The Zinc-Sensing Mechanism of Mouse MTF-1 Involves Linker Peptides between the Zinc Fingers

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Mouse metal response element-binding transcription factor-1 (MTF-1) regulates the transcription of genes in response to a variety of stimuli, including exposure to zinc or cadmium, hypoxia, and oxidative stress. Each of these stresses may increase labile cellular zinc, leading to nuclear translocation, DNA binding, and transcriptional activation of metallothionein genes (*MT* genes) by MTF-1. Several lines of evidence suggest that the highly conserved six-zinc finger DNA-binding domain of MTF-1 also functions as a zinc-sensing domain. In this study, we investigated the potential role of the peptide linkers connecting the four N-terminal zinc fingers of MTF-1 in their zinc-sensing function. Each of these three linkers is unique, completely conserved among all known vertebrate MTF-1 orthologs, and different from the canonical Cys₂His₂ zinc finger TGEKP linker sequence. Replacing the RGEYT linker between zinc fingers 1 and 2 with TGEKP abolished the zinc-sensing function of MTF-1, resulting in constitutive DNA binding, nuclear translocation, and transcriptional activation of the *MT-I* gene. In contrast, swapping the TKEKP linker between fingers 2 and 3 with TGEKP had little effect on the metal-sensing functions of MTF-1, whereas swapping the canonical linker for the shorter TGKT linker between fingers 3 and 4 rendered MTF-1 less sensitive to zinc-dependent activation both in vivo and in vitro. These observations suggest a mechanism by which physiological concentrations of accessible cellular zinc affect MTF-1 activity. Zinc may modulate highly specific, linker-mediated zinc finger interactions in MTF-1, thus affecting its zinc- and DNA-binding activities, resulting in translocation to the nucleus and binding to the *MT-I* gene promoter.

Vertebrate metal response element-binding transcription factor-1 (MTF-1) coordinates gene expression in response to metal ions (zinc and cadmium), hypoxia, oxidative stress, and elevated temperature (2, 39, 44, 54). *MTF-1* is also essential for the development and differentiation of embryonic hepatocytes (27), and it has been implicated in malignant progression of cancer cells (43). Thus, MTF-1 integrates a diverse set of environmental signals and modulates, either directly or indirectly, a wide array of genes (31, 40). How it accomplishes these tasks is suggested by the recent demonstration that MTF-1 directly interacts with or cooperates with a diverse set of factors, including NF- κ B (16), HIF-1 α (45), USF (3), SP1 (46), HSF-1 (54, 57), and ribosomal protein S35 (1).

Central to the mechanism of action of MTF-1 is its ability to function as a zinc sensor (2). This essential metal is the second most abundant in the body, next to iron. It has catalytic functions in hundreds of enzymes, serves structural roles in many proteins, and may function as a signaling molecule (42). A deficiency of this essential metal results in a wide spectrum of physiological effects (58). Regulatory functions of zinc ions in cellular signaling pathways have been proposed (42). Thus, an

increase in available zinc may be a unifying theme during many stresses (54, 56, 64).

How MTF-1 senses available zinc has been examined in some detail (2, 25, 39, 60), although a molecular mechanism for this process has not been elucidated. Zinc induces the rapid nuclear translocation and binding of MTF-1 to the *MT-I* promoter (18, 19, 24, 53, 55), resulting in activation of *MT-I* transcription (20). Zinc also induces a dramatic and reversible increase in MTF-1 DNA-binding activity in vivo and in vitro (8, 17, 18, 33, 47, 62). Two putative zinc sensing domains of MTF-1 have been identified: the zinc finger domain (17, 52), which consists of a cassette of six highly conserved Cys₂His₂ zinc fingers and a less-characterized small cysteine cluster near the C terminus of MTF-1 (13). In addition, the presence of a zinc-sensitive inhibitor of MTF-1 activity has been hypothesized (48).

We have focused on elucidating the roles of the zinc finger domain in the zinc-sensing functions of MTF-1 (2). Mutagenesis experiments in which individual zinc fingers were removed and the protein assayed for function in vivo provided evidence that the metal-induced recruitment of MTF-1 to the *MT-I* promoter and formation of a stable MTF-1–chromatin complex constitute a rate-limiting step in zinc- and cadmium-induced activation of gene expression by MTF-1 and that MTF-1 zinc fingers 1, 5, and 6 play an essential role in this process (29). In contrast to other non-zinc-sensing zinc finger proteins, such as SP1, the formation of a ternary (or higher-order) complex between MTF-1, zinc, and DNA requires the addition of high nanomolar to low micromolar exogenous zinc (8, 17, 18). Moreover, MTF-1 finger 1 appears to play a role in this un-

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usual exogenous zinc dependence of DNA binding in vitro (9). The six zinc fingers of MTF-1 display functional heterogeneity (9, 11, 12, 29, 32), although recent structural studies of the purified recombinant mouse MTF-1 zinc finger domain revealed that each zinc finger adopts a typical $\beta\beta\alpha$ finger fold in the presence of stoichiometric zinc (50).

