levels in the untreated control cells were slightly reduced and enhanced by the expression of MKP-1 and MKP-1-CS, respectively (Fig. 7). Conversely, the over-expression of either wild-type MKP-1 or MKP-1-CS had no effect on MKK1/2 activation by Pb(II) (Fig. 7A). These results indicate that supplementing exogenous MKP-1 can dephosphorylate ERK1/2 in CL3 cells.

In an earlier study we demonstrated that Pb(II)-elicited ERK1/2 activity is required for the induction of c-Fos protein levels and the prevention of cytotoxicity (25). To investigate whether exogenous expression of MKP-1 affects c-Fos and cytotoxicity, CL3 cells were transfected with MKP-1, MKP-1-CS, or control vectors. The cells were then exposed to Pb(II) or left untreated as described above. The c-Fos levels were then measured by Western blot assay, and cytotoxicity was determined by a colony-forming ability assay. As shown in Fig. 8, the expression of MKP-1 but not MKP-1-CS in CL3 cells decreased the c-Fos protein levels and enhanced cytotoxicity caused by Pb(II). These results indicate that forced expression of functional MKP-1 dephosphorylates ERK1/2 and thereby decreases downstream c-Fos levels in Pb(II)-treated cells, which may contribute to Pb(II) cytotoxicity.

**DISCUSSION**

Sustained ERK1/2 activity is frequently observed in many types of cancers when they progress to more advanced stages and has been associated with high levels of upstream Ras-Raf-MKK1/2 (20–22). However other reports indicate that constitutive activation of ERK1/2 can be independent of MKK1/2 and Raf-1 (23, 24) and accompanied by low expression of MKP-1.
More recently, decreased expression of MKP-1 has been strongly associated with the advanced progression of human ovarian cancers (37). Yet, the regulatory mechanisms underlying sustained ERK1/2 activation and MKP-1 down-regulation have not been well characterized. In an earlier study we demonstrated that Pb(II) specifically stimulates sustained ERK1/2 without activating JNK, p38, and ERK5 (25). In the present study, we found sustained ERK1/2 activation to be correlated with proteolysis of MKP-1 in Pb(II)-treated cells. By using a MKK1/2 inhibitor, PD98059, we demonstrated that the ERK1/2 signal is vital for ubiquitination and proteolysis of MKP-1 stimulated by Pb(II). Moreover, forced expression of constitutively active MKK1/2 in several cells results in MKP-1 ubiquitination and proteolysis. Together, these results indicate that ERK1/2 signaling can trigger degradation of MKP-1 via the ubiquitin-proteasome pathway, thus facilitating sustained kinase activation for long periods (Fig. 9).

Upon mitogenic stimulation of quiescent cells, the ERK1/2 signal is rapidly activated, promoting cell cycle progression through the R point of the G1 phase by phosphorylation of downstream kinases such as ribosomal S5 kinase (RSK) and several immediate early gene products such as c-Fos (1–5). The mitogen-activated ERK also triggers expression of MKP-1 and MKP-2 (38), which may form an inhibitory feedback loop to fine tune the activity of ERK1/2 for G1 progression. In the present study, we also demonstrate that, like mitogens, Pb(II) can stimulate MKP-1 expression at transcriptional and translational levels even after a long exposure time, during which MKP-1 degradation occurs. Expression of a dominant active Ras or Raf substantially blocks the early activation of ERK1/2 induced by Pb(II). These results suggest that early activation of ERK1/2 by Pb(II) is dependent on Ras-Raf-MKK1/2 signaling and may enhance MKP-1 to dephosphorylate ERK1/2, resulting in net transient activation/inactivation of the ERK1/2 signal (Fig. 9). The foregoing suggests that ERK1/2 may play dual and opposing roles in controlling its own activity through up-

FIG. 7. Overexpression of MKP-1 in CL3 cells decreases ERK1/2 phosphorylation elicited by Pb(II). A, cells were transfected with 5 μg of Myc-tagged vectors containing wild-type MKP-1 or the catalytically inactive mutant MKP-1CS. The cells were then allowed for 24 h before exposure to 100 μM Pb(II) for 24 h or left unexposed. Whole cell extracts were subjected to Western blot analysis using specific antibodies against phospho-ERK1/2, ERK2, phospho-MKK1/2, MKK1, and Myc. B, quantitative analysis of results obtained from three independent experiments; bars represent the S.E. Student’s t test was used to compare cells expressing MKP-1 and pSG5 empty vectors under Pb(II) exposure (*, p < 0.05; **, p < 0.01).

FIG. 8. Overexpression of MKP-1 decreases c-Fos protein levels and enhances cytotoxicity in Pb(II)-exposed CL3 cells. A, MKP-1 or MKP-1CS was transiently transfected into cells that were then exposed to Pb(II) as described in the Fig. 7 legend. Whole cell extracts were subjected to Western blot analysis using specific antibodies against c-Fos and α-tubulin. The relative c-Fos levels shown were calculated by averaging the results of three experiments. B, after transfection and Pb(II) treatment, the cells were subjected to a colony-forming ability assay. Results were obtained from four independent experiments; bar represents the S.E. Student’s t test was used to compare cells expressing MKP-1 and pSG5 empty vectors under Pb(II) exposure (*, p < 0.05; **, p < 0.01).
Ser364, which enhances MKP-1 stabilization without affecting controls of ERK1/2 and MKP-1. Activation of ERK1/2 can either down-regulate MKP-1 via the ubiquitin-mediated proteasome degradation pathway or up-regulate MKP-1 by increasing gene expression, which correlates with the duration or strength of the activated kinase. Serum-activated ERK1/2 has been shown to increase MKP-1 expression and phosphorylate the extreme MKP-1 C-terminus, thereby elevating phosphatase activity and stabilizing MKP-1, which leads to de-phosphorylation of ERK1/2 and the net transient activation of ERK signaling. Overexpression of MKK1/2 can increase the duration or strength of ERK activation, which may phosphorylate MKP-1 at sites other than the two extreme C-terminal Ser residues and facilitate subsequent ubiquitination and degradation. By triggering MKP-1 down-regulation, ERK achieves sustained activation.

