Mitogen-Activated Protein Kinase Phosphatase-1 Is Required for Cisplatin Resistance

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Abstract

Mitogen-activated protein kinase (MAPK) phosphatase (MKP)-1 is a member of the MKP family that negatively regulates MAPK signaling. MKP-1 has been implicated in cell survival in response to stressful stimuli, including anticancer treatment, but its role in cisplatin resistance is not fully understood. Here, we show that cisplatin induces MKP-1 in several human cancer cell lines. Induction of MKP-1 by cisplatin was through the transcriptional mechanism regulated by extracellular signal-regulated kinase (ERK). Overexpression of MKP-1 rendered human lung cancer cells resistant to cisplatin. Conversely, down-regulation of MKP-1 by small interfering RNA silencing sensitized primary mouse embryonic fibroblasts (MEF) from MKP-1 knockout mice, whereas such change was minimal in MEFs, whereas such change was minimal in MEFs, whereas such change was minimal in MEF-1/+MEFs. More importantly, cisplatin-induced cell death is inhibited by blocking JNK but not ERK and p38 activities. Collectively, our results establish a critical role of JNK in cisplatin-induced apoptosis and suggest that MKP-1 is required for cisplatin resistance. (Cancer Res 2006; 66(17): 8870-7)

Introduction

Cis-diaminedichloroplatinum (cisplatin) is effective against several solid tumors, including lung and ovarian cancers. The mechanism underlying its anticancer activity is incompletely defined, but it is generally accepted that cisplatin is a DNA-damaging agent that forms cisplatin-DNA adducts that kill cells via several mechanisms, including induction of apoptosis (1). Apoptosis may be activated through the death receptor pathway via activation of caspase-8 or through the mitochondrial pathway via activation of caspase-9 (2). Both pathways trigger activation of a set of cysteine proteases called caspases, leading to a caspase cascade and apoptosis (2). It has been shown that cisplatin can activate the mitogen-activated protein kinase (MAPK) pathways, leading to apoptotic cell death (1).

The MAPK signal pathway mainly consists of three subfamilies: the stress-activated protein kinase (JNK)/stress-activated protein kinase, the p38 MAPK, and the extracellular signal-regulated kinase (ERK). MAPK activities are regulated by two opposing events (i.e., phosphorylation and dephosphorylation). On one hand, MAPKs are activated through the reversible phosphorylation of both threonine and tyrosine residues of the TXY motif in the catalytic domain by upstream dual-specificity kinases called MAPK kinases (MKK). These upstream MKKs include MKK1/2, MKK3/6, and MKK4/7. MKK3/6 and MKK4/7 activate p38 and JNK, respectively, whereas MKK1/2 activate ERK (3–5). Once activated, MAPKs can phosphorylate several cellular substrates [e.g., c-Jun and cyclic AMP–responsive element binding protein (CREB)] that can trigger diverse signal cascades, leading to several cellular responses, including apoptosis. It is believed that activation of ERK favors cell proliferation, whereas activation of JNK and p38 causes cell death. On the other hand, MAPKs can be inactivated through dephosphorylation by members of the MAPK phosphatase (MKP) family.

The MKPs are a family of dual-specificity protein phosphatases that include MKP-1, MKP-2, MKP-3, MKP-4, MKP-5, VHR, PAC1, hVH2, hVH3, Pyst1, and Pyst2 (6). These phosphatases can dephosphorylate both phosphorylated threonine and phosphorylated tyrosine residues and inactivate MAPK signaling (6). MKP-1 was the first member of this family to be identified as a MKP. MKP-1 was originally cloned as a growth factor-inducible gene implicated in the G0-G1 transition (7, 8). It has been shown that MKP-1 can be induced by stresses (9–11). It has also been shown that MKP-1 can inactivate all three major MAPKs, including ERK, JNK, and p38 (10, 12–14). Because JNK, p38, and ERK are capable of inducing either apoptosis or cell proliferation, MKP-1 is believed to be involved in regulating the cell cycle (15–18) or apoptosis (19, 20). Consistent with this notion, a recent study suggested that MKP-1 could protect cells from anisomycin-induced apoptosis (21).