The interdomain flexibility between adjacent MTF-1 zinc fingers appears to be restricted compared to what has been reported previously for most other multifinger proteins (21, 28, 37). This "quasi-order" within the MTF-1 DNA-binding domain may be related to the unusual composition of the amino acid linkers connecting the four N-terminal fingers (zinc finger linkers). Although over 70% of all known multi-zinc finger domains are connected by TGEK(R)P peptide linkers (37), only the linkers between fingers 4 and 5 and fingers 5 and 6 of MTF-1 obey this consensus. Since linker peptides in DNA-bound zinc finger proteins play a role in stabilizing the α -helix in the finger (21, 37), orienting adjacent fingers along the DNA major groove (49, 63), interfinger packing interactions (63), and ultimately DNA-binding affinity (14), the unusual linker sequences within MTF-1 fingers 1 through 4 may affect the DNA-binding properties of MTF-1. Several studies suggest that the metal-binding affinities of the MTF-1 fingers are similar but not identical (12, 26, 50). The zinc fingers of MTF-1 appear to have unusual structural and functional properties, but what makes this DNA-binding domain a zinc sensor is not understood.

To further address this question, we examined the effects of mutations in the linker peptides between adjacent zinc fingers of MTF-1 on its zinc-sensing functions. Our results suggest that the linker between fingers 1 and 2 plays a key role in the zinc-sensing ability of MTF-1 with regard to formation of the ternary (MTF-1–zinc–DNA) complex in vivo and in vitro and the activation of transcription of the *MT-I* gene.

MATERIALS AND METHODS

Mutagenesis of mouse MTF-1. FLAG-tagged *MTF-1* cDNA was cloned into the selectable mammalian expression vector pcDNA3.1/Hygro(+), as previously described (29, 55), and this construct was used as the template for mutagenesis of the zinc finger linkers. Mutagenesis of MTF-1 linkers was generated using the QuickChange site-directed mutagenesis kit (Stratagene) with the mutation primers as listed below. PCR was performed using two antiparallel primers with the required nucleotide substitutions, and the PCR products were used to transform XL1-Blue supercompetent cells. The complete sequence of each mutated construct was verified by DNA sequencing.

Sequences of sense oligonucleotides used in site-directed mutagenesis were as follows: L12TGEYT, CAG AAG ACT CAC ACA GGA GAG TAC ACC; L12RGEYP, CGA GGA GAG TAC CCA TTC GTC TGT AAT C; L12RGEKT, CAC CGA GGA GAG AAG ACC TTC GTC TG; L12TGEYP, C ACA GGA GAG TAC CCC TTC GTC TGT AAT C; L12TGEKT, CAC ACA GGA GAG AAG ACC TTC GTC TG; L12RGEKP, CAC CGA GGA GAG AAA CCC TTC GTC TGT AAT C; L12TGEYP, CAC ACA GGA GAG AAG CCC TTC GTC TGT AAT C; L23TGEKP, CGA GTG CAC ACA GGG GAG AAG CCA TTT G; L34TGEKT, CTT CAC ACA GGG GAG AAA ACG TTT AAC; L34TGEKP, C ACA GGG GAG AAA CCG TTT AAC TGT GAA TC. Mutated codons are italicized. L12TGEYT, L12TGEKT, and L12TGEKP used L12TGEYT as a template; L34TGEKP used L34TGEKT as a template.

Transfection of MTF-1 knockout cells. *MTF-1* knockout mouse embryo fibroblasts (KO-MEF) derived from *MTF-1*^{-/-} mice were cultured in Dulbecco's modified Eagle's medium-high glucose supplemented with 10% fetal bovine serum (29, 30). Flag-tagged MTF-1 (MTF-1_{Flag}) and its zinc finger linker mutant vectors were transfected by electroporation into these KO-MEF cells (4×10^6 cells) at 70% confluence. Cells were trypsinized and collected, washed once with HeBS buffer (25 mM HEPES, 140 mM NaCl, 0.75 mM Na₂HPO₄, pH 7.05), and

then resuspended in 0.5 ml HeBS buffer. After the addition of plasmid DNA (5 μ g of DNA when cells were used for Northern blotting, 15 μ g when they were used for chromatin immunoprecipitation [ChIP], or 30 μ g DNA when cells were used for electrophoretic mobility shift assay [EMSA] or Western blotting) cells were electroporated at 200 V and 950 μ F with a Genepulser (Bio-Rad). Transfected cells were grown for 24 h (48 h for ChIP) in Dulbecco's modified Eagle's medium-high glucose supplemented with 10% Chelex-treated fetal bovine serum (38) prior to treatment with zinc or cadmium.

