— Review —

Mechanisms of Heavy Metal Sensing by Metal Response Element-binding Transcription Factor-1

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Heavy metal homeostasis and detoxification systems are often regulated by changes in gene transcription. In higher eukaryotes, metal response element (MRE)-binding transcription factor-1 (MTF-1) is the only known metal-sensing transcription factor and zinc is the only heavy metal which can reversibly and directly activate the DNA-binding activity of MTF-1, leading to its nuclear retention, promoter binding and induction or repression of transcription. Although, cadmium, copper and oxidative stresses can cause the activation of the DNA-binding activity of MTF-1 *in vivo,* they apparently do so, at least in part, by causing the redistribution of intracellular zinc. MTF-1-dependent metal-sensing transcription mechanisms are not fully understood but clearly involve zinc binding to its unique zinc finger domain. Recently, zinc has been shown to induce the formation of a co-activator complex containing MTF-1 and the histone acetyltransferase p300 which plays an essential role in the activation of mouse *metallothionein-I* (MT-I) gene transcription. In this review, we focus on current understanding of the mechanisms by which MTF-1 senses heavy metals and activates gene expression.

Key words —— metal response element-binding transcription factor-1, metallothionein, zinc, cadmium, heavy metal

INTRODUCTION

Zinc and other essential metal ions play diverse role in many biological processes by serving as structural components of proteins, as essential cofactors in enzymes and as modulators of signal transduction cascades within the cell.[1] However, all metal ions are cytotoxic at high intracellular concentrations. Metal-responsive control of gene expression allows organisms to regulate the available concentration of essential metal ions (such as zinc, copper, and iron), and restricts intracellular concentration of toxic metals (such as cadmium and mercury) within an acceptable range.

Metal response element (MRE)-binding transcription factor-1 (MTF-1) is a zinc finger transcription factor, that regulates metal-responsive gene expression.[2–4] MTF-1 has been identified in a wide range of eukaryotes including human (Entrez GeneID: 4520), mouse (GeneID: 17764), rat (GeneID: 362591), chimpanzee (GeneID: 456766), cow (GeneID: 509960), dog (GeneID: 608127), *Drosophila melanogaster* (GeneID: 39089), *Takifugu rubripes* (GeneID: 446075), zebrafish (GeneID: 195821), trout (GeneID: 100136228) and chicken (GeneID: 42821). *Metallothionein-I* (*MT-I*) and *MT-II* gene are the well studied MTF-1 target genes. MTF-1 is the essential factor for zinc-induced *MT-I* and *MT-II* gene expression.[5] MTs are small, cysteine-rich proteins that can bind many divalent heavy metal ions such as zinc, cadmium, copper and mercury. MT is thought to be an important intracellular storage site for zinc and possibly other essential trace elements.[6,7] In addition, tolerance to heavy metal toxicity is known to be due to the induction of MT, which sequesters heavy metals and lowers its concentra-

*To whom correspondence should be addressed: Department of Toxicology, Faculty of Pharmaceutical Sciences, Setsunan University, 45–1 Nagaotoge-cho, Hirakata, Osaka 573–0101, Japan. Tel.: +81-72-866-3107; Fax: +81-72-866-3107; E-mail: tomoki@pharm.setsunan.ac.jp

tion at critical intracellular sites.[8–10] MTF-1 also up-regulates *Znt1* (*Slc30a1*, a zinc efflux transporter gene).[11] These findings firmly establish a role for mammalian MTF-1 plays in zinc homeostasis and tolerance to heavy metal toxicity. In this article, we focus on current understanding of the mechanisms by which MTF-1 senses heavy metals and activates gene expression.