In this article, we report that MKP-1 is an important determinant of cisplatin resistance. We found that cisplatin induces MKP-1, which correlated with inactivation of JNK. Overexpression of MKP-1 protected human lung cancer cells from cisplatin-induced death. Importantly, knockdown of MKP-1 by small interfering RNA (siRNA) silencing sensitized human lung cancer cells to cisplatin-induced death. In addition, we found that mouse embryonic fibroblasts (MEF) from MKP-1 knockout mice were more sensitive than MEFs from wild-type mice to cisplatin-induced cell death and that this sensitization was due to activation of the caspase-mediated apoptotic pathway. Moreover, blocking JNK, but not ERK and p38 activities, could protect MKP-1 knockout cells from cisplatin-induced death, suggesting that the JNK-mediated apoptotic pathway may be critical for cisplatin-induced cell killing. Taken together, our results suggest that MKP-1 is required for cisplatin resistance.

Materials and Methods

Reagents. Cisplatin and actinomycin D were purchased from Sigma (St. Louis, MO). Rabbit polyclonal anti-human MKP-1 (C-19) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Small interfering RNA (siRNA) silencing sensitized human lung cancer cell lines to cisplatin. Conversely, down-regulation of MKP-1 by small interfering RNA silencing sensitized human lung cancer cell lines to cisplatin-induced cell death. In addition, we found that MKP-1 can inactivate all three major MAPKs, including ERK, JNK, and p38 (10, 12–14). Because JNK, p38, and ERK are capable of inducing either apoptosis or cell proliferation, MKP-1 is believed to be involved in regulating the cell cycle (15–18) or apoptosis (19, 20). Consistent with this notion, a recent study suggested that MKP-1 could protect cells from anisomycin-induced apoptosis (21).

In this article, we report that MKP-1 is an important determinant of cisplatin resistance. We found that cisplatin induces MKP-1, which correlated with inactivation of JNK. Overexpression of MKP-1 protected human lung cancer cells from cisplatin-induced death. Importantly, knockdown of MKP-1 by small interfering RNA (siRNA) silencing sensitized human lung cancer cells to cisplatin-induced death. In addition, we found that mouse embryonic fibroblasts (MEF) from MKP-1 knockout mice were more sensitive than MEFs from wild-type mice to cisplatin-induced cell death and that this sensitization was due to activation of the caspase-mediated apoptotic pathway. Moreover, blocking JNK, but not ERK and p38 activities, could protect MKP-1 knockout cells from cisplatin-induced death, suggesting that the JNK-mediated apoptotic pathway may be critical for cisplatin-induced cell killing. Taken together, our results suggest that MKP-1 is required for cisplatin resistance.
purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies against total and phosphorylated ERK, p38, JNK, CREB, and c-jun were purchased from Cell Signaling Technology (Beverly, MA). Anti-actin antibody (AC-74) was purchased from Sigma. The MAPK/ERK kinase (MEK) inhibitor U0126 and the p38 inhibitor SB203580 were purchased from Promega (Madison, WI). The JNK inhibitor SP600125 was purchased from Calbiochem (San Diego, CA). Doxorubicin and etoposide were obtained from the Oncology Outpatient Pharmacy at the Karmanos Cancer Institute (Detroit, MI).

Cell lines, culture conditions, and treatment. The human lung cancer cell line H460 was maintained in RPMI 1640 as described previously (16). The human ovarian cancer cell line OVCAR3 was obtained from American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640. H460 cells conditionally expressing MKP-1 (H460-pMEP-MKP, -MKP-1) or vector control cells (H460-pMEP-vector) were described previously (16). Because MKP-1 in the pMEP-MKP-1 vector is controlled by the human metallothionein IIA promoter, addition of CdSO4 can induce MKP-1 (16). These cells were supplemented with 10% fetal bovine serum (FBS) and antibiotics in DMEM containing 10% FBS and antibiotics in a humidified atmosphere consisting of 5% CO2 and 95% air. Cells were treated with various concentrations of cisplatin for different intervals of time as indicated in each figure legend.

Isolation of RNA and Northern blot analysis. Total cellular RNA was purified using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA (20 μg) was separated in a 1.5% formaldehyde agarose gel and blotted to Hybond-N+ membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The blots were hybridized with radioactive human MKP-1 cDNA as described previously (16). Radioactive signals were analyzed by autoradiography.