Northern blot detection of MT-I mRNA induction. Transfected or control KO-MEF cells were treated with 100 μ M ZnSO₄ or 10 μ M CdCl₂ for 6 h prior to harvest. These concentrations of metals cause maximal induction of *MT-I* gene expression (17, 18, 29). Total RNA, isolated using TRIzol reagent (Invitrogen), was separated by denaturing agarose-formaldehyde gel electrophoresis, transferred, and UV cross-linked to nylon membranes and hybridized with an *MT-I* cRNA probe, as previously described (18). Hybrids were detected by autoradiography at -70°C with intensifying screens. A β -actin cRNA probe was used as a control to normalize for RNA amount, integrity, and transfer efficiency.

Preparation of whole-cell, nuclear, and cytoplasmic extracts. Whole-cell extracts from transfected cells were prepared by snap-freezing and thawing cell pellets in nuclear extraction buffer followed by clearing the extract by ultracentrifugation, as previously described (18, 29). Cytoplasmic and nuclear extracts for Western blotting were prepared as described previously (55) from transfected KO-MEF cells that were incubated for 1 h in medium containing 100 μ M ZnSO₄ or 20 μ M CdCl₂. Protein concentrations were determined using a protein assay reagent (Bio-Rad) with bovine serum albumin as the standard.

In vitro transcription/translation of mouse recombinant MTF-1. Wild type and zinc finger linker mutants of MTF-1_{Flag} were synthesized in vitro using a TnT coupled transcription/translation reticulocyte lysate system (Promega) containing 1 μ g of the MTF-1_{Flag} plasmids and T7 RNA polymerase according to the manufacturer's suggestions (8, 17). Protein synthesis in the TnT lysate was monitored by Western blotting with an anti-Flag antibody, as described below.

EMSA. EMSA was performed using whole-cell extracts or aliquots from the TnT lysate reactions and the end-labeled double-stranded oligonucleotide called MRE-d, under conditions described previously (17, 18). Binding reactions were assembled with 20 μ g of whole-cell extract or 0.5 μ l of the TnT lysate and concentrations of exogenous zinc ranging from 0 to 30 μ M (8, 17). Binding reaction mixtures were incubated at 37°C for 15 min with or without the addition of exogenous zinc and then placed on ice. Labeled MRE-d oligonucleotide was added, and after incubation at 4°C for 15 min, the reaction mixtures were fractionated at 4°C for 1.5 h at 15 V/cm in a 4% polyacrylamide gel prepared in Tris-glycine-EDTA running buffer without zinc (17, 18). The gel was dried and subjected to autoradiography.

ChIP assay. ChIP assays were carried out as described previously (19, 29). Transfected or control KO-MEF cells were treated for 1 h with ZnSO₄ (100 μ M) or CdCl₂ (20 μ M) before fixation in 1% formaldehyde. Cross-linking was stopped by the addition of glycine to a final concentration of 0.125 M. After homogenization of the cells, as described previously (18), the nuclei were lysed in sonication buffer (29) and the chromatin was sonicated to an average length of 600 to 1,000 bp and clarified by centrifugation at $12,000 \times g$ for 10 min at 4°C . The chromatin was diluted 10-fold in ChIP dilution buffer (10), precleared with protein G-agarose (Sigma), and immunoprecipitated with anti-Flag M2-agarose affinity gel (Sigma). The beads were washed, the immune complexes eluted, and the cross-links reversed as described previously (10). The DNA was phenol-chloroform extracted, reconstituted in Tris-EDTA buffer (pH 8.0), and analyzed by PCR using primers that span bp -264 to $+43$ (relative to the transcription start site) of the mouse *MT-I* gene. PCR products (28 cycles) were separated by electrophoresis in Tris-acetate-EDTA-agarose gels, stained with SYBR green I (Sigma), and normalized to input products. For initial experiments, input and immunoprecipitated DNA samples were analyzed at various cycles (27 to 33 cycles) to ensure that the PCR products were obtained in the linear range of amplification.

Western blotting. MTF-1_{Flag} in whole-cell, nuclear, and cytoplasmic extracts (50 μ g) or TnT lysates (1 μ l) was detected by Western blotting using an anti-FLAG antibody (Santa Cruz Biotechnology), followed by a goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase, as previously described (55).

RESULTS

Sequence alignment comparisons of MTF-1 zinc finger peptides from several species revealed that the finger linker, i.e., the four or five amino acids found adjacent to the second

TABLE 1. Sequence alignment of highly conserved zinc finger-linker peptides among vertebrate MTF-1 peptides

