Gene- and cell-type-specific effects of signal transduction cascades on metal-regulated gene transcription appear to be independent of changes in the phosphorylation of metal-response-element-binding transcription factor-1

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Post-translational modification of MTF-1 (metal-response-element-binding transcription factor-1) was suggested to play a role in its metalloreulatory functions. In the present study, pulse labelling and two-dimensional electrophoresis–Western blotting were used to demonstrate that, although MTF-1 is highly modified *in vivo*, its phosphorylation level does not rapidly change in response to metals, nor does its overall modification pattern. Recombinant MTF-1 was found to serve as an *in vitro* substrate for casein kinase II, c-Jun N-terminal kinase and protein kinase C, but inhibition of these kinases *in vivo* did not significantly change the modification pattern of MTF-1. Northern blotting revealed that inhibitors of casein kinase II and c-Jun N-terminal kinase severely attenuate the metal-induced transcription of the native chromatin-packaged *metallothionein-1* and *zinc transporter-1* genes, whereas protein kinase C inhibitors exerted gene- and cell-type-specific

effects on the metal regulation and basal expression of these two genes. A chromatin immunoprecipitation assay was used to demonstrate that none of these inhibitors prevent the metal-dependent recruitment of MTF-1 to the *MT-1* promoter. In brief, results of the present study suggest that protein kinases may not alter the phosphorylation state of MTF-1 during the rapid-response phase to metals, nor do they regulate the metal-dependent formation of a stable MTF-1–chromatin complex. Instead, protein kinases may exert their interdependent effects on metal-induced gene expression by acting on cofactors that interact with MTF-1.

Key words: metal, metallothionein, metal-response-element-binding transcription factor-1 (MTF-1), phosphorylation, signal transduction, zinc transporter-1.

INTRODUCTION

Heavy metal homeostasis and detoxification systems in higher eukaryotes are often regulated at the transcriptional level by metal-sensing transcription factors. Metal-induced expression of MT (metallothionein), a class of metal-chelating proteins that facilitate storage and transfer of metal ions, and ZnT-1 (zinc transporter-1), a plasma membrane protein that effluxes excess zinc, are regulated by MTF-1 (MRE-binding transcription factor-1, where MRE stands for metal-response element) [1,2]. MTF-1 is an evolutionarily conserved transcription factor that regulates gene expression in response to heavy metals, oxidative stress and hypoxia (see [3,4] for reviews). Its target genes include, but are not limited to, *MT* [1], *ZnT-1* [2] and *glutamate–cysteine ligase heavy chain* (*γGCS_{hc}*) genes [5]. Homozygous knockout of the mouse *MTF-1* gene in cultured cells abolishes both basal and heavy-metal-induced expression of *MT* and *ZnT-1* genes [1,2], whereas homozygous knockout of this gene in mice abrogates embryonic expression of the *MT-1* gene, significantly attenuates the expression of the *ZnT-1* and *γGCS_{hc}* genes in the embryo and causes fetal death due to liver degeneration [2–5].

MTF-1 contains six Cys₂His₂ zinc fingers and three transactivation domains [6–8]. The six zinc fingers are structurally

and functionally heterogeneous [9–15] and play an essential role in the metalloreulatory functions of MTF-1 [7,8,13–15]. However, the molecular mechanisms by which MTF-1 activates gene transcription in response to heavy metals are not completely understood. A current model of the mechanisms of MTF-1 action suggests that direct interactions between zinc and a subset of the zinc fingers of MTF-1 reversibly modulate MTF-1 DNA-binding activity [16–18], promote its rapid translocation into the nucleus [19,20] and facilitate the formation of a stable MTF-1–*MT-1* promoter–chromatin complex [15]. The three C-terminal transactivation domains of MTF-1 then modulate *MT-1* gene transcription (see [3,4] for reviews). A conserved cysteine-rich region near these transactivation domains of MTF-1 is also essential for the transactivation of gene expression by MTF-1 in response to metals [21].

Activation of *MT-1* gene expression by cadmium, a more potent inducer compared with zinc, has been postulated to utilize a distinct MTF-1-dependent mechanism. This is based on the following observations: (i) cadmium is less effective than zinc at driving MTF-1 to the nucleus [19,20], (ii) cadmium has little effect on DNA-binding activity of MTF-1 *in vitro* [18] and (iii) mouse MTF-1 can function as a zinc sensor but not as a cadmium sensor in yeast [22]. However, the formation of a stable

Abbreviations used: BIM I, bisindolylmaleimide I; ChIP, chromatin immunoprecipitation; CKII, casein kinase II; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; IEF, isoelectric focusing; JNK, c-Jun N-terminal kinase; MEF, mouse embryo fibroblast; MRE, metal-response element; MT, metallothionein; MTF-1, MRE-binding transcription factor-1; MTF-KO, MTF-1 knockout; PKC, protein kinase C; poly(A)⁺, polyadenylated; RNA Pol II, RNA polymerase II; ZnT-1, zinc transporter-1.

