Metal Response Element-binding Transcription Factor-1 Is Activated by Degradation of Metallothionein

Tomoki Kimura,[a] Fumika Okumura,[a] Ikuyo Oguro,[b] Tsuyoshi Nakanishi,[b,1] Tomomichi Sone,[a] Masakazu Isobe,[a] Keiichi Tanaka,[b,2] and Norio Itoh*,[b]

[a]Department of Toxicology, Faculty of Pharmaceutical Sciences, Setsunan University, 45–1 Nagaotoge-cho, Hirakata, Osaka 573–0101 Japan and [b]Department of Toxicology, Graduate School of Pharmaceutical Sciences, Osaka University, 1–6 Yamada-oka, Suita, Osaka 565–0871, Japan

(Received July 28, 2008; Accepted December 2, 2008; Published online December 5, 2008)

Cytosolic zinc-binding protein, metallothionein (MT), is normally saturated with Zn. It is thought that Zn-saturated MT (Zn-MT) acts as a major intracellular Zn pool. Metal-response element-binding transcription factor-1 (MTF-1) plays an important role in Zn-mediated *MT* transcription. Here, we showed that degradation of Zn-MT activates MTF-1. We measured activated MTF-1 using an electrophoretic mobility shift assay. Interleukin-6 induced *MT* expression and increased MTF-1 activity. MTF-1 activation was not observed in MT-overexpressing cells. MT-dependent MTF-1 activation was observed only after treating MT-overexpressing cells with cycloheximide (CHX), a protein synthesis inhibitor. CHX-treatment increased the degradation/synthesis ratio of protein. An increase in the degradation/synthesis ratio for the MT protein is expected to increase the level of labile Zn and activate MTF-1. Recombinant MTF-1 was activated by H_2O_2 only in the presence of Zn-MT. Oxidative stress activated MTF-1 DNA-binding activity in primary cultured hepatocytes but not in MT-deficient hepatocytes. These findings suggest that degradation of Zn-MT activates MTF-1, and that MT plays an important role in zinc-mediated signal transduction.

Key words —— metal response element-binding transcription factor-1, metallothionein, zinc, oxidative stress

INTRODUCTION

Zn is an essential micronutrient involved in structural and regulatory cell functions.[1)] Metallothionein (MT), the cytosolic Zn-binding protein, is normally saturated with Zn,[2)] and is thought to maintain Zn homeostasis.[3,4)] In mammals, four *MT* genes have been cloned, *MT-I* to *IV*. *MT-I* and *-II* are expressed in many cell types in various organs and tissues, and in most cultured cells. By comparison, *MT-III* and *-IV* show a very restricted cell-type-specific expression pattern.[5)] The present study focuses on *MT-I* and *-II*. Transcription of *MT* is induced by heavy metals, including Zn, and is mediated by metal response element (MRE), a *cis*-element.[6–8)] MRE-binding transcription factor-1 (MTF-1) is a highly conserved Zn-finger transcription factor that regulates the transcription of *MT*.[9–11)] The overloading of Zn in MT activates MTF-1, and then induces MT synthesis. Newly synthesized metal-free MT (apo-thionein) binds to Zn. Finally, a decrease in available Zn causes inactivation of MTF-1. In summary, MT synthesis induces MTF-1 inactivation, although, it is known that MT activates nuclear factor-κB (NF-κB), which requires zinc for activation.[12,13)] Independent findings suggest that Zn-MT acts as a Zn donor for some enzymes[14,15)] and transcription related factors, including Sp1 and the estrogen receptor.[16–18)] The synthesis and degradation of MT, especially the degradation of Zn-MT, have implications for the availability of Zn and Zn-mediated signal transduction. In this study, we examined whether degradation of MT activates MTF-1.

