ERK-Dependent MKP-1–Mediated Cisplatin Resistance in Human Ovarian Cancer Cells

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Abstract
Mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1) is the MAPK phosphatase family member that negatively regulates MAPK signaling. Our previous study showed that MKP-1 is involved in cisplatin resistance, but the mechanism underlying its resistance is not understood. Here, we show that ERK2-mediated MKP-1 expression is critical for cisplatin resistance. Specifically, we showed that in the human ovarian cancer cell lines, cisplatin induces MKP-1 through phosphorylation. We also showed that inhibition of ERK2 activity by the MEK1/2 inhibitor U0126 or by small interfering RNA silencing decreases MKP-1 induction, leading to an increase in cisplatin-induced cell death, which mimicked the results obtained with cells in which MKP-1 is downregulated. Importantly, down-regulation of ERK2 decreased cisplatin-induced MKP-1 phosphorylation, suggesting that MKP-1 phosphorylation depends on ERK2 activity. Furthermore, down-regulation of ERK2 or MKP-1 enhanced cisplatin-induced apoptosis. In addition, we showed that down-regulation of ERK2 or MKP-1 decreases the basal level of Bcl-2 protein and that inhibition of Bcl-2 activity sensitizes ovarian cancer cells to cisplatin. Collectively, our results indicate that induction of MKP-1 by cisplatin is through phosphorylation involving ERK signaling and that MKP-1 plays a critical role in ERK-mediated cisplatin resistance. Thus, our results suggest that targeting ERK-MKP-1 signaling could overcome cisplatin resistance in human ovarian cancer. [Cancer Res 2007;67(24):11933–41]

Introduction
Ovarian carcinoma is the fifth most common cause of cancer deaths among women in the United States. The current available therapies that are for the most part palliative are not very effective, but some progress is being made with neoadjuvant approaches including the use of cisplatin to improve the survival of patients with locally advanced disease. The major obstacle in ovarian cancer chemotherapy including cisplatin is the development of resistant cell populations following the relapse of an initially responsive malignancy. Therefore, there is an urgent need to understand chemoresistance including cisplatin resistance. Cisplatin is the first line drug for treating epithelial ovarian cancer. Although the mechanism underlying its anticancer activity is incompletely defined, it is generally accepted that cisplatin is a DNA-damaging agent that forms cisplatin-DNA adducts that kill cells by the activation of cell cycle arrest and apoptosis. It is believed that cisplatin-induced cancer cell apoptosis involves the activation of the mitogen-activated protein kinase (MAPK) pathways (1).

Mammalian MAPK pathways mainly consist of three subfamilies: the p38 MAPK, the extracellular signal-related kinase (ERK), and the stress-activated protein c-fos NH2-terminal protein kinase (JNK)/SAPK. These MAPKs can be activated by diverse stimuli including growth factors and cellular/extracellular stresses. In response to stimuli, 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. These upstream MAPK kinases (MKK) include MKK1/2, MKK3/6, and MKK-4/7, which are in turn activated by MAPK kinase kinases 2–4. Specifically, MKK3/6 activate p38 and MKK4/7 activate JNK, whereas MKK1/2 activate ERK (3). It is believed that activation of ERK tends to the induction of cell proliferation, whereas activation of JNK and p38 favors the induction of cell death. Because phosphorylation is a critical event for the activation of MAPKs, dephosphorylation of MAPKs by members of the MAPK phosphatase (MKP) family plays a critical role in negatively regulating MAPK signaling. The MAPK phosphatases are a family of dual-specificity protein phosphatases (DUSP; ref. 7). Eleven members of MKPs have been identified thus far, including MKP-1, MKP-2, MKP-3, MKP-4, MKP-5, MKP-7, MKP-X, PAC1, hVH3, M3/6, and MK-STYX (8). These MKPs can dephosphorylate both phosphorylated threonine and phosphorylated tyrosine residues and thus inactivate MAPK activities (7). MKP-1 is the founding member of the DUSP family. MKP-1 was originally cloned as a growth factor–inducible gene (9, 10), which could also be induced by stresses (11–13). It has been shown that MKP-1 can inactivate ERK, JNK, and p38 (12, 14–16). Because the activation of JNK, p38, and ERK can induce apoptosis or cell proliferation, MKP-1 is shown to be involved in regulating the cell cycle (17–20) and apoptosis (21–23). For example, MKP-1 could protect cells from anisomycin-induced apoptosis (24). Our recent study showed that MKP-1 is induced by cisplatin in human lung cancer cell lines and that the induction of MKP-1 plays an important role in cisplatin resistance (25).

