# Activation of a 36-kDa Myelin Basic Protein Kinase during Cadmium-Induced Apoptosis in Human Leukemia HL-60 Cells

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(Received December 24, 2003; Accepted January 19, 2004)

**Cadmium (Cd) is a well-known modulator of intracellular signal transduction including mitogen-activated protein kinases and protein kinase C. In this study, we investigated activation of a kinase using myelin basic protein (MBP) as a substrate during Cd-induced apoptosis in human leukemia HL-60 cells. To detect a kinase during Cd-induced apoptosis in HL-60 cells, we performed an in-gel kinase assay using MBP as a substrate and found that Cd induced the activation of a kinase with an apparent molecular mass of 36 kDa. The time-course of appearance of DNA ladders induced by Cd was consistent with that of activation of this kinase. The kinetics of activation of p36 MBP kinase was different from that of p38 mitogen-activated protein kinase (p38MAPK). Activation of p36 MBP kinase was also observed with kinetics distinct from that of activation of p38MAPK during mercuric chloride-induced apoptosis. This is the first report on activation of p36 MBP kinase during Cd- or Hg-induced apoptosis.**

**Key words** —— cadmium, mercury, apoptosis, p36 myelin basic protein kinase

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## INTRODUCTION

Cadmium (Cd) is a systemic poison affecting many cellular functions *via* direct and indirect interactions with cellular proteins, including cell surface receptors and intracellular proteins involved in intracellular signaling and gene expressions.[1] Treatment with Cd induced an increase in intracellular free calcium (Ca) concentration followed by activation of Ca-dependent intracellular signaling such as protein kinase C[2,3] and calmodulin.[4,5] Moreover, Cd can modulate cell growth, death and differentiation *via* its activation of mitogen-activated protein kinases (MAPKs).[6–8] Exposure to Cd results in immunotoxicity and injury of the liver and kidneys, and recent studies have indicated that the toxicity of Cd is partly involved in induction of apoptosis.[9–12] Although induction of apoptosis by Cd is triggered by activation of caspases which are initiators and executors of apoptosis,[8,13,14] association of the stress-induced signaling cascade with Cd-induced apoptosis remains to be proved.

A mammalian Ste20-like kinase family, including MST1 and MST2, is a unique kinase family to play a role in apoptosis both upstream and downstream of caspase activation.[15] MST1 is cleaved by caspase, and the cleaved Mst1 translocates from the cytoplasm to the nuclear followed by induction of chromatin condensation.[16] MST1 also activates some MAPKs.[17,18] These reports suggest the involvement of a mammalian Ste20-like kinase family in apoptosis induced by Cd. A substrate of a mammalian Ste20-like kinase family is myelin basic protein (MBP).[15–18]

In the present study, we employed a previously established Cd-induced HL-60 promyelocytic cell apoptosis system in combination with a kinase zymographic technique using MBP as a substrate to examine candidate signaling components that may be involved in the apoptosis signaling cascade. Activation of a MBP kinase with a molecular mass of 36 kDa (p36MBP kinase) was observed during Cd-induced apoptosis. Furthermore, p36 MBP kinase was activated in HL-60 cells treated with mercury chloride but not zinc. Thus, p36 MBP kinase appears to be a common component of the apoptotic signal transduction pathway that is induced some heavy metals.

## MATERIALS AND METHODS

**Materials** —— Cadmium acetate, mercuric chloride, zinc acetate, and zinc pyrithione were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and were dissolved in sterile water. MBP was purchased from Sigma. Antibodies for poly (ADP-ribose) polymerase (PARP), p38 and phosphorylated p38 were obtained from Cell Signaling Technology (Beverly, MA, U.S.A.).

**Cell Culture** —— Human promyelocytic leukemia HL-60 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum in a 5% $CO_2$ atmosphere at 37°C.

**DNA Fragmentation Assay** —— DNA fragmentation assays were performed as described previously.[14] Briefly, total DNA extracted from cells was electrophoresed on a 1.8% agarose gel, and DNA ladders were visualized by ethidium bromide staining.

**Immunoblot Analysis** —— Immunoblot analysis was carried out as described previously.[14,19] Briefly, cells were lysed in lysis buffer [1% Nonidet P-40, 20 mM Tris–HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM ethylenediaminetetraacetic acid]. Cell lysates were prepared by sonication, and then the lysates were centrifuged. Equal amounts of the resultant supernatant were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer onto polyvinylidene difluoride membranes. Immunoreactive bands were revealed by ECL-based detection (Amersham Pharmacia Biotech).