[1]Present address: Laboratory of Hygienics, Gifu Pharmaceutical University, 5–6–1 Mitahora-higashi, Gifu 502–8585

[2]Present address: Laboratory of Toxicology, Faculty of Pharmacy, Osaka Ohtani University, 3–11–1 Nishikiori-kita, Tondabayashi, Osaka 584–8540

*To whom correspondence should be addressed: Department of Toxicology, Graduate School of Pharmaceutical Sciences, Osaka University, 1–6 Yamada-oka, Suita 565–0871, Japan. Tel.: +81-6-6879-8232; Fax: +81-6-6879-8234; E-mail: n-itoh@phs.osaka-u.ac.jp

MATERIAL AND METHODS

Cells and Cell Culture —— Rat H4IIEC3 hepatoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2/95% air. For MT stable expression, Chinese Hamster Lung (CHL) cells were transfected with pBPVGRPMT or pBPVGRPTM by the calcium phosphate precipitation method as previously described.[19] After G418 selection and cloning, the presence of the transgene was detected by PCR. The concentration of MT was determined using the ^{109}Cd/hemoglobin affinity assay. Mouse hepatocytes were isolated by the *in situ* two step collagenase perfusion method as previously described.[20] The hepatocytes were plated in Williams' medium E containing 5% FBS, 1 μM dexamethasone (Dex) and 1 μM insulin.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays —— Nuclear extracts were prepared by the method of Dalton *et al.*[3] Binding reactions in the electrophoretic mobility shift assays (EMSA) were performed using binding buffer containing 12 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.9), 5 mM $MgCl_2$, 60 mM KCl, 0.5 mM dithiothreitol, 2 μg of poly(dI-dC), 2.5 pmol of mutant MRE oligonucleotide (mutMREs; 5′-GATCCAGGGAGCTAATTACTCCGCCCGAAAAGTA-3′), and 5–50 fmol of ^{32}P-labeled MRE oligonucleotide (MREs; 5′-GATCCAGGGAGCTCTGCACACGGCCCGAAAAGTA-3′), with 0.2–0.5 μl of recombinant human MTF-1 protein (1.0 pmol) in a total volume of 20 μl. After incubation on ice for 30 min, protein-DNA complexes were separated electrophoretically at 4°C on 10% nondenaturing polyacrylamide gels. The gels were dried and the labeled complexes were detected by radioluminography.

***In Vitro* Transcription/Translation** —— Recombinant human MTF-1 proteins were synthesized *in vitro* with the TnT® T7 quick coupled transcription/translation system (Promega, Madison, WI, U.S.A.) using 1.0 μg of plasmid (pRc/CMV-MTF) per reaction as previously described.[21]

Preparation of Zinc-saturated MT —— Zn-MT was prepared by adding zinc (10 μmol) to the apo-MT solution (0.1 μmol). The reaction mixture was subjected to dialysis to purify Zn-MT. The apo-MT was prepared from Cd, Zn-MT (Sigma, St. Louis, MO, U.S.A.) by adding 1 mM HCl. The reaction mixture was subjected to dialysis to purify the apo-MT.[22]

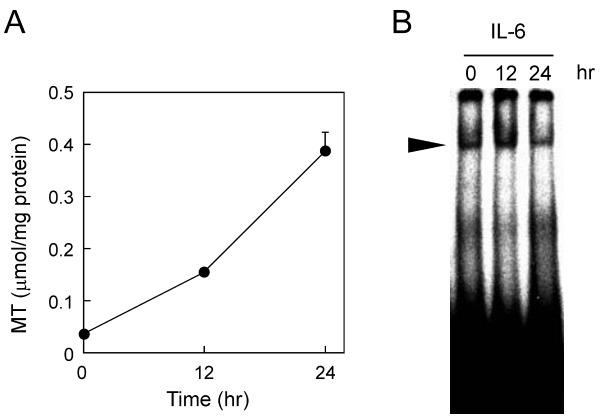

Fig. 1. Effect of IL-6 on MTF-1 DNA-binding Activity

H4IIEC3 cells were treated with rhIL-6 (500 U/ml) plus Dex (1 μM). Cells were harvested at the indicated times and then MT concentration (A) and MTF-1 DNA-binding activity (B) were measured. The arrowhead indicates the specific binding of MREs. Experiments were reproduced three times with similar results. Results from a representative experiment are shown.