MTF-1 BINDS TO *cis*-ELEMENT TERMED MRE IN RESPONSE TO HEAVY METALS

Induction of *MT* genes through MTF-1 is mediated by multiple copies of a 12 base pair *cis*-element termed the MRE.[12–16] The MRE consensus (5′-TGCRCNC-3′) is the highly conserved (Fig. 1) and is found in other MTF-1-regulated genes, including *Znt1*, *Gclc* (glutamate-cysteine ligase catalytic subunit gene), *Ndrg1* (N-myc downstream regulated 1 gene), *Sepw1* (selenoprotein W, muscle 1 gene) and *Zip10* (*Slc39a10*, a zinc influx transporter gene).[11,17,18] Zinc can reversibly and directly activate the DNA-binding activity of MTF-1 as measured in an electrophoretic mobility shift assay.[19] MTF-1 binds to the MRE in response to zinc, and then induces transcription. In comparison, the DNA-binding activity of MTF-1 is activated *in vitro* by zinc, but not by cadmium, copper or other heavy metals.[20] In addition, mammalian MTF-1 can act as a zinc sensor in yeast, but not as a sensor of cadmium.[21] Genomic footprinting and chromatin immunoprecipitation (ChIP) assay show that zinc, cadmium and copper activate the DNA-binding activity of MTF-1 *in vivo* in mammalian cells. The mechanisms of cadmium and copper-induced gene transcription via MTF-1 were examined using a cell-free, MTF-1-dependent transcription system.[22] Transcriptional induction by zinc

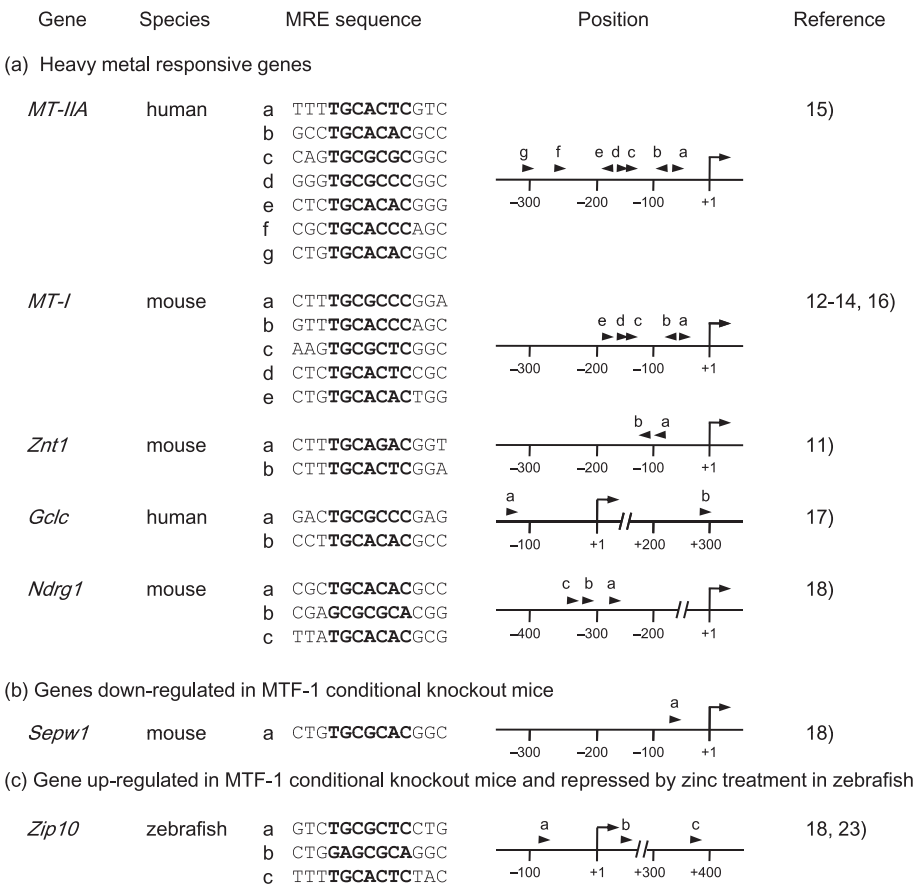

Fig. 1. MREs in the Proximal Promoters of MTF-1 Regulated Genes

The sequence of each MRE is shown and the core sequence (5′-TGCRCNC-3′) is shown in bold. MREs in the proximal promoter region (+1 designates the transcription start site) are indicated by arrows.

was achieved by elevated zinc concentration alone in the cell-free transcription system. Induction by cadmium and copper additionally required the presence of zinc-saturated MT suggesting that these metals cause the release of zinc from MT. This can be explained by the preferential binding of cadmium or copper to zinc-saturated-MT. Although cadmium and copper-induced MTF-1 activation is caused by increase of an available zinc concentration, other mechanisms are also involved in the heavy metal-dependent MTF-1 activation. We discuss the possibility that post-translational modification of MTF-1 is involved in the heavy metal-dependent MTF-1 activation, later. It is interesting to note that a gene, *Zip10*, has MRE consensus sequences, which can form an MTF-1 DNA complex, in the promoter.[18] But, transcription of *Zip10* is not activated in response to zinc.[18,23] The reason why MTF-1 does not activate transcription of *Zip10* is unknown.