**In-Gel Kinase Assay** —— The in-gel kinase assay was performed essentially as described previously.[20,21] Cells were lysed in the lysis buffer, and the lysate was centrifuged. Equal amounts of the supernatant (50–100 $\mu$g) were electrophoresed in 10% SDS-polyacrylamide gel containing MBP (0.5 mg/ml) as a substrate. Following electrophoresis, SDS was removed from the gel, protein was renatured, and a kinase assay was carried out by incubating the gel in buffer containing [$\gamma$-$^{32}$P]ATP. Gels were washed, and incorporated radioactivity was quantified using an image analyzer (BAS2000, Fuji Film Co. Ltd., Japan).

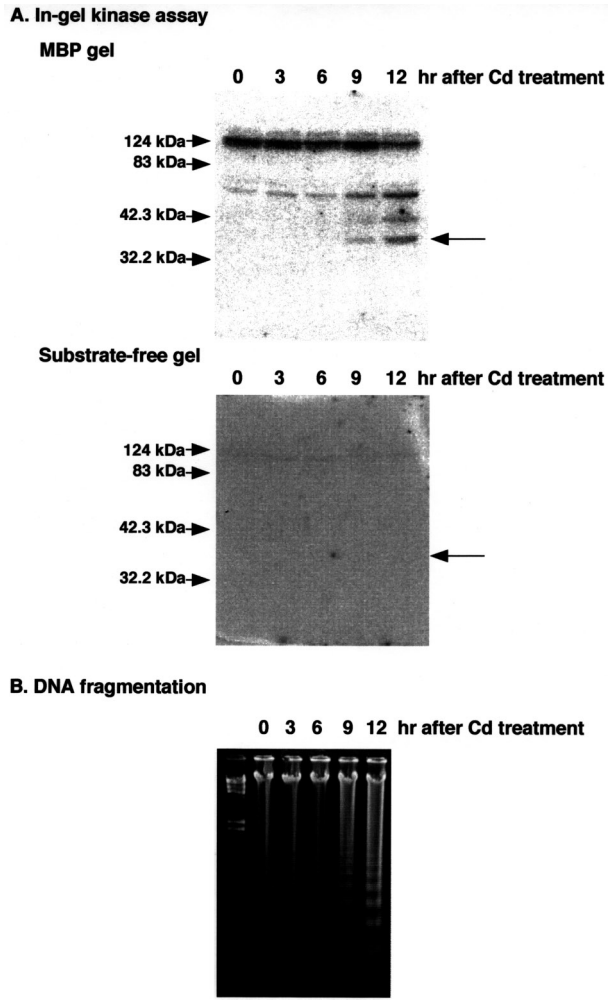


**Fig. 1.** Activation of p36 MBP Kinase during Cd-Induced Apoptosis in HL-60 Cells

Cells were treated with Cd (100 $\mu$M) for the indicated periods. A) In-gel kinase assay. The cell lysates were prepared as described in Materials and methods, and kinase activity was detected by in-gel kinase assays using MBP (0.5 mg/ml) as a substrate or not using MBP. The phosphorylated bands were detected by autoradiography. The arrow indicates a molecular mass of p36 kDa. B) Induction of DNA fragmentation by Cd. DNA extracted from the cells was electrophoresed on an agarose gel, and DNA ladder formation was visualized by ethidium bromide staining.

## RESULTS

Since MAPK family, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK, have been shown to be activated in the human promonocytic cell line U937 and in the human non-small lung carcinoma cell line CL3,[6,8] we performed an in-gel kinase assay using MBP as a substrate to determine whether Cd can activate MAPs (Fig. 1A). Cd induced a kinase with an apparent molecular mass of 36 kDa (p36 MBP kinase). When MBP was omitted from the gel, this