RESULTS AND DISCUSSION

To determine the effect of MT on MTF-1 activity, we compared the DNA-binding activity in MT-induced cells and in cells stably overexpressing MT. The MT concentration increased in Interleukin (IL)-6 treated cells (Fig. 1A). MTF-1 DNA-binding activity transiently increased after 12 hr of IL-6-treatment (Fig. 1B). In previous experiments using the luciferase reporter assay we observed activation of MTF-1.[20] By comparison, MTF-1 DNA-binding activity did not increase in MT-overexpressing cells (approximately 300-fold MT concentration, Fig. 2A and 2B). The IL-6-induced transient activation of MTF-1 was not observed in MT-deficient (MT-KO) cells (Fig. 4). This result indicates that the IL-6-induced activation of MTF-1 correlates positively with induction of MT. It is possible that activation of MTF-1 required a dramatic change in the concentration of MT, but not a high concentration of MT. A drastic change of the concentration of MT may bring a drastic change of labile intracellular zinc pool. The activation of MTF-1 after IL-6 treatment may be caused by an increase of labile zinc pool. To confirm this possibility, an increase of labile zinc pool was caused by cycloheximide (CHX)-treatment, which blocked protein synthesis (included MT synthesis). CHX-treatment causes a decrease in the concentration

of MT in IL-6 treated cells but not in IL-6-free cells (Fig. 3A). CHX-induced activation of MTF-1 was observed only in IL-6 treated cells (Fig. 3B). By comparison, activation of MTF-1 was not observed in MT-KO cells (Fig. 4A and 4B). The activation of MTF-1 was striking in cells overexpressing MT (Fig. 3C). IL-6-mediated MT induction occurred during the inflammation reaction. Once the MT synthesis/degradation balance is disrupted, the activity of MTF-1 will be altered. It is known that MT-KO mice are hypersensitive to lipopolysaccharide (LPS)-induced coagulatory disturbance[23] and LPS/D-galactosamine-induced lethality.[24, 25] It is possible that this hypersensitivity is related to a lack of MT-mediated MTF-1 activation.

The accelerated degradation of MT is observed under oxidative stress conditions.[26, 27] For this reason, we analyzed the effect of the oxidative agents H_2O_2 and *tert*-butyl hydroperoxide (*t*BH), on the activity of MTF-1. Incubation with zinc induced the formation of MTF-1/MRE complexes (Fig. 5A). By comparison, incubation with H_2O_2 alone was not able to induce the formation of MTF-1/MRE complexes. Incubation with H_2O_2 and Zn-saturated MT induced the formation of MTF-1/MRE complexes in a dose-dependent manner. These results

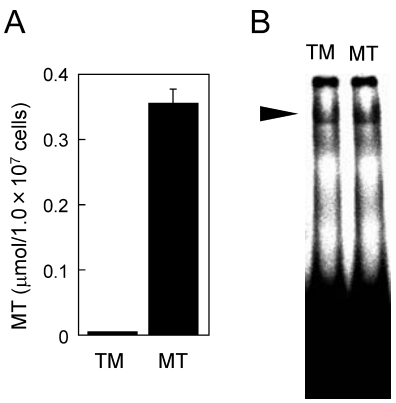

Fig. 2. Effect of MT-I Overexpression on MTF-1 DNA-binding Activity

CHL cells were stably transfected with pBPVGRMT (CHL-MT) and pBPVGRTM (CHL-TM). Cultured cells were harvested and then MT concentration (A) and MTF-1 DNA-binding activity (B) were measured. Experiments were reproduced three times with similar results. Results from a representative experiment are shown.

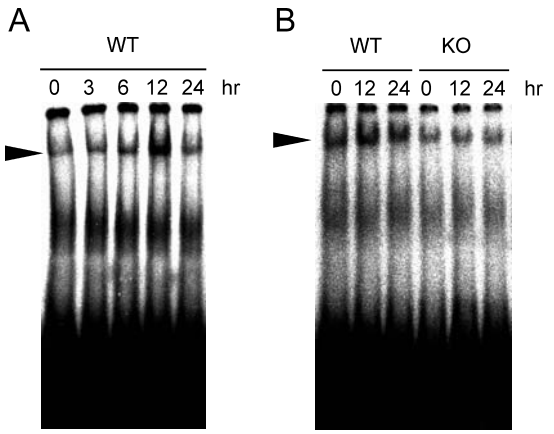

Fig. 4. Effect of IL-6 on MTF-1 DNA-binding Activity in Hepatocytes Derived from wild type (WT) and MT-KO Mice

Primary hepatocytes were treated with rhIL-6 (500 U/ml) plus Dex (1 μM). Hepatocytes were harvested at the indicated times and then MTF-1 DNA-binding activity were measured. Experiments were reproduced three times with similar results. Results from a representative experiment are shown.