ZINC-SENSING MECHANISMS BY MTF-1 THROUGH ITS METAL BINDING DOMAINS

MTF-1 has a DNA-binding domain composed of six Cys_2His_2 zinc fingers. It also possesses three transcriptional activation domains, namely an acidic domain, a proline-rich region and a serine/threonine-rich region (Fig. 2A).[24] When these three domains fused to the DNA-binding domain of yeast factor GAL4, zinc-independent transactivation was observed. The acidic domain showed very strong transactivation. In human and mouse MTF-1, there are no apparent sensing modules for heavy metals such as the MT-like cysteine cluster structure discovered in Ace1 and Mac1,[25–27] a copper-responsive transcription factor for the yeast *MT* gene. Cu-activation of Ace1 occurs through formation of a tetracopper thiolate cluster in one

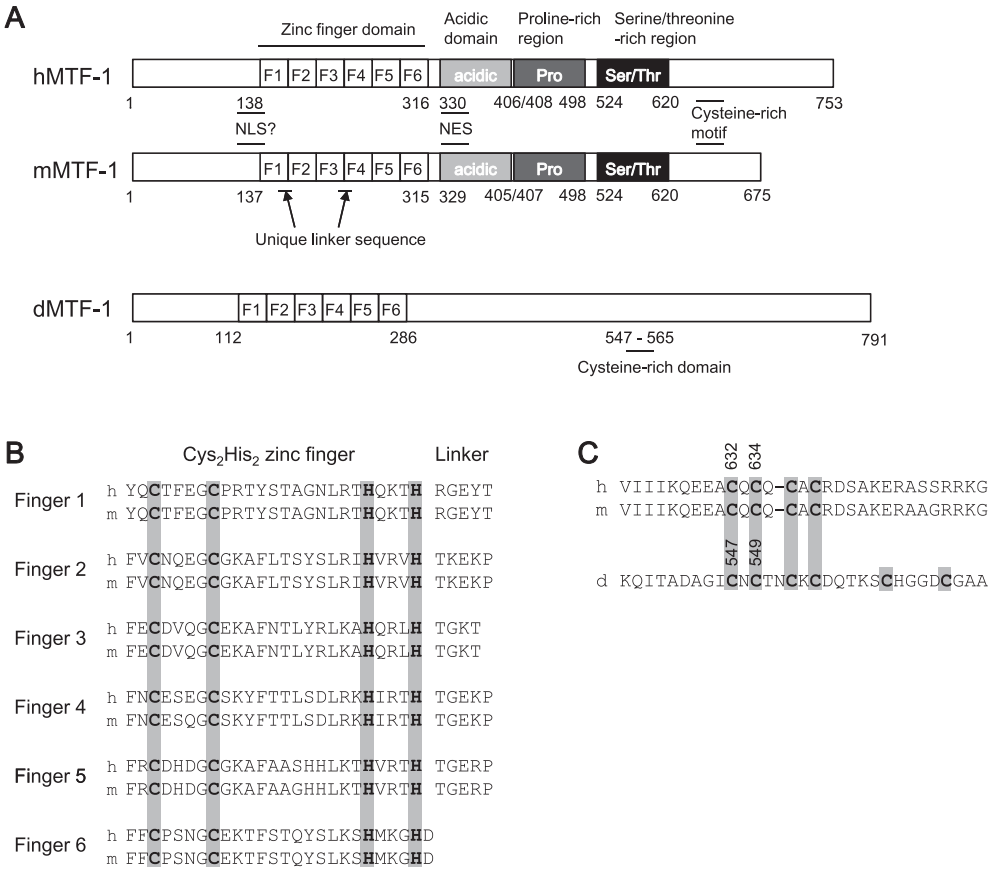

Fig. 2. Schematic Representation of Human, Mouse and *Drosophila* MTF-1

A, The regions of the six-zinc fingers, acidic, proline-rich, and serine/threonine-rich domains are indicated by boxes and the amino acid numbers. B, The highly conserved six-zinc finger domains and the unique finger-linker sequences are shown. C, The cysteine-rich motif, which plays a metal regulatory role in transcriptional activation in human and mouse MTF-1, is shown. The copper-sensing cysteine-rich domain of dMTF-1 binds copper and forms a Cu_4-Cys_6 polynuclear cluster.