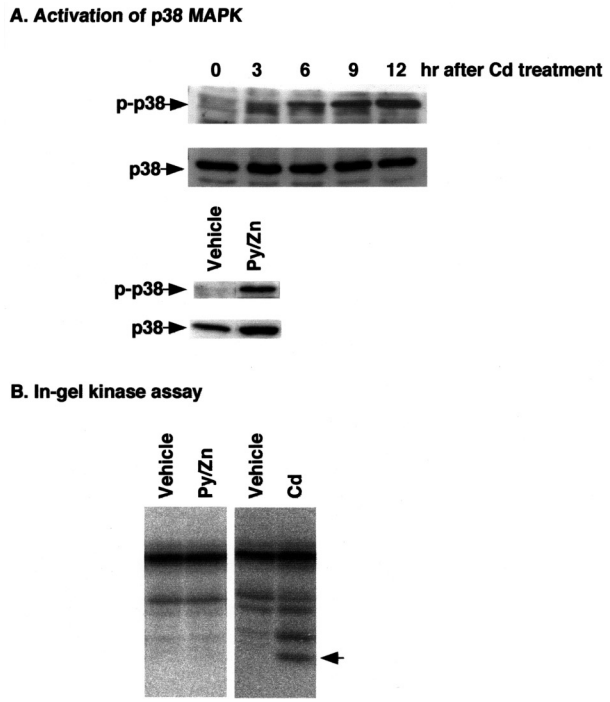


**Fig. 2.** Activation of p38 MAPK and p36 MBPK by Cd or Zn

A) Cells were treated with Cd (100 $\mu$M) for the indicated periods or 1 $\mu$M pyrithione (Py) and 25 $\mu$M Zn for 6 hr. The cell lysates were prepared as described in Materials and methods. Immunoblot analysis for p38 and active form of p38, phosphorylated p38 MAPK (p-p38), was performed using each specific antibody. B) Cells were treated with 1 $\mu$M pyrithione and 25 $\mu$M Zn (Py/Zn) for 6 hr, and in-gel kinase assay was performed as described above (Fig. 1). The arrow indicates a molecular mass of p36 kDa.

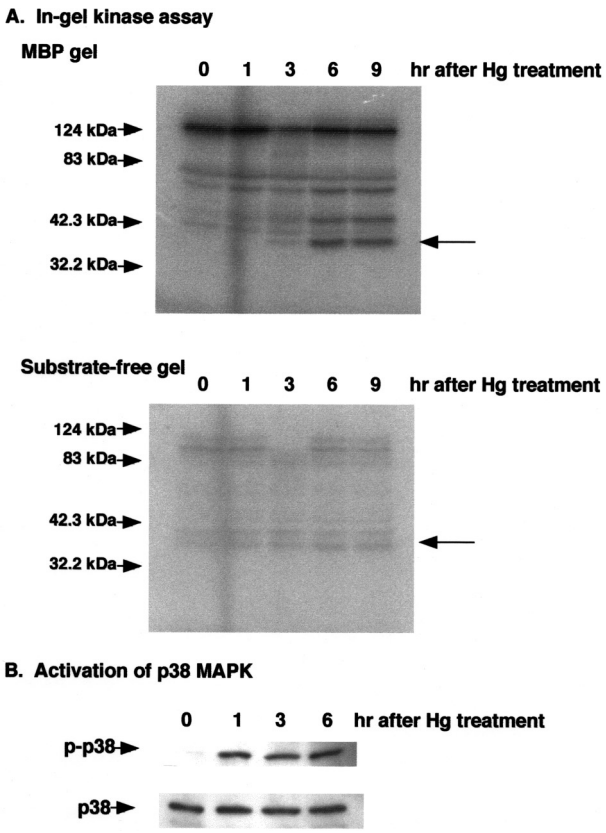


**Fig. 3.** Activation of p36 MBP Kinase during Hg-Induced Apoptosis in HL-60 Cells

Cells were treated with Hg (30 $\mu$M) for the indicated periods. In-gel kinase assay (A) was performed as described in Fig. 1. Activation of p38 MAPK (B) was examined as described above (Fig. 2). The arrow indicates a molecular mass of p36 kDa.

