The Role of Mitogen-Activated Protein Kinase Phosphatase-1 in Oxidative Damage–Induced Cell Death

Jun-Ying Zhou, Yusen Liu, and Gen Sheng Wu

Abstract
Mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1) is a member of the MAPK phosphatase family that functions as a negative regulator of MAPK signaling. MKP-1 is induced by oxidative stress, but the role of its induction in cell death is not fully understood. Here, we show that hydrogen peroxide (H₂O₂) induces MKP-1 and activates MAPKs. Induction of MKP-1 by H₂O₂ correlated with inactivation of p38 and c-Jun-NH₂-kinase (JNK). Overexpression of MKP-1 increased cell resistance to H₂O₂-induced death. Furthermore, we show by small interfering RNA silencing that down-regulation of MKP-1 increases phosphorylated p38 and JNK and subsequent cell death induced by H₂O₂. More importantly, primary embryonic fibroblasts from mice lacking MKP-1 had a higher level of phosphorylated p38 and JNK and were more sensitive to H₂O₂-induced cell death compared with corresponding cells with MKP-1, indicating that p38 and JNK pathways may play important roles in H₂O₂-mediated cell death. Thus, these results suggest that activation of MKP-1 is a survival mechanism against oxidative damage. (Cancer Res 2006; 66(9): 4888-94)
Results

\( \text{H}_2\text{O}_2 \) induces MKP-1 that correlates with inactivation of MAPKs. To determine the role of MKP-1 in \( \text{H}_2\text{O}_2 \)-mediated cellular responses, the breast cancer cell MCF-7 was treated with varying doses of \( \text{H}_2\text{O}_2 \) for 3 hours, and induction of MKP-1 and activation of ERK, p38, and JNK were determined by Western blot. We chose a 3-hour treatment, because at this time point \( \text{H}_2\text{O}_2 \) induced MKP-1 and activated MAPKs in preliminary experiments. As shown in Fig. 1A, MKP-1 was induced at 100 \( \mu \text{mol/L} \), increased to a much higher degree at 200 \( \mu \text{mol/L} \), and peaked at 400 \( \mu \text{mol/L} \) \( \text{H}_2\text{O}_2 \) in a dose-dependent manner. To determine the effects of such treatments on MAPK pathways, we analyzed the appearance of phosphorylated (active) forms of ERK, p38, and JNK. Similar to the results obtained with MKP-1, \( \text{H}_2\text{O}_2 \) activated all three MAPKs by increasing phosphorylated ERK, JNK, and p38 (Fig. 1B). However, the levels of total ERK, JNK, and p38 proteins remained unchanged (data not shown). These data are consistent with the role of oxidative damage in activation of MAPKs and MKP-1.

It is well known that MKP-1 is able to dephosphorylate and inactivate ERK, p38, and JNK. Because both MKP-1 and MAPKs are induced by \( \text{H}_2\text{O}_2 \), we asked if induction of MKP-1 by \( \text{H}_2\text{O}_2 \) is able to inactivate MAPK activities. To this end, MCF-7 cells were treated with \( \text{H}_2\text{O}_2 \) at 200 \( \mu \text{mol/L} \) for different time points, and induction of MKP-1 and phosphorylation of ERK, p38, and JNK were analyzed. Figure 1B shows that phosphorylation of p38, JNK, and ERK was detectable at 30 minutes, significant at 1 hour, and then returned to normal around 4 hours. Coincidentally, induction of MKP-1 was detected after a 2-hour exposure to \( \text{H}_2\text{O}_2 \) and then stayed at a higher level for at least 4 more hours (Fig. 1B). Thus, a correlation of MKP-1 induction with the disappearance of phosphorylated MAPKs suggests that MKP-1 might play a physiologic role in inactivation of oxidative damage–induced MAPK activities.