Moreover, transient activation of ERK1/2 can stimulate MKP-1 degradation in response to sustained ERK activation. This docking theory satisfactorily correlates with our current observation that sustained ERK1/2 activation by Pb(II) increases the stability of c-Fos, which appears to be antipodal to the capability of this activated kinase to trigger MKP-1 degradation. Overexpression of functional MKP-1 decreases the protein levels of phosphorylated-ERK1/2 and c-Fos and increases the cytotoxicity induced by Pb(II). This finding is consistent with the induction of MKP-1 expression by proteasome inhibitors resulting in inactivation of ERK1/2 and cell death (18). The results presented here suggest that degradation of MKP-1 mediated by sustained ERK1/2 may occur earlier than phosphorylation and stabilization of c-Fos to prevent cell death in response to Pb(II) stress.

IEX-1, a novel substrate for ERK1/2 that prolongs ERK1/2 activation through docking interaction with a DEF motif, was recently identified (45). IEX-1 is an early gene product induced by growth factors, cytokines, and various stress stimuli, and it may be located on the membranes of subcellular organelles. Upon docking interaction with ERK1/2, IEX-1 is phosphorylated on an (S/T)P site upstream from the DEF motif, which is required for its survival effect (45). Thus, IEX-1 functions as both a positive regulator of ERK1/2 activation and an ERK1/2 downstream mediator that prevents cell death. Whether IEX-1 also participates in the sustained ERK1/2 activation induced by Pb(II) deserves further exploration.

Several recent reports have indicated that ERK1/2 activity participates in proteolysis. ERK1/2 signaling is essential for phosphorylation and activation of microphthalmia, a melanocyte-specific transcription factor that regulates differentiation, and phosphorylation is also required for the subsequent proteasome-mediated degradation of microphthalmia (46). Proteolysis of microphthalmia induced by sustained ERK1/2 is accompanied by a decrease in MKP-1 expression, which triggers melanocyte de-differentiation and transformation (36). On the other hand, MKK1/2-ERK1/2 signaling stimulates proteasome-catalyzed processing of hHR23B (47), which functions in the initiation step of nucleotide excision repair (48). Moreover, forced expression of constitutively active MKK1/2 can up-regulate a proteasome-ζ-chain protein, suggesting that this signaling may have a general role in regulating the proteasome machinery (47).

It is of interest to discover the specific E3 ubiquitin ligase for MKP-1 degradation in response to sustained ERK activation. Intriguingly, MEKK1, an MKKK upstream of ERK1/2 and JNK, has recently been shown to possess an E3 ubiquitin ligase activity along with its known kinase activity (49). Overexpression of MEKK1 or hyperosmotic stress can elicit polyubiquitination and degradation of ERK1/2, but not JNK, via the proteasome pathway. ERK1/2 degradation induced by MEKK1

![Diagram](http://www.jbc.org/)

**Fig. 9.** Scheme depicting the dual and opposing feedback controls of ERK1/2 and MKP-1. Activation of ERK1/2 can either down-regulate MKP-1 via the ubiquitin-mediated proteasome degradation pathway or up-regulate MKP-1 by increasing gene expression, which correlates with the duration or strength of the activated kinase. Serum-activated ERK1/2 has been shown to increase MKP-1 expression and phosphorylate the extreme MKP-1 C-terminus, thereby elevating phosphatase activity and stabilizing MKP-1, which leads to de-phosphorylation of ERK1/2 and the net transient activation of ERK signaling. Overexpression of MKK1/2 can increase the duration or strength of ERK activation, which may phosphorylate MKP-1 at sites other than the two extreme C-terminal Ser residues and facilitate subsequent ubiquitination and degradation. By triggering MKP-1 down-regulation, ERK achieves sustained activation.
ERK1/2 Triggers MKP-1 Degradation

ERK1/2 relies on both the kinase and E3 ligase activities of MEKK1 and the physical association of MEKK1 with the CD domain of ERK1/2. This novel finding suggests that upstream MAPK signals can also act as an E3 ligase to negatively regulate downstream substrates.

In summary, we have demonstrated here for the first time that ERK1/2 can trigger MKP-1 degradation via the ubiquitin/proteasome pathway to achieve long-term ERK1/2 activation. This function is antipodal to previous reports indicating that transient ERK1/2 activation can increase the function of MKP-1 at the levels of gene expression, de novo protein synthesis, catalytic activation, and protein stabilization, which then attenuates ERK1/2 activation. The dual and opposing feedback roles of ERK1/2 in controlling MKP-1 are elicited in Pb(II)-treated cells. Sustained ERK1/2 induced by Pb(II) is accompanied by enhancement of c-Fos levels; also, overexpression of functional MKP-1 can suppress ERK1/2 signaling and c-Fos levels and enhance Pb(II) cytotoxicity. Results obtained in this study also imply that sustained activation of ERK1/2 could be a cause rather than a result of the decreased MKP-1 observed during advanced progression of human cancers.

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