MTF-1 type	Sequence ^a						
	Finger 1	Linker	Finger 2	L1-2	L2-3	L4-5	L5-6
Mouse	YQCTFEGCPRTYSTAGNLRTHQKTH	<i>RGEYT</i>	FVCNQEGCGKAFLTSYSLRIHVRVH	<i>RGEYT</i>	<i>TKEKP</i>	<i>TGEKP</i>	<i>TGERP</i>
Human	YQCTFEGCPRTYSTAGNLRTHQKTH	<i>RGEYT</i>	FVCNQEGCGKAFLTSHSLRIHVRVH	<i>RGEYT</i>	<i>TKEKP</i>	<i>TGEKP</i>	<i>TGERP</i>
Chicken	YQCTFEGCPRTYSTAGNLRTHQKTH	<i>RGEYT</i>	FVCNQEGCGKAFLTSYSLKIHVRVH	<i>RGEYT</i>	<i>TKEKP</i>	<i>TGEKP</i>	<i>TGERP</i>
Fugu	YKCMFEGCTRITYSTAGNLRTHQKTH	<i>RGEYT</i>	FVCNQQCGCGKAFLTSYSLKIHVRVH	<i>RGEYT</i>	<i>TKEKP</i>	<i>TGEKP</i>	<i>TGEKP</i>
Zebra fish	YQCLFEGCTRITYSTAGNLRTHQKTH	<i>RGEYT</i>	FVCNQQCGCGKAFLTSYSLKIHVRVH	<i>RGEYT</i>	<i>TKEKP</i>	<i>TGEKP</i>	<i>TGEKP</i>
Drosophila	YRCNYENCYRSYSTIGNLRTHLKTH	<i>TGDYS</i>	FKCPEDGCHKAFLLTSYSLKIHVRVH	<i>TGDYS</i>	<i>TKVKP</i>	<i>TQERP</i>	<i>TGEKP</i>

^a Residues conserved in the MTF-1 linkers are in italics. Boldface letters indicate a conservative change from lysine to arginine.

histidine of each zinc finger, was highly conserved among these vertebrate peptides, but much less so in *Drosophila* MTF-1 (Table 1). Interestingly, the amino acid sequence of each linker was unique to each finger pair, and only the linkers between zinc fingers 4 and 5 (L4-5) and fingers 5 and 6 (L5-6) conformed to the Cys₂His₂ zinc finger consensus sequence TGEKP (36, 37). In humans and mice, there is a conservative change with arginine replacing the lysine in L5-6. L2-3 differed from the consensus peptide by a single amino acid (TKEKP), whereas the other linker peptides were much less similar.

The combination of unique amino acid sequences and high evolutionary conservation of linker peptides between the first four zinc fingers of MTF-1 suggests that they may play a role in its metalloregulatory function. To examine this possibility, each of the linker peptides between these zinc fingers was individually mutated into the canonical TGEKP linker peptide. These mutations were made in an otherwise intact full-length mouse MTF-1 that was Flag tagged at the C terminus (MTF-1_{Flag}), and these proteins were expressed in vitro in a TnT lysate system (Fig. 1A) or in vivo in transfected mouse cells that lack endogenous MTF-1 (Fig. 1B). The ability of MTF-1 and its finger linker mutants to bind in vitro to a metal-response element (MRE-d) binding site was then assayed using an EMSA (Fig. 1). In the absence of the addition of exogenous zinc to the binding reaction, little specific MTF-1-binding activity was detected in the TnT lysate or in whole-cell extracts from transfected cells expressing native MTF-1_{Flag}, as reported previously (8, 17), and a similar result was obtained after mutation of L2-3. In sharp contrast, replacing the unusual RGEYT linker L1-2 resulted in constitutive DNA-binding activity that is independent of exogenous zinc, whereas replacing the short TGKT linker L3-4 resulted in diminished DNA-binding activity in response to zinc. That these results did not reflect the levels of expression of these proteins was confirmed by Western blotting (Fig. 1).

The biological relevance of the results given above was examined by monitoring the ability of MTF-1_{Flag} and the corresponding finger linker mutants to activate transcription of the endogenous chromatin packaged *MT-I* gene (Fig. 2). This was accomplished by electroporation of mutant and wild-type *MTF-1_{Flag}* expression vectors into mouse embryo fibroblasts which lack endogenous MTF-1. Transfected cells were then incubated with zinc or cadmium for 6 h and changes in *MT-I* mRNA were measured by Northern blotting. Very low levels of *MT-I* mRNA were present in the nontransfected cells, and this gene was not responsive to zinc or cadmium, but metal responsiveness was restored by native MTF-1_{Flag} and by the protein

FIG. 1. EMSA analysis of the zinc-dependent DNA-binding activity of MTF-1_{Flag} and finger linker mutants of MTF. The effects of mutating zinc finger linker peptides in MTF-1_{Flag} into the canonical linker TGEKP were examined using an EMSA. This replacement mutation was introduced between fingers 1 and 2 (L12), between fingers 2 and 3 (L23), or between fingers 3 and 4 (L34). (A) MTF-1 peptides were synthesized in vitro in a TnT lysate (of the coupled transcription/translation system), and the effects of the indicated concentrations of zinc on the MRE-d-binding activity of an aliquot of the lysate were measured. The arrow indicates the specific MTF-1-MRE-d-zinc complex. The lower panel shows Western blots of the MTF-1_{Flag} protein in each TnT lysate reaction. (B) Whole-cell extracts were prepared from KO-MEF cells that had been transfected with an expression vector for MTF-1_{Flag} or its zinc finger linker mutants described above. The effects of the indicated concentrations of zinc on the MRE-d-binding activity of an aliquot of the whole-cell extract were measured and the arrow indicates the specific MTF-1-MRE-d-zinc complex. The lower panel shows Western blots of the MTF-1_{Flag} proteins in each whole-cell extract.