We previously showed that MKP-1 expression could be induced by p53 overexpression (16). To determine whether \( \text{H}_2\text{O}_2 \)-induced MKP-1 is dependent on p53, we treated both HCT116 p53\(^{−/−} \) (wt p53) and HCT116 p53\(^{−/−} \) (no p53; ref. 28) with 200 \( \mu \text{mol/L} \) \( \text{H}_2\text{O}_2 \) for varying time points, and induction of MKP-1 was determined by Northern blot analysis. Figure 1C shows that MKP-1 is induced in both cell lines. However, there was a higher level of MKP-1 in HCT116 p53\(^{−/−} \) than HCT116 p53\(^{+/−} \) after 1-hour treatment. In addition, we found that MKP-1 is induced by \( \text{H}_2\text{O}_2 \) in both H460-Neo and H460-E6 cells.

Of note, kinetics of MKP-1 induction was slightly different between H460-Neo and H460-E6 cells (Fig. 1C). Therefore, these results suggest that induction of MKP-1 by \( \text{H}_2\text{O}_2 \) could be p53 independent.

\textbf{Overexpression of MKP-1 inhibits \( \text{H}_2\text{O}_2 \)-mediated cell death.}

We have shown that induction of MKP-1 correlates with inactivation of MAPK activity. We asked if MKP-1 plays an inhibitory role in regulating MAPK signaling in response to \( \text{H}_2\text{O}_2 \) treatment. To this end, we transfected MCF-7 cells with either the empty vector pIRE2-EGFP, or the MKP-1-expressing vector pIRE2-EGFP-MKP-1 and then selected with G418 for individual clones that stably express MKP-1 by Western blot with MKP-1 antibody. As shown in Fig. 2A, three clones (clones 3, 12, and 15) overexpressed MKP-1 protein compared with cells transfected with the empty vector. To determine whether transfected MKP-1 is functional in cells, we treated these cells with \( \text{H}_2\text{O}_2 \) to induce MAPK activity and then determined the levels of phosphorylated MAPKs. As shown in Fig. 2B, \( \text{H}_2\text{O}_2 \) activated ERK, p38, and JNK in vector control cells, which is consistent with the results obtained in Fig. 1A. In contrast, activation of all three MAPKs, particularly phosphorylated JNK, was substantially inhibited in cells overexpressing MKP-1, compared with vector control cells (Fig. 2B). These results suggest that overexpression of MKP-1 is able to inactivate MAPK signaling induced by \( \text{H}_2\text{O}_2 \).
cells were left untreated or treated with 50, 100, 200, and 400 μM H_2O_2 for 5 hours, and detached dead cells and attached surviving cells were determined by trypan blue exclusion and Coomassie blue staining, respectively. As shown in Fig. 2C, upon H_2O_2 treatment, the majority of MKP-1-overexpressing cells (clones 12 and 15) remained attached (alive cells), compared with the vector control cells in which fewer cells remained attached. Furthermore, there were many more dead cells detected in H_2O_2-treated vector control cells over the MKP-1-overexpressing cells (Fig. 2D). Thus, these data suggest that overexpression of MKP-1 could inhibit MCF-7 cell death induced by oxidative damage.

**siRNA knockdown of MKP-1 enhances MCF-7 cell death induced by H_2O_2.** Although overexpression of MKP-1 is able to inhibit H_2O_2-induced cell death, it is possible that the results obtained from the overexpression system may not reflect the physiologic condition. To directly address the role of MKP-1 in oxidative damage–induced cell death, we used siRNA silencing to knockdown MKP-1 expression and then determined the effects of knockdown of MKP-1 on cell death. Using a computer-designed program, we identified four regions that could be targeted by siRNA and two of which were tested for their ability to knockdown MKP-1 expression (data not shown). Our preliminary data indicated that 5'-CCAAUGUGCCCGACUAAUUG-3' is the better one for knockdown of MKP-1 (data not shown) and thus this duplex was used in this study. As shown in Fig. 3A, induction of MKP-1 by H_2O_2 in cells transfected with MKP-1 siRNA was abolished compared with cells transfected with control oligos. Consistent with activation of MAPKs by H_2O_2, p38, JNK, and ERK were activated in cells transfected with control oligos. In contrast, by knockdown of MKP-1, activation of these three MAPKs was obvious, particularly for JNK and p38 (Fig. 3A). These data confirm the role of MKP-1 in negative regulation of MAPK activity and support that MKP-1 could play an important role in inhibition of p38 and JNK activation upon H_2O_2 treatment.