In this article, we showed that cisplatin activates ERK and induces MKP-1 expression. Induction of MKP-1 by cisplatin is through phosphorylation. Inhibition of ERK activity by the pharmacologic inhibitor U0126 abolished MKP-1 induction. Furthermore, knockdown of ERK2 by small interfering RNA (siRNA) decreased MKP-1 phosphorylation caused by cisplatin, leading to an increase in cisplatin-induced ovarian cancer cell death, which is consistent with the result obtained with cells in which MKP-1 is down-regulated. Importantly, we showed that knockdown of ERK2 or MKP-1 decreases the basal level of Bcl-2.
expression, which may suggest the underlying mechanism of cisplatin resistance. Taken together, our results identify a new mechanism by which ERK2-MKP-1 signaling is responsible for cisplatin resistance and suggest that targeting ERK-MKP-1 signaling may overcome cisplatin resistance in human ovarian cancer.

Materials and Methods

Reagents. Cisplatin and antiactin antibody (AC-74) were purchased from Sigma. The MEK inhibitor U0126 and the p38 inhibitor SB203580 were purchased from Promega. The JNK inhibitor SP600125 and mouse anti-Bcl-2 monoclonal antibody were purchased from Calbiochem. Gossypol was obtained from AXXORA. Rabbit polyclonal anti-human MKP-1 antibody was purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibodies against total and phosphorylated ERK, p38, JNK, cyclic AMP–responsive element-binding (CREB) protein, c-Jun, caspase-3, PARP, and phosphorylated MKP-1 at Ser359 were purchased from Cell Signaling Technology.

Cell lines, culture conditions, and treatment. The human ovarian cancer cell lines OVCA432, TOV112D, CAOV3, OVCA420, OV433, and RMG-1 were kindly provided by Dr. Samuel Mok (Brigham and Women’s Hospital, Boston, MA), as described previously (26). All cell lines were maintained in MCDB105/M199 except CAOV3, which was maintained in DMEM. These cells were supplemented with 10% fetal bovine serum and maintained in a humidified atmosphere consisting of 5% CO2 and 95% air. Cells were treated with various concentrations of cisplatin for different periods of time as indicated in each figure legend.

siRNA transfection for knockdown of MKP-1 and ERK2. On TARGETplus SMARTpool siRNAs for MKP-1, ERK2, and corresponding control siRNA were purchased from Dharmacon Research. The transfection was performed as suggested by Dharmacon with slight modifications, as described previously (25). Briefly, OV433 cells were plated at 6 × 103 cells per well in six-well plates. The next day, cells were transfected with ERK2, MKP-1 siRNA oligonucleotides or non-target control oligonucleotides using LipofectAMINE 2000 (Invitrogen). After 3 days, transfected cells were left untreated or treated with cisplatin (50 μg/mL) for 6 h and then harvested to examine the expression of ERK2 and MKP-1 protein using Western blot analysis. To determine cisplatin sensitivity, transfected cells were placed at 8,000 cells per well in 96-well plates and then treated with or without cisplatin (20 or 50 μg/mL) for 24 h, and cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

MTT assay. The MTT assay was done as previously described (27). Briefly, cells were left untreated or pretreated with 10 μmol/L of SP600125, 10 μmol/L of U0126, or 10 μmol/L of SB203580 for 30 min, and then treated with cisplatin. After incubation with MTT solution, isopropanol was added to dissolve the formazan crystals. Absorbance was measured using a Vmax Microplate Reader ( Molecular Devices) at 590 nm. Survival was calculated from the mean of pooled data from three separate experiments with five wells (27).

Western blot analysis. Cell lysates were prepared as described previously (28), and protein concentration was determined using the Protein Assay Kit (Bio-Rad). Cell lysates (100 μg) were electrophoresed through 12% or 15% denaturing polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (Millipore). The blots were probed or reprobed with the antibodies, and bound antibody was detected using enhanced chemiluminescence or ECLplus Reagent (Axxora Pharmacia Biotech) according to the manufacturer’s protocol.