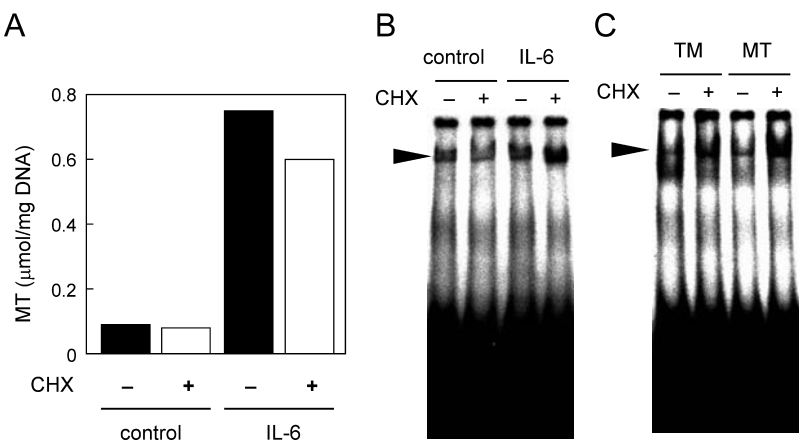

Fig. 3. Effect of CHX on MTF-1 DNA-binding Activity

H4IIEC3 cells (A, B), which incubated with rhIL-6 plus Dex for 12 hr, and CHL-MT cells (C) were treated with CHX (10 μg/ml) for 3 hr. Cells were harvested and then MT concentration (A) and MTF-1 DNA-binding activity (B, C) were measured. Experiments were reproduced three times with similar results. Results from a representative experiment are shown.

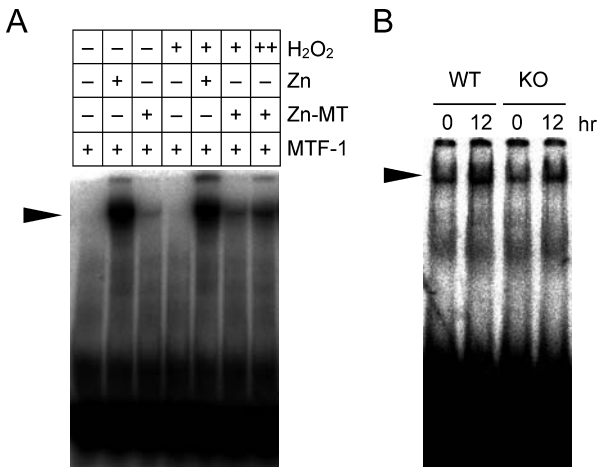

Fig. 5. Effect of Oxidative Agents on MTF-1 DNA-binding Activity

(A) Incubation of MTF-1 (1.0 pmol) was carried out in buffer containing 50 μM Zn and 1 μM (+) or 10 μM (++) H_2O_2 at 30°C for 15 min, and then binding reactions for EMSA were performed. (B) Nuclear extracts were prepared from hepatocytes treated with 20 μM *t*BH for 12 hr. MTF-1 DNA-binding was analyzed by EMSA. Experiments were reproduced three times with similar results. Results from a representative experiment are shown.

suggest that H_2O_2 induces the degradation of MT and increases the level of free Zn. This hypothesis is supported by the observation that *t*BH-induced MTF-1 activation does not occur in MT-KO cells (Fig. 5B). In summary, MT acts as a sensor for oxidative stress. Under oxidative stress conditions, Zn is released from Zn-MT and induces the activation of MTF-1. Oxidative stress-induced *MT* transcription is mediated by certain *cis*-elements, MRE, and the antioxidant response element (ARE).[28] The identity of the transcriptional factors involved in oxidative stress-induced ARE-mediated MT transcription is unclear, although, activation by MTF-1 is reported to induce this process.[29] Using an *in vitro* transcription system, Zhang *et al.* showed that Zn-saturated MT is required for oxidative stress-mediated activation of MT transcription.[29] It is unclear which proteins are the main zinc sources for activation of MTF-1. As shown in Fig. 5B, *t*BH-induced MTF-1 activation was not observed in MT-KO cells. Our result suggests that MT is the main zinc source for MTF-1 activation in response to oxidative stress.