Isolation of primary MEFs. The generation of MKP-1 knockout mice and the preparation of MEFs were described previously (22–24). Briefly, E12 to E16 embryos were minced and digested with trypsin/EDTA at 37°C for 30 minutes. After washing with PBS, isolated cells were collected by centrifugation and plated at a density of 2 × 10^6 in a 150-mm dish in DMEM containing 10% FBS and antibiotics in a humidified atmosphere consisting of 5% CO2 and 95% air.

siRNA transfection for knockdown of MKP-1. siRNA duplex oligonucleotides were purchased from Dharmacon Research (Lafayette, CO). The targeted sequence for MKP-1 siRNA was 5'-CCAAUUUGGCCCAACCCAUUUG-3'. The transfection was done as suggested by Dharmacon with slight modifications. Briefly, H460 cells were plated at 4 × 10^5 per well in six-well plates and then transfected with MKP-1 siRNA oligonucleotides or scrambled oligonucleotides using Oligofectamine (Invitrogen). After 3 days, transfected cells were harvested for examining the expression of MKP-1 protein by Western blot analysis or subjected to cisplatin treatment. To determine cisplatin sensitivity, transfected cells were placed at 8,000 per well in 96-well plates and then treated with or without cisplatin (12.5 or 25 μg/mL) for 24 hours, and cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays.

MTT assays. MTT assay was described previously (25). Briefly, cells were treated with cisplatin at different doses for various times. In the experiments involving the MAPK inhibitors, cells were pretreated with 20 μmol/L SP600125, 10 μmol/L U0126, or 10 μmol/L SB203580 for 30 minutes and then treated with cisplatin in the presence or absence of these inhibitors. After incubation with MTT solution, isopropanol was added to dissolve the formazan crystals. Absorbance was measured using a Vmax microplate reader ( Molecular Devices, Sunnyvale, CA) at 490 nm. The survival was calculated from the mean of pooled data from three separate experiments with six wells (25).

Western blot analysis. Whole-cell lysates were prepared as described previously (26), and protein concentration was determined using the Protein Assay kit (Bio-Rad, Hercules, CA). Cell lysates (100 μg) were electrophoresed through 12% denaturing polyacrylamide gels and transferred to a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH). The blots were probed or reprobed with the antibodies, and bound antibody was detected using enhanced chemiluminescence reagent (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

Assay of caspase-3 activity. The enzymatic activity of caspase-3 was assayed using the caspase-3 colorimetric assay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. Briefly, cells were left untreated or treated with 50 μg/mL cisplatin for 24 hours and then lysed in lysis buffer for 10 minutes on ice. The lysed cells were centrifuged at 14,000 rpm for 5 minutes, and protein (200 μg) was incubated with 50 μL reaction buffer and 5 μL caspase-3 substrate at 37°C for 1 hour, and the absorbance was measured at a wavelength of 405 nm on a plate reader.

Statistical analysis. Statistical analyses were done using Student’s t test. The data were presented as the mean ± SD, and P < 0.05 was considered significant.

Results

Cisplatin induces MKP-1 through the ERK pathway. Although MKP-1 is a stress-inducible protein (9), it is unclear if cisplatin can induce MKP-1. To this end, we treated H460 cells with 50 μg/mL cisplatin for different periods, and induction of MKP-1 protein was determined by Western blot analysis. As shown in Fig. 1A, MKP-1 protein started to increase at 3 hours, became abundant at 4 hours, and then stayed at a higher level at least for 2 more hours. Such induction was also observed in the ovarian cancer cell line OVCAR3 (Fig. 1A) and in the breast cancer cell line MDA-231 (data not shown). Consistent with induction of MKP-1 protein, MKP-1 mRNA was increased on cisplatin treatment in H460 cells and such increase was detected in OVCAR3 on a 6-hour treatment (Fig. 1B), which suggests that cisplatin induces MKP-1 through a transcriptional mechanism. To further gain insight into the regulation of MKP-1 expression by cisplatin, we treated H460 cells with cisplatin in the presence or absence of the transcription inhibitor actinomycin D, and the expression of MKP-1 was examined at 4 hours. As expected, the levels of MKP-1 protein and mRNA were significantly increased on cisplatin treatment, but such increases were inhibited in the presence of actinomycin D (Fig. 1C). In addition, we found that cycloheximide, a protein synthesis inhibitor, can block induction of MKP-1 protein by cisplatin (data not shown). Thus, our results indicate that induction of MKP-1 by cisplatin is regulated at the transcriptional level.

It has been shown that the expression of MKP-1 can be regulated by ERK and p38 (6, 10). To test whether ERK and p38 are involved in cisplatin-mediated MKP-1 induction, we pretreated H460 cells with U0126, SB203580, or both and then treated with 50 μg/mL cisplatin for 4 hours, and induction of MKP-1 was then examined. Figure 1D shows that induction of MKP-1 protein by cisplatin was partially blocked by SB203580 and completely abolished by U0126 or both inhibitors, whereas SB203580 had little effect on the basal level of MKP-1. Interestingly, U0126 was able to decrease the basal level of MKP-1 (Fig. 1D). Similar results were obtained with induction of MKP-1 mRNA; cisplatin induced MKP-1 mRNA, and U0126 blocked cisplatin-induced MKP-1 mRNA, whereas SB203580 had no effect (Fig. 1D). Of note, because SB203580 had no effect on induction of MKP-1 mRNA but partially blocked MKP-1 protein, we speculate that p38 may play a role in MKP-1 induction by cisplatin through a post-translational mechanism. Nevertheless, these results indicate that the ERK pathway is involved in induction of MKP-1 by cisplatin.