Assay of MKP-1 phosphatase activity. MKP-1 activity was measured using immunoprecipitated MKP-1. Immunoprecipitation was performed as described previously (29) with slight modification. Briefly, cells were lysed in lysis buffer, and 500 μg of supernatant protein was incubated with 2 μg of antiphosphorylated MKP-1 antibody or IgG at 4°C for 2 h, followed by incubation with protein A/G PLUS for 1 h. Immunoprecipitates were washed four times with cell lysis buffer. The precipitates were used to measure MKP-1 phosphatase activity. Phosphatase activities of immunoprecipitated MKP-1 were analyzed as described previously (30) using pNPP protein phosphatase assay kit from AnaSpec according to manufacturer’s instructions. Briefly, 50 μL of the pNPP reaction mixture was added to 50 μL of MKP-1 immunoprecipitates and the reaction was performed at 30°C for 1 h. Absorbance measured at 405 nm was obtained in a microplate reader. Nonspecific hydrolysis of pNPP was assessed in IgG immunoprecipitates, which served as a control.

Assay of caspase-3 activity. The enzymatic activity of caspase-3 was assayed using the caspase-3 colorimetric assay kit (R&D Systems) according to the manufacturer’s protocol. Briefly, cells left untreated or treated with cisplatin for 24 h and then lysed in lysis buffer for 10 min on ice. The lysed cells were centrifuged at 14,000 rpm for 5 min, and 100 μg of protein was incubated with 20 μL of reaction buffer and 10 μL of caspase-3 substrate at 37°C for 1 h, and absorbance was measured at a wavelength of 405 nm on a plate reader.
Statistical analysis. Statistical analyses were performed 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 in a panel of human ovarian cancer cell lines. Our previous study indicated that cisplatin induces MKP-1, which plays an important role in cisplatin resistance in the lung cancer cell line H460 (25). Because cisplatin is the first-line chemotherapy for the treatment of ovarian cancer, it is possible that such a mechanism also applies to ovarian cancer. To this end, we treated a panel of human ovarian cancer cell lines including OVCA432, TOV112D, CAOV3, OVCA420, OV433, and RMG-1 with 50 μg/mL of cisplatin for different periods of time, and induction of MKP-1 protein was determined by Western blot analysis. As shown in Fig. 1A, MKP-1 protein was increased in all the cell lines tested with different kinetics. MKP-1 was dramatically increased 3 h after cisplatin treatment in TOV112D, CAOV3, OVCA420, and RMG-1 cells. Such an induction was observed only after 1- to 6-h cisplatin treatments in OVCA432 cells, whereas MKP-1 was gradually increased by cisplatin in OV433 cells. These data suggest that induction of MKP-1 by cisplatin is a general event in human ovarian cancer cell lines. To determine their cisplatin sensitivities, we treated these cell lines with various doses of cisplatin for 24 h, and the effect of cisplatin on growth was determined by MTT assays. Figure 1B shows that cisplatin inhibits the growth of all the cell lines in a dose-dependent manner; OV433 and RMG-1 were the most resistant lines, whereas CAOV3 was the most sensitive line (Fig. 1B).

The role of MAPKs in MKP-1 induction by cisplatin. It has been shown that MKP-1 expression can be regulated by ERK, p38, and JNK (12, 31, 32). Our previous study indicated that ERK might be involved in MKP-1 induction by cisplatin in human lung cancer cell line H460 (25). To determine which MAPK is involved in cisplatin-induced MKP-1 expression in ovarian cancer cells, six ovarian lines were left untreated or treated with the MEK1/2 inhibitor U0126 (10 μmol/L), the p38 inhibitor SB203580 (10 μmol/L), or the JNK inhibitor SP600125 (10 μmol/L) for 30 min and then treated with 50 μg/mL of cisplatin for 6 h, and the expression of MKP-1 was determined by Western blot analysis. As expected, cisplatin induced MKP-1 in all cell lines tested and the induction of MKP-1 was inhibited or abrogated by either U0126 or SB203580 (Fig. 2A). U0126 inhibited MKP-1 induction in OV433 and RMG-1 cells and reduced its induction in CAOV3, OVCA420, and TOV112D cells (Fig. 2B), suggesting that the induction of MKP-1 in these lines is dependent on ERK. In addition, we found that MKP-1 induction was inhibited or abrogated by SB203580 in CAOV3, OVCA432, and RMG-1 cells (Fig. 2B). Interestingly, SP600125 had no effects on MKP-1 induction by cisplatin (Fig. 2A), suggesting that JNK may not play a role in regulating MKP-1 expression under this condition.