Here, we showed that MT induces MTF-1 activity. In IL-6 treated cells, it is possible that activation of MTF-1 is induced by a dramatic change in the concentration of MT. Oxidative stress induced the activation of MTF-1 via degradation of Zn-MT. IL-6-mediated MT induction is observed during endotoxemia. Oxidative stress mediated MT induction occurs in response to a change in environmental conditions. For this reason, there are many candidate target genes for MTF-1. A change in MTF-1 activity may be an adaptive response to changes in environmental conditions. Further studies are necessary to reveal the mechanisms controlling MT-mediated activation of MTF-1.

REFERENCES

1) Vallee, B. L. and Falchuk, K. H. (1993) The biochemical basis of zinc physiology. *Physiol. Rev.*, **73**, 79–118.
2) Krezel, A., Hao, Q. and Maret, W. (2007) The zinc/thiolate redox biochemistry of metallothionein and the control of zinc ion fluctuations in cell signaling. *Arch. Biochem. Biophys.*, **463**, 188–200.
3) Dalton, T., Fu, K., Palmiter, R. D. and Andrews, G. K. (1996) Transgenic mice that overexpress metallothionein-I resist dietary zinc deficiency. *J. Nutr.*, **126**, 825–833.
4) Kelly, E. J., Quaife, C. J., Froelick, G. J. and Palmiter, R. D. (1996) Metallothionein I and II protect against zinc deficiency and zinc toxicity in mice. *J. Nutr.*, **126**, 1782–1790.
5) Ghoshal, K. and Jacob, S. T. (2001) Regulation of metallothionein gene expression. *Prog. Nucleic Acid Res. Mol. Biol.*, **66**, 357–384.
6) Searle, P. F., Stuart, G. W. and Palmiter, R. D. (1985) Building a metal-responsive promoter with synthetic regulatory elements. *Mol. Cell. Biol.*, **5**, 1480–1489.
7) Stuart, G. W., Searle, P. F. and Palmiter, R. D. (1985) Identification of multiple metal regulatory elements in mouse metallothionein-I promoter by assaying synthetic sequences. *Nature*, **317**, 828–831.
8) Culotta, V. C. and Hamer, D. H. (1989) Fine mapping of a mouse metallothionein gene metal response element. *Mol. Cell. Biol.*, **9**, 1376–1380.
9) Radtke, F., Heuchel, R., Georgiev, O., Hergersberg, M., Gariglio, M., Dembic, Z., Schaffner, W. (1993) Cloned transcription factor MTF-1 activates the mouse metallothionein I promoter. *EMBO J.*, **12**, 1355–1362.
10) Brugnera, E., Georgiev, O., Radtke, F., Heuchel, R., Baker, E., Sutherland, G. R. and Schaffner, W. (1994) Cloning, chromosomal mapping and characterization of the human metal-regulatory transcription factor MTF-1. *Nucleic Acids Res.*, **22**, 3167–3173.
11) Otsuka, F., Iwamatsu, A., Suzuki, K., Ohsawa, M., Hamer, D. H. and Koizumi, S. (1994) Purification