of two DNA binding domains. Cu(I) binding to Ace1 induces a conformation that enables Ace1 to bind to its cognate DNA binding site. In contrast, Cu(I) binding to Mac1 inhibits its function as a transcriptional activator. A simple metalloregulatory model in which constitutively expressed MTF-1, with an intrinsically low binding affinity for zinc, would bind zinc only under conditions of zinc excess. Other zinc-requiring, but not zinc-regulated, factors (*e.g.*, Sp1) would be saturated with zinc under most physiological conditions. Specific zinc fingers in MTF-1 apparently have zinc binding affinities in the nanomolar to sub-micromolar range.[28, 29] By contrast, canonical Cys_2His_2 zinc fingers typically bind zinc with higher affinity (10^{-9}–10^{-12} M). Structural and functional heterogeneity among the six zinc fingers of MTF-1 was reported. Fluorescence polarization DNA-binding studies by Giedroc *et al.* showed that a subset of zinc fingers (F3–F4) plays a structural role in folding and high-affinity binding to zinc, while one or more additional fingers have biochemical properties potentially consistent with a metalloregulatory role.[28, 30] Bittel *et al.* showed, using zinc finger deletion constructs, that zinc finger F1 plays a role in zinc-sensing and that zinc fingers F2–F4 constitute a core DNA-binding domain, whereas F5 and F6 appear to be unnecessary for DNA binding *in vitro* but play a role in the formation of a stable MTF-1 chromatin complex.[31] Koizumi *et al.* also reported that, using deletion mutants and point-mutants of individual zinc fingers, the N-terminal four fingers (F1–F4) play roles in metal response.[32] Interestingly, Bittel *et al.* found that deletion of F1 resulted in a protein that bound DNA poorly but constitutively *in vitro*.[31] Transfer of the F1 to a position immediately preceding the three zinc fingers of Sp1 resulted in a chimeric protein that required exogenous zinc to activate DNA binding *in vitro*, unlike native Sp1, which binds DNA constitutively.[31] In contrast, independent NMR studies suggest that each zinc finger adopts a stable $\beta\beta\alpha$ fold in the presence of stoichiometric zinc and the zinc affinities of all fingers are similar (less than 10–50-fold of each other).[29, 33, 34] Because two of the medium zinc affinity fingers (F1 and F3) are needed for high affinity DNA binding and concomitant MTF-1 function, Potter *et al.* proposed a model for zinc-sensing by MTF-1 in which the F1–F6 occupancies ranging from <10 to >90% of the maximum attainable will always occur over a 100-fold or less range of accessible zinc concentration. More recently we reported that a unique linker sequence (RGEYT; Fig. 2B) between zinc fingers F1 and F2 of mMTF-1 confers unique properties to the zinc finger domain that allows it to sense changes in available intracellular zinc.[35] Replacing the unique linker with canonical Cys_2His_2 zinc finger TGEKP linker sequence abolished the zinc-sensing function of MTF-1 and rendered it constitutively active. Replacing a unique linker sequence (TGKT; Fig. 2B) between F3 and F4 of mMTF-1 with the canonical TGEKP linker sequence rendered the protein significantly less sensitive to zinc-induction of DNA binding activity and *MT-I* gene activation. Thus, the zinc finger domain of MTF-1 is very unique and plays a central but not exclusive role in the zinc-sensing mechanisms of this transcription factor.