Overexpression of MKP-1 protects H460 cells from cisplatin-induced death, whereas knockdown of MKP-1 by siRNA silencing sensitizes H460 cells to cisplatin-induced death. Because MKP-1 has been implicated in cell survival in response to UV and osmotic stress (19, 21), we asked whether overexpression of MKP-1 plays a role in cisplatin resistance in human lung cancer.

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cells. To this end, we conditionally expressed MKP-1 by adding CdSO₄ to the growth medium in H460-pMEP4-MKP-1 or H460-pMEP4 cells to induce MKP-1 (16) and then treated the cells with cisplatin. As shown in Fig. 2A, addition of CdSO₄ to the medium resulted in an increase in the MKP-1 protein in H460-pMEP4-MKP-1 cells, whereas no such increase was detected in vector

Figure 1. Induction of MKP-1 by cisplatin is through the ERK-mediated transcriptional mechanism. A, induction of MKP-1 protein by cisplatin. H460 and OVCAR3 cells were treated with 50 μg/mL cisplatin for 0, 1, 2, 3, 4, and 6 hours, and total protein was then extracted for assaying MKP-1 protein by Western blot analysis. Actin was used as a loading control. B, induction of MKP-1 mRNA by cisplatin. H460 and OVCAR3 cells were treated with 50 μg/mL cisplatin for 0, 4, and 6 hours, and total RNA was extracted to assay for MKP-1 mRNA expression by Northern blot analysis. rRNA was visualized as a loading control. C, transcriptional induction of MKP-1 by cisplatin. H460 cells were treated with 50 μg/mL cisplatin (Cis) in the presence or absence of actinomycin D (Act D: 5 μg/mL). Total protein and RNA were extracted at 4 hours, and the levels of MKP-1 protein and mRNA were determined by Western and Northern blots, respectively. Actin and rRNA were used as a loading control for Western and Northern blots, respectively. D, the ERK pathway is required for cisplatin-induced MKP-1 expression. H460 cells were left untreated or pretreated with U0126 (U), SB203580 (SB), or both for 30 minutes and then treated with cisplatin for 4 hours in the presence or absence of these two inhibitors. Total protein and RNA were extracted. The levels of MKP-1 protein and mRNA were determined by Western and Northern blots, respectively. Actin and rRNA were used as a loading control for Western and Northern blots, respectively.

Figure 2. Effects of MKP-1 overexpression or knockdown on cisplatin-induced cell death. A, conditional overexpression of MKP-1 in H460 cells. H460-pMEP4-MKP-1 and H460-pMEP4 cells were treated with CdSO₄ (10 μmol/L) for 0, 2, and 4 hours, and total protein was then extracted. Induction of MKP-1 was determined by Western blotting. Actin was included as a loading control. B, knockdown of MKP-1 by siRNA. H460 cells were plated at 4 × 10⁵ per well in six-well plates. The next day, cells were mock transfected or transfected with MKP-1 siRNA or control oligonucleotides using Oligofectamine. After 3 days, total protein was extracted, and the expression of MKP-1 was determined by Western blot analysis. Actin was included as a loading control. C, overexpression of MKP-1 renders cells resistant to cisplatin-induced death. H460-pMEP4-MKP-1 and H460-pMEP4 cells were left untreated or treated with cisplatin (12.5 or 25 μg/mL) for 24 hours in the presence of CdSO₄ (10 μmol/L). Cell viability was determined by MTT assays. D, knockdown of MKP-1 sensitizes cells to cisplatin-induced cell death. H460 cells were transfected with MKP-1 siRNA or control oligonucleotides as described in (B). After 3 days, cells were left untreated or treated with cisplatin (12.5 or 25 μg/mL) for 24 hours. Cell viability was determined by MTT assays. In both (C) and (D), cell survival data are expressed as percentage of untreated cells. Data are representative of three independent experiments.
control H460-pMEP4 cells, confirming that MKP-1 was induced by CdSO4 (16). After pretreatment of the cells with CdSO4 we treated these cells with or without cisplatin (12.5 or 25 μg/mL) and then determined cell viability by MTT assay. Figure 2C shows that H460-pMEP4-MKP-1 cells treated with CdSO4 were more resistant to both doses of cisplatin compared with the control H460-pMEP4 vector cells (Fig. 2C). Thus, these results indicate that overexpression of MKP-1 plays a critical role in cisplatin resistance in the H460 human lung cancer cell.