To determine the consequence of inhibition of MAPKs in cisplatin-induced death, all six cell lines were left untreated or pretreated with U0126, SB203580, or SP600125 for 30 min and then treated with 10 μg/mL of cisplatin for 24 h, and cell viability was determined by MTT assays. As shown in Fig. 2B, cisplatin inhibited the growth of all cell lines to different degrees, and U0126 alone had minimal or no effect on their growth. In contrast, inhibition of ERK signaling by U0126 enhanced cisplatin-induced death in OVCA432, OV433, and RMG-1 cells, but not in the rest of cell lines. These data indicate that ERK signaling may be important for cisplatin resistance in OVCA432, OV433, and RMG-1 cells. We also found that in both RMG-1 and TOV112D cells, SB203580 has some effect on cisplatin-induced death (Fig. 2B). In addition, inhibition of JNK signaling by SP600125 increased cisplatin-induced death in CAOV3 cells (Fig. 2B).

Cisplatin induces MKP-1 and activates MAPKs in human ovarian cancer cell lines. To determine the effect of cisplatin on both MKP-1 induction and MAPK activation, OV433 cells were treated with 50 μg/mL of cisplatin for 0, 5, 10, 15, and 20 h, and induction of MKP-1 and activation of MAPK signaling were then determined. Of note, we chose the OV433 cell line because blocking ERK in this cell line correlated with both decreased MKP-1 induction and increased cisplatin-induced cell death, whereas either U0126 or SB203580 could inhibit MKP-1 induction and increase cell death in both RMG-1 and TOV112D cells, suggesting
that ERK may be the only MAPK that plays a role in MKP-1 induction in OV433 cells (Fig. 2). Figure 3A shows that cisplatin treatment increases the levels of total and phosphorylated MKP-1 protein (Fig. 3A), suggesting that an increase in MKP-1 protein may be due to its phosphorylation by cisplatin. We also found that cisplatin treatment increases the half-life of MKP-1 protein (data not shown). Importantly, MKP-1 phosphatase activity was also increased following cisplatin treatment (Fig. 3B). In addition, cisplatin treatment caused phosphorylation of ERK, p38, JNK, CREB, and c-Jun, indicating that cisplatin activates all three MAPK pathways. Interestingly, the activation of ERK was detected after a 5-h exposure to cisplatin, whereas MKP-1 induction was evident at 10 h following cisplatin treatment while activation of both JNK and p38 occurred relatively later as compared with ERK phosphorylation (Fig. 3A). It seems that activation of ERK precedes MKP-1 induction, which suggests that ERK may be an upstream activator of MKP-1 in response to cisplatin treatment.

Blockade of ERK activation by the MEK1/2 inhibitor U0126 abrogates MKP-1 induction, leading to an increase in cisplatin-induced cell death. To determine the role of ERK in MKP-1 induction, OV433 cells were left untreated or treated with U0126, SB203580, or SP600125 for 30 min and then treated with 50 μg/mL of cisplatin for 6 h, and induction of MKP-1 and activation of MAPKs were determined. As shown in Fig. 4A, cisplatin induced

Figure 3. Effect of cisplatin treatment on the level of MKP-1 protein and phosphatase activity and activation of MAPK pathways. A, induction of MKP-1 and activation of MAPK pathways by cisplatin. OV433 cells were left untreated or treated with 50 μg/mL of cisplatin for 5, 10, 15, and 20 h. Total protein was extracted and the levels of MKP-1, phosphorylated MKP-1, ERK, phosphorylated ERK, p38, phosphorylated p38, CREB, phosphorylated CREB, JNK, phosphorylated JNK, c-Jun, and phosphorylated c-Jun were determined by Western blot analysis. Actin was used as a loading control. B, cisplatin treatment increases MKP-1 phosphatase activity. OV433 cells treated with cisplatin were lysed in lysis buffer, and subjected to immunoprecipitation using antiphosphorylated MKP-1 antibody as described in Materials and Methods. Immunoprecipitated MKP-1 was incubated with 50 μL of pNPP reaction mixture using pNPP protein phosphatase assay kit at 30 °C for 1 h. Absorbance measured at 405 nm was obtained in a microplate reader. Nonspecific hydrolysis of pNPP was assessed in IgG immunoprecipitates.