FIG. 2. Determination of the effects of finger linker mutations in MTF-1_{Flag} on the induction of *MT-I* expression in response to zinc and cadmium. The effects on the biological activity of MTF-1_{Flag} of mutating zinc finger linker peptides into the canonical linker TGEKP were examined in transfected KO-MEF cells. The replacement linker mutation was introduced between fingers 1 and 2 (L12), between fingers 2 and 3 (L23), or between fingers 3 and 4 (L34). (A) Cells were transfected with vector alone (lanes 1 to 3) or with the indicated MTF-1_{Flag} expression vectors and then treated with 100 μ M ZnSO₄ (Zn) or 10 μ M CdCl₂ (Cd) for 6 h. *MT-I* mRNA and β -actin gene mRNAs were detected by Northern blotting. (B) Whole-cell extracts from the transfected cells were analyzed by Western blotting using an anti-Flag antibody.

with the TGEKP replacement mutation of L2-3 (TKEKP). Consistent with the *in vitro* DNA-binding results, the linker 1 and 2 replacement mutation (L1-2; RGEYT to TGEKP) resulted in constitutive activation of the *MT-I* gene, whereas the linker 3 and 4 replacement mutation (L3-4, TGKT to TGEKP) was inactive under these conditions (Fig. 2). These results did not reflect apparent differences in MTF-1_{Flag} expression levels among the transfected cells.

The mechanisms of action of MTF-1 include metal-induced nuclear translocation and formation of an MTF-1–chromatin complex on the *MT-I* promoter. The nuclear translocation of MTF-1_{Flag} and the finger linker mutants in transfected cells was examined by Western blot analysis of cytoplasmic and nuclear extracts from control and metal-treated cells (Fig. 3), and the formation of the MTF-1–chromatin complex was monitored using a ChIP assay (Fig. 4). Native MTF-1_{Flag}, as well as

FIG. 3. Western blot analysis of metal-induced nuclear translocation of MTF-1_{Flag} and finger linker mutants of MTF. As described in the legend to Fig. 1, cells were transfected with the indicated MTF-1_{Flag} expression vectors and then incubated with 100 μ M ZnSO₄ (Zn) or 20 μ M CdCl₂ (Cd) for 1 h. –, incubation alone; L12, linker between fingers 1 and 2; L23, linker between fingers 2 and 3; L34, linker between fingers 3 and 4. Cytoplasmic (CE) and nuclear (NE) extracts prepared from the transfected cells were analyzed by Western blotting using an anti-Flag antibody.

FIG. 4. ChIP analysis of the interaction of MTF-1_{Flag} and finger linker mutants of MTF with the *MT-I* promoter. Cells were transfected with expression vector alone or with the indicated native MTF-1_{Flag} or MTF-1_{Flag} finger linker mutation vectors (see legend to Fig. 1). Transfected cells were incubated in medium containing 100 μ M ZnSO₄ (Zn) or 20 μ M CdCl₂ (Cd) for 1 h, and then chromatin was fixed *in vivo*, sheared, and immunoprecipitated using anti-Flag agarose beads. The *MT-I* promoter was amplified by PCR in the precipitated DNA and in the input DNA from treated and untreated cells. L12, linker between fingers 1 and 2; L23, linker between fingers 2 and 3; L34, linker between fingers 3 and 4.

this protein in which L2-3 or L3-4 was replaced by the canonical linker peptide, was detected in both the cytoplasmic and nuclear extracts in untreated cells. It should be noted that there is about three times more total protein per cell in the cytoplasmic extract than in the nuclear extract, but an equal amount of protein for each extract was analyzed by Western blotting. Therefore, the vast majority of MTF-1 is in the cytoplasm in these cells before treatment, and in all three cases, zinc and cadmium caused an almost complete loss of detectable MTF-1 in cytoplasmic extracts and an increased signal in the nuclear extracts (Fig. 3). Thus, metal-induced nuclear translocation of MTF-1_{Flag} was unimpaired by these finger linker replacement mutations, even though induction of *MT-I* gene expression was abolished by the canonical replacement of the shorter TGKT L3-4, under these conditions (Fig. 2). Interestingly, the L1-2 canonical replacement mutation resulted in constitutive nuclear localization of MTF-1_{Flag} (Fig. 3), consistent with the constitutive DNA-binding activity and activation of the *MT-I* gene (Fig. 1 and 2).