Although conditional overexpression of MKP-1 confers cisplatin resistance in H460 cells, overexpression in this system may not reflect the physiologic condition. To directly address the role of MKP-1 in cisplatin resistance, we used siRNA silencing to knockdown MKP-1 expression and then determined the effects of knockdown of MKP-1 on cisplatin-induced cell death. To this end, H460 cells were transfected with either control oligos or oligos against MKP-1. The effects of siRNA-mediated MKP-1 knockdown on cisplatin sensitivity were determined. As shown in Fig. 2B, the levels of MKP-1 in cells transfected with MKP-1 siRNA decreased significantly compared with cells transfected with either control oligos or mock-transfected cells, indicating that MKP-1 siRNA is sufficient to knockdown MKP-1 expression. To determine the effects of knockdown of MKP-1 on cisplatin sensitivity, H460 cells transfected with either MKP-1 siRNA or control siRNA were treated with cisplatin (12.5 or 25 μg/mL) for 24 hours and cell viability was determined by MTT assays. As shown in Fig. 2D, on 25 μg/mL cisplatin treatment, ~70% of treated cells transfected with control siRNA survived. In contrast, there was a 20% increase in death in cells transfected with MKP-1 siRNA over cells transfected with control siRNA (Fig. 2D). Enhanced cisplatin sensitivity in cells transfected with MKP-1 siRNA was also observed on 12.5 μg/mL cisplatin treatment (Fig. 2D). Taken together, these data suggest that, in the human lung cancer cell H460, MKP-1 is a determinant of cisplatin resistance.

Role of MKP-1 in cisplatin-induced MAPK signaling. The ability of MKP-1 to dephosphorylate and inactivate ERK, p38, and JNK plays an important role in regulating MAPK signaling (6). We have shown that down-regulation of MKP-1 by siRNA against MKP-1 sensitizes H460 cells to cisplatin-induced cell death (Fig. 2). Because siRNA could not completely eliminate MKP-1 protein in cells, the results obtained with this approach may not clearly reflect the role of MKP-1 in cisplatin resistance. Therefore, we examined

![Figure 3. Activation of MAPK pathways by cisplatin in primary MEFs. MKP-1+/+ and MKP-1−/− MEFs were treated with 50 μg/mL cisplatin for 0, 1, 2, 3, 4, and 6 hours. Total protein was extracted and then assayed for the levels of MKP-1, JNK, phosphorylated JNK (p-JNK), ERK, phosphorylated ERK (p-ERK), p38 (p-p38MAPK), phosphorylated p38 (p-p38MAPK), c-Jun, phosphorylated c-Jun (p-c-Jun), CREB, and phosphorylated CREB (p-CREB) by Western blot analysis.](image-url)
the role of MKP-1 in cisplatin-induced cell death using MKP-1 knockout MEF cells. To this end, MKP-1+/+ and MKP-1−/− cells were treated with 50 μg/mL cisplatin, and activation of MAPKs and induction of MKP-1 were analyzed. As expected, MKP-1 was treated with 50 μg/mL cisplatin (Fig. 3), confirming the absence of MKP-1 in MKP-1−/− cells, and total c-Jun protein remained unchanged, which is consistent between MKP-1+/+ and MKP-1−/− cells (Fig. 3). In contrast, in MKP-1+/+ cells, no caspase-3 cleavage was observed, whereas PARP cleavage was barely detectable (Fig. 5A). More importantly, a 24-hour cisplatin treatment caused an 18-fold increase in caspase-3 activity in MKP-1−/− cells compared with untreated cells (Fig. 5B). In contrast, MKP-1+/+ cells were equally sensitive to doxorubicin treatment. Additionally, phosphorylation of p38 by cisplatin was significant after 4 hours in MKP-1−/− cells versus a slower increase in MKP-1+/+ cells (Fig. 3). In contrast, activation of JNK was significantly different between MKP-1+/+ and MKP-1−/− cells on cisplatin treatment; an earlier more robust increase in MKP-1+/+ versus a slower increase in MKP-1−/− cells (Fig. 3). Interestingly, there was a different kinetics of ERK2 phosphorylation between MKP-1+/+ and MKP-1−/− cells on cisplatin treatment; an earlier more robust increase in MKP-1+/+ versus a slower increase in MKP-1−/− cells (Fig. 3). In contrast, activation of JNK was significantly different between MKP-1+/+ and MKP-1−/− cells on cisplatin treatment. In MKP-1−/− cells, JNK phosphorylation was robust and prolonged to 6 hours on cisplatin treatment compared with MKP-1+/+ cells, in which phosphorylated JNK started to decrease at 4 hours (Fig. 3), suggesting that MKP-1 plays an important role in negatively regulating cisplatin-induced JNK activation. In addition, phosphorylation of p38 by cisplatin was comparable between MKP-1+/+ and MKP-1−/− cells (Fig. 3), although p38 was shown to be a significant target of MKP-1 in anisomycin-induced cell death (21). Importantly, total ERK, JNK, and p38 proteins remained unchanged in both cell lines (Fig. 3). Thus, these results indicate that MKP-1 specifically targets the JNK pathway in response to cisplatin treatment.