Figure 4. Effect of MAPK inhibitors on MKP-1 induction and MAPK activation and cisplatin sensitivity. A, role of MAPK inhibitors in the induction of MKP-1 and activation of MAPKs by cisplatin. OV433 cells were left untreated or pretreated with 10 μmol/L of U0126 (U), 10 μmol/L of SB203580 (SB), or 10 μmol/L of SP600125 (SP) for 30 min and then treated with 50 μg/mL of cisplatin (Cis) for 6 h. Total protein was extracted and the levels of MKP-1, ERK, phosphorylated ERK, CREB, phosphorylated CREB, c-Jun, and phosphorylated c-Jun were determined by Western blot analysis. Actin was used as a loading control. B, role of MAPK inhibitors in cisplatin-induced cell death. OV433 cells were left untreated or treated with three MAPK inhibitors as described in A and then treated with 50 μg/mL of cisplatin for 24 h, and cell viability was determined by MTT assays. Cell viability data are expressed as a percentage of untreated cells and are representative of three independent experiments. **, P < 0.001, statistically significant.
MKP-1 and such an induction was blocked by U0126, whereas neither SB203580 nor SP600125 had any effects on MKP-1 induction. As expected, cisplatin treatment resulted in the activation of all three MAPK signaling pathways, as exemplified by the appearance of phosphorylated ERK, CREB, and c-JUN (Fig. 4A). To determine the effect of MAPK inhibitors on cisplatin sensitivity, OV433 cells pretreated with U0126, SB203580, or SP600125 were treated with cisplatin (50 μg/mL) for 24 h, and cell viability was determined by MTT assays. As shown in Fig. 4B, following 50 μg/mL of cisplatin treatment, >70% of cells treated with cisplatin survived. In contrast, there was a 20% decrease in survival in cells pretreated with U0126 over cells treated with cisplatin alone (Fig. 4B). Similar results were obtained with cells treated with 20 μg/mL of cisplatin (data not shown). However, increased cisplatin sensitivity was not observed in cells pretreated with SB203580 or SP600125. Taken together, these data suggest that ERK-MKP-1 signaling plays a critical role in cisplatin resistance.

Knockdown of either ERK2 or MKP-1 by siRNA silencing increases cisplatin-induced death. Although U0126 is a specific inhibitor for MEK1/2, it is possible that U0126 may affect other kinases that indirectly affect ERK activation. To directly address the role of ERK in MKP-1–mediated cisplatin resistance, OV433 cells were transfected with either non-target control siRNA or siRNA against ERK2, and the effect of ERK2 knockdown on cisplatin sensitivity was determined. As shown in Fig. 5A, total ERK2 in cells transfected with ERK2 siRNA was decreased significantly as compared with cells transfected with control siRNA. Furthermore, cisplatin-induced ERK2 phosphorylation was abrogated in cells transfected with ERK2 siRNA (Fig. 5A). Importantly, phosphorylated MKP-1 was decreased in ERK2 siRNA-transfected cells as compared with cells transfected with control siRNA following cisplatin treatment (Fig. 5A), suggesting that ERK2 indeed plays an important role in MKP-1 phosphorylation and induction by cisplatin. As expected, cisplatin treatment caused ERK, p38, and JNK phosphorylation in cells transfected with control siRNA (Fig. 5A). Interestingly, knockdown of ERK2 seemed to have no effect on both p38 and JNK phosphorylation by cisplatin (Fig. 5A).