We next asked if blockade of MKP-1 by siRNA could sensitize cells to oxidative damage–induced cell death. To this end, MCF-7 cells were transfected with either MKP-1 siRNA or control siRNA and then treated with 300 μmol/L H_2O_2 for 5 hours, and cell viability was determined by trypan blue exclusion. As shown in Fig. 3B, H_2O_2 caused ~30% of death in cells transfected with control siRNA. In contrast, there was a 20% increase in death in cells transfected with MKP-1 siRNA than cells transfected with control siRNA (Fig. 3B). These data suggest that MKP-1 plays an inhibitory role in oxidative damage–induced cell death and that inhibition of MKP-1 could enhance H_2O_2-mediated cell death, possibly through activation of the JNK and p38 death pathways.

**M KP-1** knockout MEF cells are more sensitive than MKP-1+/+ cells to H_2O_2-induced cell death. We have shown that down-regulation of MKP-1 by siRNA against MKP-1 sensitizes MCF-7 cells to H_2O_2-induced cell death. Because siRNA could be a transient effect on MKP-1 knockdown, the results obtained with this approach may not completely reflect the role of MKP-1 in oxidative damage–induced cellular responses. Therefore, we thought to test the role of MKP-1 in oxidative damage–induced cell death using MKP-1 knockout MEF cells. Primary MEFs were obtained from wild-type (MKP-1+/+) embryos and embryos in which MKP-1 gene was

It is well known that activation of MAPK signaling can lead to either cell proliferation or cell death in part depending on cell types, which can be inhibited by members of the MAPK phosphatase family, including MKP-1 (8). To determine whether overexpression of MKP-1 affects cell survival in response to oxidative stress, MCF-7 cells were treated with H_2O_2 at 300 μmol/L for 5 hours, and detached dead cells and attached surviving cells were determined by trypan blue exclusion and Coomassie blue staining, respectively. As shown in Fig. 2C, upon H_2O_2 treatment, the majority of MKP-1-overexpressing cells (clones 12 and 15) remained attached (alive cells), compared with the vector control cells in which fewer cells remained attached. Furthermore, there were many more dead cells detected in H_2O_2-treated vector control cells over the MKP-1-overexpressing cells (Fig. 2D). Thus, these data suggest that overexpression of MKP-1 could inhibit MCF-7 cell death induced by oxidative damage.

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disrupted by a neomycin cassette in exon 2 (25). MKP-1+/+ and MKP-1−/− cells were treated with 300 μmol/L H₂O₂ and expression of MAPKs and MKP-1 was analyzed. As expected, MKP-1 was induced by H₂O₂ in MKP-1+/+ MEFs but not in MKP-1−/− MEFs (Fig. 4A), confirming the absence of MKP-1 in MKP-1−/− cells. We then analyzed activation of MAPKs in these cells. As shown in Fig. 4A, the levels of phosphorylated ERK were not significantly different between MKP-1+/+ and MKP-1−/− MEFs upon H₂O₂ treatment. In contrast, activation of JNK and p38 was significantly different between MKP-1+/+ and MKP-1−/− MEFs upon H₂O₂ treatment. In MKP-1−/− cells, JNK activation was detected at 2 hours compared with MKP-1+/+ cells in which the level of phosphorylated JNK returned to the basal level (Fig. 4A). Consistent with the role of MKP-1 in regulating p38, activation of p38 was not only more robust but also prolonged to 4 hours after H₂O₂ treatment in MKP-1−/− cells, compared with MKP-1+/+ cells in which the levels of phosphorylated p38 started to decrease by 2 hours and returned to basal levels by 3 hours (Fig. 4A). However, total ERK, JNK, and p38 proteins remained unchanged following H₂O₂ treatment in both cell lines (data not shown). These data further suggest that MKP-1 plays a physiologic role in negatively regulating p38 and JNK pathways in response to oxidative stress.