We have shown that loss of MKP-1 enhances JNK, but not p38 activation, in response to cisplatin treatment (Fig. 3). Because CREB and c-Jun are the downstream targets of p38 and JNK, respectively, we asked if the alterations of p38 and JNK activities affect phosphorylation of c-Jun and CREB. As shown in Fig. 3, c-Jun phosphorylation was more robust in MKP-1+/+ cells than MKP-1−/− cells, and total c-Jun protein remained unchanged, which is consistent with the results obtained with JNK phosphorylation (Fig. 3). However, phosphorylation of CREB was comparable between MKP-1+/+ and MKP-1−/− cells (Fig. 3), which is consistent with the results obtained with p38 phosphorylation (Fig. 3). Thus, our results suggest that loss of MKP-1 enhances the JNK signaling pathway, leading to increased c-Jun activity.

**MKP-1 modulates the sensitivities of cells to anticancer drugs.** Because activation of the MAPK pathways, including JNK, has been implicated in anticancer drug-mediated apoptosis, we investigated the effects of loss of MKP-1 on cisplatin resistance in these MEFs. We treated MKP-1+/+ and MKP-1−/− MEFs with different doses of cisplatin for 24 hours and then assessed cell viability. As shown in Fig. 4A, MKP-1−/− cells were more sensitive than MKP-1+/+ cells to cisplatin; ~50% of MKP-1−/− cells versus 90% of MKP-1+/+ cells survived following 50 μg/mL cisplatin treatment. Extending the exposure of cisplatin to 48 or 72 hours showed that there was much more death in MKP-1−/− cells than MKP-1+/+ cells (Fig. 4B). To determine whether loss of MKP-1 can sensitize cells to other anticancer drugs, we treated MKP-1+/+ and MKP-1−/− cells with doxorubicin and etoposide for 24 hours and assessed cell viability. As shown in Fig. 4C, MKP-1−/− cells were more sensitive than MKP-1+/+ cells to etoposide-induced cell death. However, MKP-1+/+ and MKP-1−/− cells were equally sensitive to doxorubicin (Fig. 4D). Thus, these results suggest that MKP-1 may be involved in the cellular resistance to a subset of anticancer drugs, such as cisplatin and etoposide in this study and anisomycin in a previous study (21).

**Loss of MKP-1 sensitizes cells to cisplatin-induced apoptosis.** Because cisplatin is believed to kill cells by apoptosis via activation of the JNK pathway (1), we reasoned that loss of MKP-1 could sensitize cisplatin-induced JNK-mediated apoptosis. To this end, MKP-1+/+ and MKP-1−/− MEFs were treated with cisplatin at 50 μg/mL for 0, 1, 2, 3, 4, and 6 hours, and total cells were then collected for analyzing cleavage of caspase-3 and poly(ADP-ribose) polymerase (PARP), hallmarks of apoptosis. As shown in Fig. 5A, cleavage of caspase-3 and PARP by cisplatin was significant after 4 hours in MKP-1+/+ cells. In contrast, in MKP-1+/+ cells, no caspase-3 cleavage was observed, whereas PARP cleavage was barely detectable (Fig. 5A). More importantly, a 24-hour cisplatin treatment caused an 18-fold increase in caspase-3 activity in MKP-1−/− cells compared with untreated cells (Fig. 5B). In contrast, there was only...
a 7-fold increase in caspase activity in MKP-1+/+ cells following cisplatin treatment (Fig. 5B). These results show that loss of MKP-1 sensitizes cells to cisplatin-induced caspase activation and apoptosis.