A cysteine-rich motif (Fig. 2A and C) in MTF-1 is located just C-terminal to a serine/threonine-rich transactivation domain, and also apparently plays a metal regulatory role in transcriptional activation.[36] It has been suggested but not demonstrated[36] that this cysteine-rich motif expressed in bacteria, can bind zinc and cadmium. In that regard, *Drosophila* MTF-1 (dMTF-1) has a more extensive cysteine-rich domain that is required for sensing copper.[37] The copper-sensing cysteine-rich domain of dMTF-1 binds copper and forms Cu_4-Cys_6 polynuclear cluster. The cysteine-rich domain of dMTF-1 has 6 cysteine residues although the domain of mammalian MTF-1 has 4 cysteine residues (Fig. 2C). Copper-sensing via the formation of Cu_4-Cys_6 polynuclear cluster is a specific property of dMTF-1.

POST-TRANSLATIONAL MODIFICATION OF MTF-1

Independent studies suggest that post-translational modification of MTF-1 may play a role in its mechanism of action.[38–42] Numerous evolutionarily conserved consensus phosphorylation sites in mouse MTF-1 were reported.[42] Inhibitor studies indicated that multiple kinases and signal transduction cascades, including those mediated by casein kinase II, c-Jun N-terminal kinase, protein kinase C (PKC), and tyrosine kinase were essential for zinc- and cadmium-inducible transcriptional activation. Moreover, recombinant MTF-1 was a substrate for some kinases *in vitro*.[42] But, Jiang *et al.* reported that inhibition of several kinases *in*

vivo did not significantly change the modification pattern of MTF-1 on two-dimensional electrophoresis. MTF-1 appears to be highly modified in mouse cells. The effects of PKC signaling were found to be gene- and cell-type-specific and ChIP assay showed that none of these kinase inhibitors prevent the metal-dependent recruitment of MTF-1 to the *MT-I* promoter. These studies suggest that protein kinases may exert their interdependent effects on metal-induced gene expression by acting on cofactors that interact with MTF-1.

EPIGENETIC MECHANISMS IN HEAVY METAL-INDUCED *MT-I* GENE EXPRESSION

Many nuclear processes require that DNA is recognized by sequence-specific DNA binding proteins. The packaging of DNA into chromatin inhibits DNA binding by most proteins, and influences the efficiency of transcription. Chromatin structure can be characterized by using micrococcal nuclease (MNase). Increased susceptibility to MNase digestion suggests that the DNA is loosely associated with nucleosomes. Koropatnick *et al.* showed that the susceptibility of the mouse *MT-I* gene to nuclease cleavage was increased in the mouse liver in response to cadmium.[43] This result suggests that MTF-1 brings chromatin remodeling factors to the promoter. The chromatin remodeling complexes has been demonstrated to alter nucleosome structure in an ATP-dependent manner. Most of these complexes can be classified into four groups: homologues of the yeast switch 2-sucrose non fermentable 2 (SWI2-SNF2) ATPase subunit, homologues of the Drosophila imitation-switch ATPase gene, Mi-2 and Ino80.[44] The involvement of human SWI-SNF (hSWI-SNF) complexes can be gene specific and the hSWI-SNF complexes are not required for cadmium-induced *MT-I* gene transcription.[45] In that study, the hSWI-SNF complexes were not required for cadmium-induced *heme oxygenase* (*HO*)*-1* gene transcription. But, the role of BRG1, a catalytic subunit of SWI2/SNF2-like chromatin remodeling complexes, in Nrf2-mediated *HO-1* gene transcription was reported.[46] It seems that BRG1 remodels the nucleosomes to generate Z-DNA formation in the *HO-1* promoter. The roles of chromatin remodeling factors at the *MT-I* promoter is still unknown.

The regulation of gene expression in the chromatin context involves dynamic changes in post-translational modifications of nucleosomal histones. Histones are the targets of numerous signal transduction pathways resulting in a variety of post-translational modifications of these proteins. Among these modifications, the role of acetylation, phosphorylation, and methylation in gene expression are extensively explored. Methylation of DNA at position 5 of cytosine in CpG dinucleotides by DNA-methyltransferase (DNMT) is the predominant epigenetic modification in the regulation of gene expression. Methyl CpG binding protein 2 (MeCP2) condenses the chromatin structure and forms a complex with histone deacetylase (HDAC). The major outcome of promoter methylation appears to be long-term silencing of the associated genes. In some solid and liquid tumors, mouse *MT-I* promoter is highly methylated and *MT-I* gene expression is highly suppressed.[47,48] Although MTF-1 protein was expressed, basal and zinc-induced *MT* expression was not detected in mouse lymphosarcoma cells. It seems that MTF-1 can not activate the *MT* promoter if it is methylated and in a closed chromatin structure. Once the *MT* promoter attained a more open chromatin structure by treatment with inhibitors of DNMT and HDAC, *MT* expression was induced in response to zinc treatment.