To test the effect of loss of MKP-1 on oxidative damage–induced cell death, we treated MKP-1+/+ and MKP-1−/− MEFs with H₂O₂ at 300 μmol/L for 24 hours and then determined cell viability. As shown in Fig. 4B, MKP-1+/+ MEFs remained attached and alive (Fig. 4B). Cell viability of MKP-1−/− MEFs decreased to 33% whereas >89% of MKP-1+/+ MEFs remained alive as determined by trypan blue exclusion (Fig. 4C). These results indicate that blockade of p38 and JNK signaling by MKP-1 may be critical for

Figure 2. Effects of MKP-1 overexpression on activation of MAPKs and cell viability. A, protein analysis of transfected MCF-7 cells. MCF-7 cells were transfected with either vector alone (lanes 1-3) or MKP-1-expressing vector (lanes 4-6), followed by selection with G418 (1 mg/mL) for 2 weeks. Individual clones were isolated and their cell lysates were analyzed with a polyclonal antibody to MKP-1. Clones 3, 12, and 15 expressed a transfected MKP-1 protein (lanes 4-6 versus lanes 1-3). B, activation of MAPKs by H₂O₂ in MCF-7 cells stably transfected with either vector (clone 1) or MKP-1 (clones 12 and 15). MCF-7 transfectants were treated with 200 μmol/L H₂O₂ for the indicated time points. Whole-cell protein lysates were prepared, and phosphorylated ERK, JNK, and p38 proteins were detected with Western blot analysis. Actin expression was used as a loading control. C, morphologic changes in MCF-7 cells transfected with either vector (V1) or MKP-1 (12 and 15) following H₂O₂ treatment. MCF-7 cells were either left untreated or treated with 300 μmol/L H₂O₂ for 5 hours and attached cells were stained with Coomassie blue. Original magnification, ×100. D, quantification of cell death. Floating cells were collected from MCF-7-V1 or MCF-7-MKP-1#12 and MCF-7-MKP-1#15 treated with H₂O₂ in (C) and dead cells were determined using the trypan blue exclusion assay. Representative of three independent experiments.
protecting cells from oxidative damage–induced death. To determine if loss of MKP-1 enhances H2O2-induced cell death through apoptosis, we tested cleavage of PARP, a hallmark of apoptosis, in cells with or without H2O2 treatment. As shown in Fig. 4D, in MKP-1−/− cells, H2O2 treatment increased PARP cleavage compared with untreated cells. In contrast, cleavage of PARP was not detected in MKP-1+/− cells treated with 300 μmol/L H2O2. Thus, these results suggest that loss of MKP-1 sensitizes cells to oxidative damage–induced death in part through apoptosis.

Discussion

MKP-1 is an early response gene, which was cloned from cells treated with growth factors and stresses, including oxidative stress (6, 10–13), but the role of MKP-1 in oxidative stress in cells has not been established. In this study, we provide evidence that induction of MKP-1 protects cells from oxidative damage–induced cell death probably through inactivation of the p38 and JNK pathways. These findings suggest that activation of the JNK and p38 pathways plays a critical role in oxidative stress–induced cell death.