**JNK is required for cisplatin-induced apoptosis.** We have shown that JNK is hyperactivated in MKP-1−/− cells in response to cisplatin treatment. Because activation of JNK has been shown to play an important role in cisplatin-mediated apoptosis, we hypothesized that loss of MKP-1-mediated inhibition of JNK is responsible for the enhanced JNK activation and subsequent apoptosis in cisplatin-treated MKP-1−/− MEFs. To test this possibility, we pretreated both MKP-1+/+ and MKP-1−/− MEFs with the JNK inhibitor SP600125 and then exposed cells to cisplatin. As shown in Fig. 6A, in MKP-1−/− cells, there is ~10% cell death following SP600125 plus cisplatin treatment compared with 45% cell death by cisplatin alone, whereas SP600125 had a little effect. In contrast, cisplatin treatment had a little effect on MKP-1+/+ cell death, and SP600125 did not protect MKP-1+/+ cells from cisplatin-induced cell death (Fig. 6B). We confirmed that SP600125 blocks cisplatin-induced JNK phosphorylation in both MKP-1+/+ and MKP-1−/− cells (Fig. 6C). These data indicate that the JNK inhibitor SP600125 protects MKP-1−/− cells from cisplatin-induced cell death. Furthermore, pretreatment with the MEK inhibitor U0126 or the p38 inhibitor SB203580 failed to protect MKP-1+/+ cells from cisplatin-induced death (Fig. 6A), although these two inhibitors effectively blocked ERK and p38 activation (Fig. 6C), indicating that the ERK and p38 pathways do not play a significant role in cisplatin-induced cell death. Collectively, these results clearly indicate that activation of JNK is required for sensitizing cells to cisplatin-induced cell death.

**Discussion**

In this study, we showed that cisplatin induces MKP-1 through a mechanism involving the ERK pathway. Importantly, we found that loss of MKP-1 can sensitize cells to cisplatin-induced apoptosis. Furthermore, we showed that the mechanism by which loss of MKP-1 sensitizes cells to cisplatin-induced apoptosis is attributable to relief of MKP-1-mediated JNK inhibition. Thus, these findings indicate that MKP-1 plays an important role in the negative regulation of cisplatin-induced apoptosis.

MKP-1 is a MKP that can be induced by a variety of stimuli, including growth factors, oxidative damage, and UV (7, 9–11). Induction of MKP-1 by these stimuli can be regulated through both transcriptional and post-transcriptional mechanisms (6, 10). It has been shown that several transcription factors can bind to their binding sites in the MKP-1 gene to induce MKP-1 expression, including AP2, SP1, and p53 (16, 17, 27). In addition, it has been shown that JNK is hyperactivated in cells treated with cisplatin because both the MEK1/2 inhibitor and the p38 inhibitor SB203580 can block MKP-1 induction (10, 28). In the present study, we have shown that cisplatin induces MKP-1 and that such induction is inhibited by actinomycin D, suggesting that cisplatin-induced MKP-1 is through the transcriptional mechanism. We have also shown that induction of MKP-1 by cisplatin is abrogated in the presence of MEK1/2 inhibitor, indicating that ERK is involved in MKP-1 induction. In addition, we have found that the p38 inhibitor SB203580 can partially block the accumulation of MKP-1 protein in cisplatin-treated cells, although such blockade was not observed at the MKP-1 mRNA level (Fig. 1D), which suggests that p38 may play a role in translational or post-translational regulation of MKP-1. Nevertheless, our results indicate that ERK plays an important role in cisplatin-induced MKP-1 expression.

Several pieces of evidence suggest an important role of MKP-1 in protecting cells from death induced by stresses (19–21). For example, it has been shown that conditional expression of MKP-1 reduces UV-mediated apoptosis in U937 human leukemia (19). It has also been shown that overexpression of MKP-1 inhibits Fas ligand–induced apoptosis in human prostate DU145 cells (29). Using MKP-1 knockout MEFs, Wu and Bennett (21) showed that loss of MKP-1 enhances cell death in response to serum starvation, anisomycin, and osmotic stress. In addition, a previous study showed that overexpression of MKP-1 inhibits cisplatin-induced apoptosis in human embryonic kidney 293 cells (20). Consistently, we have found that overexpression of MKP-1 increases cell resistance to cisplatin (Fig. 2), which agrees with the role of MKP-1 overexpression in cisplatin resistance obtained with 293 cells (20). Furthermore, we have found that knockdown of MKP-1 by siRNA sensitizes H460 cells to cisplatin-induced cells (Fig. 2D). Thus, these observations establish that MKP-1 plays an important role in cisplatin resistance in the H460 human lung cancer cell line.