Interactions between MTF-1_{Flag} and the endogenous *MT-I* promoter were examined by ChIP analysis of fixed and sheared chromatin extracted from the transfected and control MEF cells as described above. Immunoprecipitation was carried out using an anti-Flag antibody. The *MT-I* promoter and exon I were amplified by PCR (Fig. 4). Amplification of input DNA was used to normalize between samples, and PCRs were performed in the linear range of cycle numbers (28 cycles) to assure quantitative comparisons between control and metal-treated samples. In the absence or presence of wild-type MTF-1_{Flag}, basal amounts of the *MT-I* promoter were precipitated by the anti-FLAG antibody. The background in the ChIP assay was higher than that seen using stably transfected cells due to transfection efficiency. Nonetheless, wild-type MTF-1_{Flag} was clearly recruited to the *MT-I* promoter in response to zinc and high levels of cadmium, as anticipated. The effects of the finger linker replacement mutations in MTF-1_{Flag} on metal-induced binding to the *MT-I* promoter mimicked those on *MT-I* mRNA induction, as described above (Fig. 2). That is, the L1-2 canonical replacement mutation lead to constitutive binding of MTF-1_{Flag} to the *MT-I* promoter and metals had little effect on the amount of binding. In contrast, the L2-3 replacement mutation resulted in slightly increased constitutive binding but a clear metal-induction of additional binding, whereas the L3-4

FIG. 5. Fine analysis of the effects of mutations in the zinc finger linker peptide between fingers 1 and 2 of MTF-1_{Flag} by determination of zinc-dependent DNA-binding activity in TnT lysates. The finger linker between zinc fingers 1 and 2 of native MTF-1 is RGEYT in all vertebrate species. This sequence was mutated into the indicated peptides containing single (A) or double (B) amino acid substitutions. Proteins were synthesized in vitro in a TnT lysate (of the coupled transcription/translation system) and the effects of the indicated concentrations of zinc on the MRE-d-binding activity of an aliquot of the lysate were measured using an EMSA. The arrow indicates the specific MTF-1-MRE-d-zinc complex. (C) Western blot detection of MTF-1_{Flag} protein in each TnT lysate reaction.

replacement mutation severely diminished/abolished binding to the *MT-I* promoter.

The results presented above suggest that L1-2 is a particularly important structural component of the zinc-sensing mechanism of MTF-1. This observation was explored in more detail by systematically mutating the amino acids in the native linker peptide RGEYT and then determining their effects on zinc-induced DNA-binding activity of MTF-1_{Flag} synthesized in vitro in the TnT lysate (Fig. 5) or in whole-cell extracts from transfected MEF cells (Fig. 6). The approach was to change residues in the native linker into those found in the canonical linker TGEKP. A single amino acid change of tyrosine to lysine at the penultimate residue or threonine to proline at the C-terminal end of the linker resulted in a reduced dependence on exogenous zinc for DNA-binding activity (Fig. 5A and 6A). Further mutation of the first amino acid arginine to threonine did not exacerbate the reduced dependence on exogenous zinc (Fig. 5B and 6B). In contrast, mutating both the penultimate tyrosine and C-terminal threonine residues of the native linker to lysine and proline, respectively, resulted in constitutive DNA-binding activity of MTF-1_{Flag} (Fig. 5B and 6B). Western blotting was used to confirm that nearly equal amounts of each MTF-1_{Flag} peptide were present in the TnT lysates (Fig. 5C) and the whole-cell extracts (Fig. 6C). Thus, two amino acids in L1-2 play a key role in the zinc-dependent DNA-binding activity of MTF-1.

FIG. 6. Fine analysis of the effects of mutations in the zinc finger linker peptide between fingers 1 and 2 of MTF-1_{Flag} by determination of zinc-dependent DNA-binding activity in whole-cell extracts. The finger linker between zinc fingers 1 and 2 of native MTF-1 is RGEYT in all vertebrate species. This sequence was mutated into the indicated peptides containing single (A) or double (B) amino acid substitutions. Proteins were expressed in transfected KO-MEF cells and the effects of the indicated concentrations of zinc on the MRE-d-binding activity of an aliquot of the whole-cell extract were measured and the arrow indicates the specific MTF-1-MRE-d-zinc complex. (C) Western blot detection of MTF-1_{Flag} protein in each whole-cell extract.

DISCUSSION

The finding that the peptide sequences between adjacent zinc fingers play key roles in the zinc-sensing mechanisms of MTF-1 is consistent with the remarkable conservation of all five finger linker sequences among vertebrate MTF-1 orthologs. Our studies also suggest that the finger linker sequence between zinc fingers 1 and 2 confers unique properties to the zinc finger domain that allows it to sense changes in available intracellular zinc and to regulate *MT-I* gene expression.