TRANSCRIPTION RELATED FACTORS COOPERATED WITH MTF-1

Diverse set of transcription factors, including nuclear factor-1 (NF1),[49] CCAAT/enhancer binding protein α (C/EBPα),[50] hypoxia-inducible factor 1α (HIF-1α),[51] heat shock factor 1 (HSF1),[52,53] Sp1,[54,55] upstream stimulatory factor (USF),[55,56] c-Jun[55] and nuclear factor-κB (NF-κB)[57] were reported to interact with or cooperate with MTF-1 and regulate *MT-I*, *heat shock protein 70* (*HSP70*) and *placenta growth factor* (*PlGF*) gene transcription (Table 1). We very recently reported that MTF-1 forms a complex containing Sp1 in response to zinc treatment.[58] Sp1 has been proposed to act as negative regulator of MTF-1 activation perhaps by competing for MTF-1 binding to DNA.[54] The relationship between the formation of the MTF-1–Sp1 complex and heavy zinc-induced gene expression is still not clear.

A study of dMTF-1, which mediates cop-

Table 1. Transcription Related Factors/complexes Which Affect MTF-1 Activation of Gene Expression

Protein/complex	Species	Regulated genes	Findings	Reference
(a) Transcription factor				
NF1	mouse	*MT-I*	NF1 was recruited to *MT-I* promoter in response to zinc treatment Inactive NF1 protein strongly inhibited *MT-I* promoter activity Loss of the two NF1 sites in *MT-I* promoter decreased metal-induced the promoter activity	49)
C/EBPα	human	*MT-I, MT-IIA*	Down-regulation of C/EBPα by siRNA decreased basal and zinc-dependent *MT* gene transcription	50)
Sp1	mouse	*MT-I*	Sp1 was constitutively recruited to *MT-I* promoter A complex containing MTF-1, p300 and Sp1 was formed in response to zinc treatment	55, 58)
	human	*MT-IIA*	Sp1 competed with MTF-1 for formation of the complex with MRE	54)
USF	mouse	*MT-I*	USF was constitutively recruited to *MT-I* promoter	55)
c-Jun	mouse	*MT-I*	c-Jun was recruited to *MT-I* promoter in response to zinc and cadmium treatment	55)
HIF-1α	mouse	*MT-I*	HIF-1α formed a complex with MTF-1 during hypoxia HIF-1α was recruit to *MT-I* promoter during hypoxia	51)
HSF1	human	*HSP70*	Overexpression of MTF-1 decreased zinc and cadmium-induced *HSP70* promoter activity A complex containing MTF-1, HSF1 and heat shock element was formed	52, 53)
NF-κB	mouse	*PlGF*	NF-κB was recruited to *PlGF* promoter during hypoxia	57)
(b) Coactivator				
TFIID, MED	*Drosophila*	*MtnA*	Down-regulation of TFIID and MED by siRNA affected copper-dependent *MT* gene transcription MTF-1 recruited TFIID and MED to *MtnA* promoter	59)
p300/CBP	mouse	*MT-I*	A complex containing MTF-1, p300 and Sp1 was formed in response to zinc treatment Down-regulation of p300 by siRNA decreased zinc-dependent *MT-I* gene transcription Chromium (VI) inhibited zinc-induced *MT-I* transcription and prevented zinc-dependent formation of the MTF-1–p300 complex	58, 70)
(c) Chromatin remodeling complex				
MeCP2, DNMT1 and HDAC1	mouse	*MT-I*	MeCP2 was associated with the *MT-I* promoter HDAC inhibitors increased the MTF-1 DNA binding activity HDAC inhibitors increased occupancy of MTF-1 to *MT-I* promoter Overexpression of DNMT1 and/or HDAC1 decreased basal and zinc-induced *MT-I* promoter activation	47, 48)
BRG1, BRM	mouse	*MT-I*	Mutant BRG1 or BRM proteins did not decreased the *MT-I* gene transcription in response to cadmium	45)