To further investigate the role of ERK-MKP-1 signaling in regulating MAPKs, we used siRNA silencing to knock down MKP-1 expression and the effect of knockdown of MKP-1 on cell death was determined. Figure 5A shows that the levels of MKP-1 in cells transfected with MKP-1 siRNA were decreased significantly as compared with cells transfected with control siRNA. Furthermore, induction of MKP-1 by cisplatin in cells transfected with MKP-1 siRNA was abolished as compared with cells transfected with control siRNA. More importantly, phosphorylated MKP-1 was also decreased in cells transfected with MKP-1 siRNA as compared with cells transfected with control siRNA following cisplatin treatment. As expected, cisplatin treatment resulted in an increase in ERK, p38, and JNK phosphorylation in cells transfected with control siRNA (Fig. 5A). In contrast, knockdown of MKP-1 by siRNA silencing resulted in much higher levels of phosphorylated ERK, p38, and JNK, particularly the level of JNK, as compared with that in cells transfected with control siRNA. Thus, these data strongly suggest that MKP-1 plays an important role in negatively regulating all three major MAPK activities following cisplatin treatment.

To determine the effect of knockdown of ERK2 or MKP-1 on cisplatin sensitivity, OV433 cells were transfected with ERK2 siRNA, MKP-1 siRNA, or control siRNA and then treated with cisplatin (50 μg/mL) for 15 h, and cell viability was determined by MTT assays. As shown in Fig. 5B, 50 μg/mL of cisplatin treatment caused ~20% growth inhibition in cells transfected with control siRNA. In contrast, such treatment caused ~40% growth inhibition in cells transfected with MKP-1 siRNA, which is consistent with the role of MKP-1 in cisplatin resistance (25). Increased cisplatin sensitivity in cells transfected with MKP-1 siRNA was also observed following 20 μg/mL of cisplatin treatment (Fig. 5B). Importantly, knockdown of ERK2 also increased cisplatin sensitivity to the same extent as that observed in cells transfected with MKP-1 siRNA (Fig. 5B). Thus, these data strongly suggest that the ERK-dependent MKP-1–mediated pathway is critical for cisplatin resistance in human ovarian cancer cell OV433.
Inhibition of ERK2-MKP-1 signaling increases apoptotic cell death by cisplatin. It has been shown that cisplatin kills cancer cells in part by apoptosis (1). To determine the effect of blockade of the ERK-MKP-1 pathway on apoptosis, OV433 cells were transfected with either ERK2 or MKP-1 siRNA to either down-regulate ERK or MKP-1 expression, or treated with U0126 to block ERK activation, and the effects of such treatments on cisplatin-induced apoptosis were determined. As shown in Fig. 6A, cisplatin alone did not cause PARP cleavage, a hallmark of apoptosis. However, cells pretreated with U0126, which is able to block ERK signaling, underwent cisplatin-induced apoptosis as exemplified by an increase in PARP cleavage and caspase-3 activity. These data suggest that inhibition of the ERK signaling pathway enhances cisplatin-induced apoptotic cell death. Consistent with the results obtained with U0126, knockdown of ERK2 by siRNA also increased cisplatin-induced caspase-3 and PARP cleavage and caspase-3 activity, as compared with cells transfected with control siRNA. Similarly, knockdown of MKP-1 also enhanced cisplatin-induced caspase-3 and PARP cleavage and caspase-3 activity, although such changes were not evident in cells transfected with control siRNA (Fig. 6C and D). Thus, our data indicate that ERK-MKP-1 signaling is a survival pathway that protects cells from cisplatin-induced apoptosis, which leads to cisplatin resistance.