In culture medium, the majority of MTF-1 is located in the cytosol, where it must sense changes in zinc availability (Fig. 3A). Nuclear translocation of wild-type MTF-1 is rapidly stimulated by the addition of low micromolar concentrations of exogenous zinc to the medium (Fig. 3A). Once bound to DNA, MTF-1 is significantly more resistant to EDTA inactivation and the zinc finger domain becomes resistant to proteolytic digestion (17, 18). We noted previously that zinc activation of MTF-1 does not occur efficiently at a low temperature (4°C), whereas once activated with zinc, its DNA binding was not diminished at a lower temperature (17, 18). Therefore, it seems likely that replacing L1-2 in MTF-1 with the canonical TGEKP peptide increases intrinsic zinc affinities for at least a subset of the zinc fingers. Our observation of constitutive nuclear translocation of the L1-2 canonical replacement mutant in the absence of exogenous zinc (Fig. 3B) strongly suggests that the well-established structural ordering of the MTF-1 fingers upon zinc binding (50) triggers this translocation process. The apparent lower zinc affinity for at least a subset of MTF-1

fingers suggests that the unusual RGEYT L1-2 linker “detunes” the finger affinities to a range of intracellular zinc that is optimal for biological functions.

Ultimately, the ternary complex of zinc–MTF-1–DNA in the nucleus regulates *MT-I* gene expression. Therefore the greater zinc affinity of at least some of the MTF-1 L1-2 mutants we examined is expected to shift the equilibrium toward formation of the ternary complex at lower zinc concentrations (i.e., to become a constitutively active transcription factor), as was observed (Fig. 1 and 2). As discussed in detail below, compared with similar zinc finger–DNA complexes in which adjacent fingers are separated by canonical TGEKP linkers, differences in finger–DNA interactions may also play a role in mediating the apparent detuning of the MTF-1 complex with DNA (Fig. 1). We note that our studies do not eliminate the possibility that the MTF-1 finger linker peptides are involved in other intermolecular or intramolecular interactions that govern the zinc-sensing mechanism, although no such interactions have been demonstrated. Based on nuclear magnetic resonance structural studies of the zinc finger domain, we conclude that these interactions do not include zinc binding to the linker peptides (50).

Previous finger deletion and swapping studies of MTF-1 suggested a role for zinc finger 1 in the metal-sensing function (9), but studies of the reactivity of cysteine thiol groups in the zinc fingers resulted in data that were inconsistent with this concept (4). Zinc finger 5 is hypersensitive to cysteine thiol oxidation, which indicates that the metal-binding properties of that finger may be unusual (50), and zinc fingers 5 and 6 have been suggested to play a role in the metal-sensing mechanism of MTF-1. They were reported to stabilize MTF-1–DNA complexes in vitro (12), and they are critical for MTF-1 to interact with and activate the endogenous *MT-I* gene (29). However, mutation of these fingers of MTF-1 does not alter its zinc dependence for MRE-binding activity measured in vitro using an EMSA (9, 29). Biochemical studies suggest that zinc fingers 1 through 4 play critical roles in high-affinity DNA binding (12). It is interesting that zinc fingers 1, 3, and 6 appear to have lower zinc-binding affinities relative to the other zinc fingers of MTF-1 (26, 50).

Evidence presented in the studies cited above is consistent with earlier suggestions that multiple zinc fingers participate in the zinc-sensing mechanism of MTF-1 (50). The quasi-ordered nature of the ensemble of solution states of the core DNA-binding zinc fingers of MTF-1 is further stabilized by DNA-binding (51; B. Potter, L. Feng, V. Matskevich, G. K. Andrews, and J. H. Laity, unpublished data). The mutational studies reported here demonstrate that finger–finger interactions in MTF-1 mediated by the RGEYT peptide connecting fingers 1 and 2 influence the equilibrium concentrations of free and stable chromatin–DNA-bound states, thus at least partially conferring zinc-sensing properties to the domain. Consistent with the notion of interacting fingers, recent studies of MTF-1 zinc fingers also reported cooperativity in metal binding (4, 26, 50). Finger–finger interactions also occur in the Zap1 transcription factor, which functions as a zinc-sensor in *Saccharomyces cerevisiae* (7). Zinc binding to zinc fingers 1 and 2 of Zap1 initiates conformational changes that are stabilized by interactions between these fingers (61). In contrast, earlier studies of tandem arrays of nonmetalloregulatory zinc fingers have dem-

onstrated that they can bind zinc and adopt canonical $\beta\beta\alpha$ structures independently (6, 35). Thus, zinc fingers in Zap and MTF-1, the only two eukaryotic zinc sensors described to date, are unusual in their zinc-binding and interacting properties.