per responsiveness of the fly *MT* genes, demonstrated that the co-activator complexes TFIID and Mediator (MED) interact functionally to modulate transcriptional response to heavy metal.[59] Recently, we reported that the histone acetyltransferase (HAT) p300 and its close relative cAMP-responsive-element binding protein-binding protein (CBP) are involved in MTF-1 dependent transcription.[58] In contrast, neither p300 nor CBP apparently plays a critical role in MTF-1-dependent ZnT-1 induction in response to zinc. p300/CBP are required for many transcription factors to activate gene transcription.[60] p300/CBP HAT activity is directly involved in chromatin remodeling. Co-immunoprecipitation assays revealed that zinc and cadmium induced formation of a complex containing MTF-1 and p300/CBP. Down-regulation of endogenous p300 expression by siRNA decreased zinc-dependent *MT-I* gene transcription. Using a deletion construct of MTF-1, the acidic domain of MTF-1 was found to be necessary for complex formation with p300 and *MT-I* gene activation. Furthermore, mutation of leucine residues within a predicted nuclear exclusion signal (NES) in the acidic domain of mMTF-1 impaired zinc-dependent recruitment of p300 and activation of the *MT-I* gene transcription. Analysis of NMR chemical shift comparisons of backbone $^{13}C\alpha$ shifts in a F6-acidic domain peptide revealed that zinc does not bind to the acidic domain of MTF-1. Zinc-dependent MTF-1–p300 interaction could involve zinc binding to p300 and/or an adaptor protein. It remains to be determined if p300 directly binds to MTF-1.

FUTURE IMPLICATIONS

In this review, we presented the current understanding of the mechanisms by which mammalian MTF-1 senses heavy metals and activates gene expression. By strenuous studies in the last decade, MTF-1 is well established as intracellular zinc sensor. However, the molecular mechanisms of the response to heavy metals are not fully understood. Identifying the co-activator and/or chromatin remodeling complex involved in the heavy metal-induced transcription by MTF-1 remains an important problem to address.

MTF-1 is activated and *MT* expression up-regulated in response to oxidative stress[61,62] and hypoxia.[51,63] MTF-1 can form a complex with HIF-1α. Recently, we provided evidence that MTF-1 may be activated by degradation of zinc-saturated MT.[64] Many other candidate target genes of MTF-1 have been reported but most have not been studied in any depth with regard to mechanisms of MTF-1-dependent transcription.[17,18,65] The importance of MTF-1 is underscored by its essential role in mouse liver development.[17,66] In contrast *MT-I* and *MT-II* double knock-out mice are viable and reproduce normally when reared under normal laboratory conditions.[8,9] In addition, we showed that DNA synthesis induced in hepatocytes by epidermal growth factor (EGF) was delayed by inhibition of MTF-1.[67] EGF-dependent extracellular signal-related kinase phosphorylation, an essential reaction for EGF-dependent DNA synthesis, was decreased in MTF-1-inhibited hepatocytes. It is also reported that loss of MTF-1 suppressed tumor growth through enhanced matrix deposition.[68] An MTF-1 polymorphism, that alters the amino acid at position 424 from a serine in susceptibility strains to a proline in resistant strains, correlates with the different susceptibility to γ-ray induced mouse thymic lymphoma.[69] The MTF-1 polymorphism affects the efficiency of the transcription activation. The proline type MTF-1 shows higher metal responsiveness than the serine type MTF-1. MTF-1 may act as anti-carcinogenic factor. We showed that chromium (VI) [Cr(VI)], a human carcinogen, inhibited *MT-I* gene transcription by preventing the zinc-dependent formation of an MTF-1–p300 complex.[70] The carcinogenicity of Cr(VI) might be mediated by MTF-1 dysfunction. MTF-1 may act as a heavy metal sensor not only for MT induction, but also other cellular defense systems. To discovery of new networks of cellular defense systems, including crosstalk between stress pathways, the MTF-1 activation mechanisms should be clarified.

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