MAPK signaling can be activated by a variety of stimuli and stresses, including oxidative stress. We have shown that all three MAPK pathways, including ERK, p38, and JNK, are activated upon H2O2 treatment in MCF-7 (Fig. 1A). It has been known that activation of ERK can lead to cell proliferation, whereas activation of JNK and p38 causes cell death. The net outcome of MAPK activation is dependent on many factors, including the type of stimulus or cell context. Although H2O2 activates all three MAPK kinases (Fig. 1A and B), H2O2 caused MCF-7 cell death (Fig. 2), suggesting that ERK pathway is not important for H2O2-induced cell death. In fact, we found that inhibition of ERK by the MEK1/2 inhibitor U0126 enhances H2O2-induced cell death (data not shown). Therefore, H2O2-induced cell death is primarily through activation of the p38 and JNK pathways. Consistent with the role of JNK and p38 in H2O2-induced cell death, we have shown that both p38 and JNK were activated upon H2O2 treatment (Figs. 1, 2, and 4). Although the original study suggested the ERK is the substrate of MKP-1 (12), several studies indicated that p38 and JNK are the preferential substrates of MKP-1 in cells in response to a variety of stimuli (14, 19, 26). In this study, we found that JNK and p38 were the preferential targets for MKP-1 because loss of MKP-1 by siRNA-mediated down-regulation of MKP-1 or MKP-1 knockout MEFs resulted in a higher level of phosphorylated p38 and JNK than cells with MKP-1. We have also shown that loss of MKP-1 in MCF-7 or MEFs results in an increase in the levels of phosphorylated ERK but such increase was not significant (Figs. 3 and 4). This suggests that ERK is still a physiologic target of MKP-1 in cells in response to oxidative stress. Therefore, we conclude that under oxidative stress, induction of MKP-1 preferentially inactivates JNK and p38 pathways, leading to cell survival.

Because activation of MAPKs could lead to either cell proliferation or apoptosis, it is expected that modulation of MAPK activity could affect cell proliferation or apoptosis. It is well known that MAPKs are negatively regulated by the dual-specificity MAPK phosphatases, including MKP-1. Therefore, it is reasonable to assume that any changes in the protein level of MAPK phosphatases could lead to inactivation or activation of MAPKs, resulting in an increase or decrease in the cellular responses. We have shown that overexpression of MKP-1 inhibits H2O2-induced activation of JNK and p38 and increases cell resistance to H2O2-induced death. Consistent with this, it has been shown that conditional expression of MKP-1 confers human leukemia U937 resistant to UV-induced apoptosis (19). Because UV-induced apoptosis involves activation of the JNK pathway, overexpression of MKP-1 blocks UV-mediated JNK activation, reduces apoptosis, and thus increases cell survival (19). It has also been shown that overexpression of MKP-1 can inhibit Fas ligand–induced apoptosis in human prostate DU145 cells (29). Because a number of anticancer drugs kill cancer cells via the JNK apoptotic pathway, it is expected that blockade of JNK activation could inhibit anticancer activity. In addition, overexpression of MKP-1 has been shown to inhibit cisplatin-induced apoptosis via inactivation of the JNK pathway in human embryonic kidney 293 cells (20).

There are three forms of cell death, including apoptosis, necrosis, and autophagy (30). It has been shown that overexpression of MKP-1 can inhibit apoptosis by inhibiting p38 and JNK apoptotic

**Figure 3.** Silencing of MKP-1 expression with siRNA and H2O2-induced MAPK activation and cell death. A, effect of MKP-1 and MAPK activation by transfecting MKP-1 siRNA. MCF-7 cells were plated at 4 × 10^4 per well in six-well plates. The next day, cells were transfected with MKP-1 siRNA or control oligonucleotides using Oligofectamine (Invitrogen). After 2 days, transfected cells were equally split into three wells in 24-well plates. Thirty-six hours after transfection, cells were treated with 100 or 200 μmol/L H2O2 for 3 hours, and induction of MKP-1 and activation of MAPKs, including ERK, p38, and JNK, were determined by Western blot. B, effect of cell survival by silencing MKP-1 expression with siRNA. MCF-7 cells were transfected with MKP-1 siRNA or control oligonucleotides as described in (A). After 3 days, cells were treated with 200 μmol/L H2O2 for 5 hours. Total cells including, floating cells, were harvested and stained with trypan blue. Cell survival data are expressed as percentage of total cells. Representative of two independent experiments.
MKP-1 pathways (19–21). We have shown that overexpression of MKP-1 protects MCF-7 cells from H_{2}O_{2}-induced cell death probably through inhibition of JNK and p38 activity (Fig. 2). However, it is not clear whether MKP-1 blocks JNK- and p38-mediated apoptosis. Because MCF-7 cells contain abnormal caspase-3 owing to a 47 bp deletion within exon 3 of the caspase-3 gene (31), we suspect that H_{2}O_{2} kills MCF-7 cells through a caspase-independent mechanism. However, we have shown that PARP is cleaved in MKP-1\textsuperscript{1/2} cells by H_{2}O_{2} (Fig. 4D), which suggests that loss of MKP-1 could sensitize cells to H_{2}O_{2}-induced apoptosis. Because JNK has been linked to autophagic cell death (32), it is possible that MKP-1 protects cells from necrosis/autophagy induced by H_{2}O_{2} and this issue is under investigation. Nevertheless, MKP-1 plays an important role in protecting cells from oxidative damage-induced death.