Knockdown of ERK2 or MKP-1 correlates with down-regulation of the basal level of Bcl-2 expression. It has been shown that Bcl-2 is an ERK target that plays a role in protecting cells from apoptosis (33). We have shown that down-regulation of ERK2 or MKP-1 increases cisplatin-induced death of OV433 cells (Fig. 5B). It is possible that Bcl-2 may play a role in ERK2-MKP-1-mediated cisplatin resistance. To test this possibility, OV433 cells were transfected with control siRNA or siRNA against ERK2 or MKP-1. The effect of siRNA-mediated ERK2 or MKP-1 knockdown on Bcl-2 expression was determined. As shown in Fig. 7A, the levels of ERK2 and MKP-1 in cells transfected with corresponding siRNA were decreased compared with cells transfected with control siRNA. Importantly, the basal levels of Bcl-2 were decreased in cells transfected with either ERK2 or MKP-1 siRNA as compared with cells transfected with control siRNA. Interestingly, transfections of ERK2-siRNA resulted in a more significant decrease in the levels of Bcl-2 protein as compared with cells transfected with MKP-1 siRNA. In addition, we found that cisplatin-resistant ovarian cancer cells have a higher level of Bcl-2 protein as compared with their sensitive cells (data not shown). These data suggest that Bcl-2 may be a downstream target of ERK-MKP-1 signaling. To determine the role of Bcl-2 in cisplatin sensitivity, we treated OV433 cells with the Bcl-2 inhibitor Gossypol in the presence or absence of cisplatin for...
24 h, and cisplatin sensitivity was determined. As expected, Gossypol treatment did not affect Bcl-2 expression, as compared with untreated cells (Fig. 7B). In addition, Gossypol did not affect the levels of MKP-1 phosphorylation. Interestingly, the combination of Gossypol with cisplatin enhanced caspase-3 and PARP cleavage as compared with cells treated with either agent alone (Fig. 7B). Importantly, we showed that Gossypol increases cisplatin-induced death as compared with either cisplatin or Gossypol alone (Fig. 7C). Therefore, our results suggest that Bcl-2 plays a protective role in cisplatin-induced death in the ovarian cancer cell OV433.

Discussion

In this study, we showed that ERK2-dependent MKP-1 induction is critical for cisplatin resistance. Specifically, we showed that knockdown of ERK2 decreases MKP-1 phosphorylation and induction, leading to increased cisplatin sensitivity. Furthermore, we showed that knockdown of either ERK2 or MKP-1 results in a decrease in Bcl-2 expression. Thus, these findings indicate that the ERK2/MKP-1 pathway is important for the negative regulation of cisplatin-mediated sensitivity in ovarian cancer cells.

MKP-1 is the founding member of the MAPK phosphatase family that can be induced by a variety of stimuli, including growth factors, oxidative damage, UV, and anticancer agents (9, 11–13, 24, 28). Induction of MKP-1 by these stimuli can be regulated by MAPKs including ERK, p38, and JNK. It has been shown that MKP-1 induction in response to arsenite and lipopolysaccharide treatment was mediated by the MAPK pathways, which was blocked by the MEK1/2 inhibitor U0126 and the p38 inhibitor SB203580 (12, 31). Our previous study showed that MKP-1 was induced by cisplatin, which was blocked by U0126 in human lung cancer cells (25). In this study, we extended our study to show that MKP-1 is induced by cisplatin, which requires ERK2 because such a change was abrogated by U0126 (Fig. 4A). Importantly, we have shown that down-regulation of ERK2 results in a decrease in MKP-1 phosphorylation and induction (Fig. 5A). These data suggest that the ERK-dependent pathway is critical for cisplatin-induced MKP-1 expression.

The regulation of MKP-1 is poorly understood, but it is believed to involve both transcriptional and posttranscriptional mechanisms (7, 12). Although transcriptional regulation of MKP-1 is important for its expression and functions under certain conditions, several lines of evidence implicated that controlling MKP-1 protein degradation is critical for regulating its phosphatase activity. It has been shown that the proteasome inhibitors Z-LLL-CHO and lactacystin could increase the levels of MKP-1 protein (34), suggesting that inhibition of the proteasome degradation pathway enhances MKP-1 activity. Consistently, in human lung cancer cell CL3, the carcinogenic metal Pb(II) induced MKP-1 polyubiquitination (35). It is believed that the mechanism underlying this process involves the activation of ERK by Pb(II), which triggers MKP-1 ubiquitination, resulting in its degradation via the ubiquitin-proteasome pathway (35). This is consistent with an earlier study showing that ERK-mediated degradation is crucial for controlling MKP-1 levels (29). It is known that the majority of proteins, once phosphorylated, are resistant to ubiquitin-mediated degradation. A previous study showed that mitogen-induced ERK is able to phosphorylate MKP-1 at Ser359 and Ser364, resulting in the stabilization of the MKP-1 protein (29). Consistent with the role of ERK in MKP-1 phosphorylation, we have shown that cisplatin can activate ERK and in turn phosphorylate MKP-1 at Ser359 (Fig. 5A). We believed that cisplatin-induced MKP-1 phosphorylation is mediated by ERK because knockdown of ERK2 by siRNA interference abolished MKP-1 phosphorylation and decreased the total levels of MKP-1 protein (Fig. 5A). Thus, MKP-1 induction by cisplatin is through an ERK-dependent mechanism.
It has been known that ERK can interact with p38 and JNK but the mechanisms by which they interact remain unclear. We have shown that ERK is required for MKP-1 activation because knockdown of ERK2 abolished MKP-1 phosphorylation (Fig. 5B). Kinetically, it seems that activation of ERK occurs earlier than that of MKP-1, p38, and JNK in response to cisplatin treatment (Fig. 3A), which suggests that ERK may be upstream of MKP-1, p38, and JNK.