Although intrinsic zinc binding by MTF-1 appears to be detuned by the RGEYT linker between fingers 1 and 2, several additional factors relating to linker ordering and concomitant finger orientations upon DNA binding may also contribute to the metal-sensing function of the protein (36). The glycine at position 2 of the canonical TGEKP linker is involved in DNA-induced capping by forming hydrogen bonds with the C-terminal region of the α -helix in the preceding finger (37). All of the zinc finger linkers in MTF-1, except for the finger 2 and 3 linker, also have glycine at position 2. Moreover, lysine has been proposed as a possible glycine substitution at this linker position to still facilitate α -helix capping (5). In this context, it is not surprising that the canonical swap mutant of L2-3 had little effect on MTF-1 zinc and DNA-binding properties (Fig. 1 and 2). The lysine at position 4 can interact with the phosphate backbone of the DNA (49), but tyrosine is found in that position in the RGEYT linker connecting fingers 1 and 2 of MTF-1. The C-terminal proline of the canonical TGEKP linker orients adjacent fingers along the major groove of DNA (49). Threonine is found in this position in the linkers between fingers 1 and 2 and fingers 3 and 4. Our site-directed mutagenesis of L1-2 suggested that the two C-terminal positions of the linker are particularly important. Altering potential interactions with the phosphate backbone by replacing tyrosine with lysine and affecting the orientation of the adjacent finger by replacing the threonine with proline in zinc finger 1 of MTF-1 abolished the dependence on exogenous zinc for activity. This observation may suggest that these tyrosine and threonine residues at the C-terminal positions of the native RGEYT linker in MTF-1 contribute to finger–finger interactions which destabilize zinc binding in the zinc finger domain. In contrast, similar changes in L3-4 had the opposite effect on the dependence on exogenous zinc for activity and reduced the sensitivity of MTF-1 to zinc in vivo and in vitro. In that case, the native linker is unique among the MTF-1 finger linkers and consists of only four residues (TGKT). Replacement of that linker with the canonical linker peptide TGEKP lengthened the native linker by a residue, which also altered the net charge of the linker, and added a C-terminal proline, which is predicted to affect the orientation of the next finger (49).

An issue that requires consideration is whether the concentrations of zinc required to activate the DNA-binding activity of MTF-1 in vitro are physiologically relevant. Since the unusual requirement for exogenous zinc for wild-type MTF-1–finger–DNA complex formation in vitro strongly suggests a lower affinity for zinc and, thus, greater zinc ion concentration requirement for folding, it is reassuring that the biological activity of MTF-1 in vivo unerringly reflected the zinc dependence for DNA-binding of MTF-1 that was measured in vitro. However, the actual amount of zinc available to interact with MTF-1 in the TnT lysates and whole-cell extracts is not known because many other proteins and potential zinc-binding sites are present. Recent calorimetric studies indicate an apparent dissociation constant for zinc for the purified MTF-1 zinc finger domain in the high nanomolar to low micromolar range (L.S. Feng, G. K. Andrews, and J. H. Laity, unpublished ob-

servation). Similar studies of zinc fingers 1 and 2 in Zap1 identified a functional zinc-responsive concentration in the low-nanomolar range (apparent K_d , ~ 5 nM) (61). In contrast, canonical Cys₂His₂ zinc fingers fold independently and bind zinc with high affinity (10^{-9} to 10^{-12} M⁻¹) (34, 35). These observations are consistent with the notions presented here and suggest that Zap1 zinc fingers, like at least some MTF-1 fingers, are detuned such that their activity and structure are dependent on higher concentrations of zinc than are other zinc fingers. It was estimated that at least 100 labile atoms of zinc per yeast cell is required to occupy zinc fingers 1 and 2 of Zap1 (61).

Several recent studies have reported the uptake kinetics for ⁶⁵Zn in several different types of cultured mammalian cells (15, 22, 23, 59). In the HEK cells, the initial rate of uptake of labeled zinc from the culture medium was ~ 4 pmol zinc/min/mg protein with an accumulation of 25 pmol zinc/mg protein within 10 to 15 min of exposure to elevated concentrations of zinc (59). For the sake of this discussion, a value of 300 pg of protein per cell and a value of 3×10^{-12} liters for the volume of a "typical" cell were used (41) to calculate that $\sim 4 \times 10^6$ to 8×10^6 atoms of zinc can be taken up per HEK cell in these experiments. This would increase the concentration of zinc inside the cell by ~ 2 to 4 μ M. Similar studies of zinc uptake in K562 cells reported the accumulation per cell of an additional 0.7 μ M zinc (in complete medium) to 27 μ M zinc (in minimal medium) during a short exposure to elevated zinc (22, 23). Studies of zinc uptake in PC3 and LNCaP cells report the rapid accumulation per cell of an additional 15 μ M zinc and 200 μ M zinc, respectively, after a short (5- to 10-min) incubation in medium containing 15 μ M zinc (15). Clearly these are rough and static estimates that do not address the availability of the rapidly accumulated zinc, and the volume of a eukaryotic cell is highly dependent on the cell type and varies over 4 orders of magnitude. Nonetheless, these zinc uptake data support the concept that the concentrations of zinc required to induce the DNA-binding activity of MTF-1 in vitro fall within a range of available zinc concentrations that may occur, at least transiently, in mammalian cells acutely exposed to high levels of zinc.

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