and characterization of a protein that binds to metal responsive elements of the human metallothionein IIA gene. *J. Biol. Chem.*, **269**, 23700–23707.
12) Abdel-Mageed, A. B. and Agrawal, K. C. (1998) Activation of nuclear factor kappaB: potential role in metallothionein-mediated mitogenic response. *Cancer Res.*, **58**, 2335–2338.
13) Kanekiyo, M., Itoh, N., Kawasaki, A., Matsuda, K., Nakanishi, T. and Tanaka, K. (2002) Metallothionein is required for zinc-induced expression of the macrophage colony stimulating factor gene. *J. Cell. Biochem.*, **86**, 145–153.
14) Li, T. Y., Kraker, A. J., Shaw, C. F., 3rd and Petering, D. H. (1980) Ligand substitution reactions of metallothioneins with EDTA and apo-carbonic anhydrase. *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 6334–6338.
15) Krezel, A. and Maret, W. (2008) Thionein/metallothionein control Zn(II) availability and the activity of enzymes. *J. Biol. Inorg. Chem.*, **13**, 401–409.
16) Zeng, J., Heuchel, R., Schaffner, W. and Kagi, J. H. (1991) Thionein (apometallothionein) can modulate DNA binding and transcription activation by zinc finger containing factor Sp1. *FEBS Lett.*, **279**, 310–312.
17) Cano-Gauci, D. F. and Sarkar, B. (1996) Reversible zinc exchange between metallothionein and the estrogen receptor zinc finger. *FEBS Lett.*, **386**, 1–4.
18) Roesijadi, G., Bogumil, R., Vasak, M. and Kagi, J. H. (1998) Modulation of DNA binding of a tramtrack zinc finger peptide by the metallothionein-thionein conjugate pair. *J. Biol. Chem.*, **273**, 17425–17432.
19) Kanekiyo, M., Itoh, N., Kawasaki, A., Tanaka, J., Nakanishi, T. and Tanaka, K. (2001) Zinc-induced activation of the human cytomegalovirus major immediate-early promoter is mediated by metallothionein and nuclear factor-kappaB. *Toxicol. Appl. Pharmacol.*, **173**, 146–153.
20) Kimura, T., Itoh, N., Takehara, M., Oguro, I., Ishizaki, J., Nakanishi, T. and Tanaka, K. (2002) MRE-binding transcription factor-1 is activated during endotoxemia: a central role for metallothionein. *Toxicol. Lett.*, **129**, 77–84.
21) Kimura, T., Itoh, N., Sone, T., Tanaka, K. and Isobe, M. (2004) C-terminal deletion mutant of MRE-binding transcription factor-1 inhibits MRE-driven gene expression. *J. Cell. Biochem.*, **93**, 609–618.
22) Rana, U., Kothinti, R., Meeusen, J., Tabatabai, N. M., Krezoski, S. and Petering, D. H. (2008) Zinc binding ligands and cellular zinc trafficking: apo-metallothionein, glutathione, TPEN, proteomic zinc, and Zn-Sp1. *J. Inorg. Biochem.*, **102**, 489–499.
23) Inoue, K., Takano, H., Shimada, A., Wada, E., Yanagisawa, R., Sakurai, M., Satoh, M. and Yoshikawa, T. (2006) Role of metallothionein in coagulatory disturbance and systemic inflammation induced by lipopolysaccharide in mice. *FASEB J.*, **20**, 533–535.
24) Kimura, T., Itoh, N., Takehara, M., Oguro, I., Ishizaki, J. I., Nakanishi, T. and Tanaka, K. (2001) Sensitivity of metallothionein-null mice to LPS/D-galactosamine-induced lethality. *Biochem. Biophys. Res. Commun.*, **280**, 358–362.
25) Kimura, T., Koujitani, T., Itoh, N., Takehara, M., Oguro, I., Ishizaki, J., Nakanishi, T. and Tanaka, K. (2001) Metallothionein-null mice are sensitive to endotoxin/D-galactosamine-induced hepatotoxicity. *J. Health Sci.*, **47**, 310–313.
26) Maret, W. (1995) Metallothionein/disulfide interactions, oxidative stress, and the mobilization of cellular zinc. *Neurochem. Int.*, **27**, 111–117.
27) Maret, W. and Vallee, B. L. (1998) Thiolate ligands in metallothionein confer redox activity on zinc clusters. *Proc. Natl. Acad. Sci. U.S.A.*, **95**, 3478–3482.
28) Andrews, G. K. (2000) Regulation of metallothionein gene expression by oxidative stress and metal ions. *Biochem. Pharmacol.*, **59**, 95–104.
29) Zhang, B., Georgiev, O., Hagmann, M., Gunes, C., Cramer, M., Faller, P., Vasak, M. and Schaffner, W. (2003) Activity of metal-responsive transcription factor 1 by toxic heavy metals and H_2O_2 in vitro is modulated by metallothionein. *Mol. Cell. Biol.*, **23**, 8471–8485.