It is documented that MKP-1 is capable of dephosphorylation of ERK, p38, and JNK, thus inactivating all three MAPK signaling pathways (7). Based on our data, it is possible that MKP-1 is a mediator that links ERK to p38 and JNK, which is under investigation. In addition, we believe that, although ERK2 can phosphorylate MKP-1 to positively regulate MKP-1 activity, MKP-1-mediated ERK inactivation keeps ERK in check, which provides a negative feedback loop for tightly controlling ERK signaling.

Several lines of evidence implicated that the activation of ERK signaling protects cancer cells from chemotherapy (36, 37). It has been shown that cisplatin treatment activates ERK in ovarian cancer cells and that activation of ERK protects ovarian cancer cells from cisplatin-induced death (36, 37). Furthermore, inhibition of ERK signaling by the MEK1/2 inhibitor PD98059 blocked ERK activation and increased cisplatin sensitivity in SKOV3 cells (37). Consistent with the role of activation of ERK in modulating cisplatin sensitivity, we have shown that ERK is activated by cisplatin in the ovarian cancer cell OV343 and that inhibition of ERK activation by the MEK1/2 inhibitor U0126 or knockdown of ERK2 expression by siRNA interference sensitizes cancer cells to cisplatin (Figs. 4B and 5B). These data suggest that ERK signaling is a survival pathway that protects cells from cisplatin-induced death. Importantly, we found that knockdown of ERK2 results in a decrease in MKP-1 expression induced by cisplatin (Fig. 5A). Interestingly, knockdown of either ERK2 or MKP-1 enhanced cisplatin sensitivity (Fig. 5B), indicating that ERK-mediated cisplatin resistance is through MKP-1. Although the role of ERK in cisplatin resistance is implicated, the underlying mechanism is not clear. Thus, demonstrating the role of MKP-1 as a mediator in ERK-mediated cisplatin resistance provides a novel mechanism by which ERK signaling protects cancer cells from cisplatin-induced apoptosis.

The antiapoptotic members of the Bcl-2 family, including Bcl-2, play an important role in chemoresistance (38). It has been known that MKP-1 is involved in cisplatin resistance (23, 25). However, it is not clear whether there is a functional interaction between Bcl-2 and MKP-1. In this study, we have shown that knockdown of MKP-1 or ERK2 decreases the basal levels of Bcl-2 protein, suggesting that down-regulation of the Bcl-2 protein level lowers the cell death threshold, thereby enhancing cisplatin-induced cell death. Furthermore, we have shown that inhibition of Bcl-2 activity by its inhibitor Gossypol enhances cisplatin-induced cell death. We believe that Bcl-2 is involved in ERK-MKP-1 signaling, which protects cells from cisplatin-induced cell death. Thus, another novel finding of this study is to demonstrate the involvement of Bcl-2 in ERK-MKP-1-mediated cisplatin resistance.

In conclusion, we showed that MKP-1 is induced by cisplatin through phosphorylation by the ERK pathway. We also showed that knockdown of ERK2 decreases MKP-1 induction. Because MKP-1 is an important regulator of p38 and JNK, our study suggests that MKP-1 may be a mediator that functionally links ERK to p38 and JNK pathways. Furthermore, we showed that knockdown of either ERK2 or MKP-1 enhances cisplatin sensitivity, suggesting that the ERK-MKP-1 pathway is critical for cisplatin resistance. Importantly, we showed that knockdown of either ERK2 or MKP-1 decreases Bcl-2 expression, which suggests that Bcl-2 is involved in ERK-MKP-1–mediated cisplatin resistance. Thus, our results suggest that targeting ERK-MKP-1 signaling may overcome cisplatin resistance in ovarian cancers.

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