band was not detected, indicating that this band was not due to autophosphorylation of p36 MBP kinase. Time-course change of appearance of p36 MBP kinase was paralleled to that of formation of DNA ladders, a typical sign of apoptosis (Fig. 1B). p38 MAPK has been shown to be activated in Cd-treated cells and to have a molecular mass similar to that of p36 MBP kinase.[6,8] So we next examined activation of p38 MAPK in Cd-treated HL-60 cells. As shown in Fig. 2, phosphorylation of p38 MAPK, a sign of activation of p38 MAPK, was observed 3 hr after treatment with Cd. In contrast, p36 MBP kinase was detected 9 hr after Cd treatment, showing a kinetics pattern different to that of p38 MAPK (Figs. 1A and 2A). Increase of intracellular zinc (Zn) level induced apoptosis through activation of p38 MAPK in HL-60 cells.[19] So, we investigated the activation of p36 MBP kinase in Zn-induced apoptosis. As shown in Figs. 2A and 2B, the increase of intracellular Zn level by treatment of the cells with Zn and Zn ionophore, pyrithione, activated p38 MAPK but not p36 MBP kinase. Moreover, we also found that p36 MBP kinase was activated during mercuric chloride (Hg)-induced apoptosis. Although p38 MAPK was activated by treatment with Hg, the kinetics pattern of the activation of p36 MBP kinase was different from that of p38 MAPK (Figs. 3A and 3B).

## DISCUSSION

It has been shown that MAPK family, including ERK, JNK and p38 MAPK, were activated in Cd-treated cells, and the activation of JNK and that of p38 MAPK were partly involved in Cd-induced apoptosis.[6,8] In this study, we investigated whether Cd activates the signaling pathway that leads to the activation of members of the MAPK family. Interestingly, we found that Cd activated a kinase with an apparent molecular mass of 36 kDa detected by an in-gel kinase assay using MBP as a substrate. As demonstrated previously, JNK and ERK were acti-

vated by treatment with Cd, and both of them can phosphorylate MBP as a substrate.[6,7,21,22] p36 MBP kinase was distinct from JNK (pp46/56) and ERK (pp44/42) in molecular weight. Since different techniques were used to investigate activities of p36 MBP kinase and p38 MAPK, it is difficult to compare the involvement of p36 MBP kinase and p38 MAPK in Cd- or Hg-induced apoptosis. But, increase of intracellular Zn levels, an apoptosis-inducing stimuli,[19] activated p38 MAPK but not p36 MBP kinase. The possibility that activation of p36 MBP kinase was that of p38 MAPK could be also excluded with distinct kinetics.

Previous studies have shown that p36 MBP kinase was activated during anticancer drug-induced apoptosis and that p36 MBP kinase is a fragment of MST1/Krs2 or MST2/Krs1, which are mammalian serine/threonine kinases homologous to the budding yeast Ste-20, processed by a broad-spectrum inhibitor of caspases Z-Asp-$CH_2$-DCB sensitive caspases.[23–26] Indeed, treatment with Z-Asp-$CH_2$-DCB attenuated Cd-induced apoptosis in HL-60 cells[14] and Hg induced apoptosis *via* Z-Asp-$CH_2$-DCB-sensitive caspases in HL-60 cells[27] (our unpublished data). Although an acute increase in intracellular Zn level also induced Z-Asp-$CH_2$-DCB-sensitive apoptosis in HL-60 cells,[15] the acute increase in intracellular Zn did not activate p36 MBP kinase. Graves *et al*. indicated that functional activation of MST1 during apoptosis requires both caspase-mediated proteolysis of MST1 and phosphorylation of MST1.[18] It is true that acute elevation of intracellular Zn levels activates caspases, but the phosphorylation of MST1 may not occur. Taken together, the findings of previous studies and our study suggest that p36 MBP kinase is activated during apoptosis induced by some heavy metals. MST1/Krs2 is localized in the cytoplasm, and cleaved MST1/Krs2 by activation of caspase translocates into the nucleus.[15,16] The translocation of cleaved MST1/Krs2 promotes morphological change of apoptosis including chromatin condensation.[16] Consideration of the involvement of Z-Asp-$CH_2$-DCB-sensitive caspases in Cd- or Hg-induced apoptosis, MST1/Krs2 may play a role of apoptosis induced by the heavy metals. Although further detail investigations of the processing of MST1/Krs2 and MST2/Krs1 are needed for clarification of the biological function of p36 MBP kinase during heavy metal-induced apoptosis, this is the first report to describe the activation of p36 MBP kinase during Cd- or Hg-induced apoptosis.

**Acknowledgements** This study was supported in part by a Grant-in-Aid for General Scientific Research from the Ministry of Education, Sciences, Sports and Culture of Japan.

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