![Figure 5](image-url)
We have shown that down-regulation of MKP-1 by siRNA against MKP-1 sensitizes H460 cells to cisplatin-induced cell death. Because siRNA only partially abolishes MKP-1 expression in a transient manner, the results obtained with this approach may not clearly reflect the role of MKP-1 in cisplatin-induced cell death. Therefore, we thought to test the role of MKP-1 in cisplatin-induced cell death using MKP-1 knockout MEF cells. In agreement with the role of MKP-1 in cisplatin resistance in H460 cells, we have found that loss of MKP-1 sensitizes MKP-1−/− MEF to cisplatin-induced death (Fig. 4), which confirms a role in cisplatin resistance in another cell type. However, we have found that loss of MKP-1 does not affect doxorubicin sensitivity (Fig. 4), suggesting that the role of MKP-1 in protecting cells from chemotherapy is drug specific.

The ability of MKP-1 to inactivate MAPKs suggests that the mechanism underlying cisplatin sensitization in MKP-1−/− cells may be due to loss of MKP-1-mediated inhibition of MAPKs. Although MKP-1 could inhibit all three major MAPKs, including ERK, p38, and JNK, several studies have indicated that p38 and JNK are the preferred substrates of MKP-1 during the cellular responses to a variety of stress (14, 19, 23). Moreover, it has been found that, in MKP-1 knockout MEF cells, p38 is the preferential substrate for MKP-1 in response to serum starvation, anisomycin, and osmotic stress (21). Thus, the substrate specificity for MKP-1 is likely to depend on stimuli and cell types. Consistent with this, we have shown that ERK, p38, and JNK were induced in MEFs on cisplatin treatment. In MKP-1−/− cells, JNK was hyperactivated by cisplatin, but p38 and ERK1 were equally activated regardless of the status of MKP-1 (Fig. 3), suggesting that MKP-1 plays a more important role in regulating JNK in response to cisplatin treatment. Therefore, we conclude that, on cisplatin treatment, MKP-1 preferentially inactivates the JNK pathway, leading to cell survival.

It has been shown that the JNK pathway plays a critical role in cell death induced by anticancer drugs, including cisplatin (30, 31). We have shown that blockade of JNK activity by its inhibitor can protect MKP-1−/− cells from cisplatin-induced death (Fig. 6), suggesting that JNK is an important mediator in this process. Although SP600125 may affect other protein kinases, it is a potent JNK inhibitor (32) and has been widely used for inhibiting JNK activity (21). It is well known that activated JNK can activate its downstream substrate c-Jun, leading to cell death.

Figure 6. Blockade of JNK activity protects MKP-1−/− MEFs from cisplatin-induced death. A and B, effects of MAPK inhibitors on cisplatin-induced cell death. MKP-1−/− MEFs (A) and MKP-1+/+ MEFs (B) were left untreated or pretreated with SB203580, U0126, or SP600125 for 30 minutes and then treated with 50 μg/mL cisplatin in the presence or absence of the inhibitors. After 24 hours, cell viability was determined by MTT assays. Data are representative of three independent experiments. *, P < 0.05. C, inhibition of cisplatin-induced MAPK activation by MAPK inhibitors. MKP-1−/− and MKP-1+/+ MEFs were left untreated or treated as described in (A and B). After 6 hours, total protein was extracted, and the levels of JNK, phosphorylated JNK, ERK, phosphorylated ERK, p38, and phosphorylated p38 were determined by Western blot analysis.
via apoptosis (33). Consistent with this, cleavage of caspase-3 and PARP was robust in MKP-1−/− cells, whereas such changes were minimal in cells retaining MKP-1 (Fig. 5). Thus, our results suggest that MKP-1 can inhibit cisplatin-induced cell death mediated by JNK.

In conclusion, we showed that MKP-1 is induced by cisplatin probably via the ERK pathway. We also showed that loss of MKP-1 sensitizes cells to cisplatin-induced cell death in both human lung cancer H460 and MEF cells. Importantly, we showed that activation of the JNK pathway is required for cisplatin-mediated cell death because blockade of JNK activity protects cells from cisplatin-induced apoptotic cell death. We speculate that modulation of MKP-1 activity may be an effective approach to overcome cisplatin resistance in certain human cancers.

References
Mitogen-Activated Protein Kinase Phosphatase-1 Is Required for Cisplatin Resistance

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