Although overexpression of MKP-1 increases cell resistance to UV-, FAS ligand-, and cisplatin-induced cell death (19, 20, 29), it is possible that the levels of MKP-1 in these systems do not reflect the physiologic conditions. To this end, Wu and Bennett (21) have recently shown that using MKP-1 knockout MEFs, MKP-1 promotes cell survival in response to serum starvation, anisomycin, and osmotic stress, and the underlying mechanism of such resistance is believed to activation of the p38 apoptotic pathway. Consistent with this, we provide evidence that MKP-1 plays an important role in protecting cells from oxidative stress-induced cell death. Our conclusion was supported by the following three experiments. First, overexpression of MKP-1 increases MCF-7 cell survival in response to H_{2}O_{2} treatment (Fig. 2). Second, knockdown of MKP-1 by MKP-1 siRNA sensitized cells to H_{2}O_{2}-induced cells death (Fig. 3). Lastly and

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**Figure 4.** H_{2}O_{2}-induced MAPK activation and cell death in primary MEFs. A, induction of MKP-1 and MAPKs upon H_{2}O_{2} treatment. MKP-1\textsuperscript{1/2} and MKP-1\textsuperscript{1/2} MEFs were treated with 300 \mu mol/L H_{2}O_{2} for 0, 1, 2, 4, and 4 hours. The total protein was extracted and then assayed for the levels of MKP-1, phosphorylated ERK, p38, and JNK by Western blot analysis. Actin was included as a loading control. B, morphologic changes in MEFs treated with 300 \mu mol/L H_{2}O_{2}. Photographs were taken after a 24-hour treatment using a Nikon microscope. Original magnification, \times 100. C, quantification of cell death induced by H_{2}O_{2} in MEFs. MKP-1\textsuperscript{1/2} and MKP-1\textsuperscript{1/2} MEFs were treated with 300 \mu mol/L H_{2}O_{2} for 24 hours as shown in (B), and total cells including floating cells were collected and stained with trypan blue. Total cells including attached and floating cells were counted, and cell survival data are expressed as percent of untreated cells. Representative of two independent experiments. D, cleavage of PARP by H_{2}O_{2}. MKP-1\textsuperscript{1/2} and MKP-1\textsuperscript{1/2} MEFs were treated with 300 \mu mol/L H_{2}O_{2} for 24 hours. The total protein was extracted and then assayed for PARP cleavage by Western blot analysis. Actin was included as a loading control.
most importantly, MKP-1−/− MEF cells were much more sensitive than MKP-1+/+ cells to H2O2-induced cell death (Fig. 4). Collectively, our results, along with other studies, suggest that MKP-1 is a general survival factor to protect cells from a variety of stresses.

In conclusion, we have found that MKP-1 protects cells from H2O2-mediated cell death. We showed that induction of MKP-1 by H2O2 correlates with inactivation of MAPKs. We also showed that overexpression of MKP-1 renders MCF-7 cells resistant to H2O2-induced cell death by inhibition of p38 and JNK activation. Importantly, we showed that loss of MKP-1 by down-regulation via siRNA or deletion of MKP-1 using MKP-1 knockout MEFs sensitizes cells to H2O2-induced cell death. Further studies are needed to determine the mechanisms by which MKP-1 inhibits JNK and p38 pathways upon oxidative damage.

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References

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