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Figure 1 Delineation of conserved consensus phosphorylation sites for various protein kinases in mouse MTF-1

Mouse MTF-1 contains six consensus PKC sites (Φ), 11 consensus CKII sites (Δ) and ten consensus JNK sites (*), among other protein kinase consensus sites (not shown). These consensus protein kinase sites are distributed throughout the entire peptide, including the zinc-finger domain, the transactivation (acidic, proline-rich and serine/threonine-rich) domains and the cysteine-rich region (CR). The eight-amino-acid FLAG tag (hatched flag shape) was added to the C-terminus of MTF-1 to facilitate identification of the protein.

MTF-1–chromatin complex at the *MT-I* promoter occurs rapidly in response to both zinc and cadmium *in vivo* [15], and mutations in MTF-1 zinc fingers that block zinc-induced *MT-I* gene expression also abolish its induction by cadmium [15]. Furthermore, a recent report showed that cadmium activation of MTF-1-dependent *in vitro* transcription required Zn₇-MT as a source of zinc [23]. Thus these two metals appear to utilize a common zinc-dependent mechanism to facilitate MTF-1–DNA interactions, but may employ distinct co-activators and/or signal transduction cascades to regulate gene expression.

Recent studies suggest that post-translational modification of MTF-1 may also play a role in its mechanism of action [24–27]. A survey of the MTF-1 peptide reveals numerous evolutionarily conserved consensus phosphorylation sites, including those for CKII (casein kinase II), PKC (protein kinase C) and JNK (c-Jun N-terminal kinase) (Figure 1). Inhibitors of these protein kinases have been shown to block metal induction of *MT-I* gene expression and the MRE-dependent activation of transiently transfected reporter genes [24–27]. Effects of protein kinase inhibitors on the metal-induced expression of other MTF-1 target genes have not been reported. MTF-1 is phosphorylated *in vivo* [25–27], but its role in the metalloregulatory functions of MTF-1 has not been addressed directly.

The mechanism(s) by which the inhibition of these kinases interferes with MTF-1-regulated gene expression warrants further investigation. Protein kinase inhibitors that inhibit metal-induced expression of the *MT-I* gene do not inhibit the *in vitro* DNA-binding activity or the nuclear translocation of MTF-1 [25–27]. However, neither an increase in the *in vitro* DNA-binding activity nor the nuclear translocation of MTF-1 ensures the formation of a stable MTF-1–chromatin complex, which is a rate-limiting step in MTF-1 activation of *MT-I* gene expression [15]. The effects of these kinase inhibitors on the metal-dependent formation of a stable MTF-1–chromatin complex has not been investigated. In the present study, we demonstrate the following: (i) inhibitors of PKC, CKII and JNK attenuate the metal-induced expression of the *MT-I* and *ZnT-1* genes; (ii) inhibitors of PKC have differential effects on the transcription of the *MT-I* and *ZnT-1* genes in different cell types; (iii) although MTF-1 can serve as an *in vitro* substrate for PKC, CKII and JNK, neither metal treatment nor inhibition of these kinases causes a significant change in the phosphorylation level or modification pattern of MTF-1; and (iv) protein kinase inhibitors that block metal-induced transcription of the native *MT-I* gene do not diminish the metal-dependent recruitment of MTF-1 to the *MT-I* promoter. These results suggest that signal transduction cascades may exert their effects on metal-regulated gene transcription through mechanisms that are independent of changes in the modification pattern of MTF-1. Instead, these signalling events may target other cell-type- and gene-speci-

fic proteins that co-operate with MTF-1 to activate gene transcription.

EXPERIMENTAL

Cell culture

Mouse Hepa cells were maintained in Dulbecco's modified Eagle's medium with high glucose content, supplemented with 2% (v/v) fetal bovine serum. Two stably transfected MEF (mouse embryo fibroblast) cell lines (lines 9 and 42) that express mouse MTF-1_{flag}, as described previously [15], were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and 200 µg/ml hygromycin B (Invitrogen, Carlsbad, CA, U.S.A.). These culture conditions were termed 2 and 10% basal medium respectively. Cells were treated at 75–90% confluency.

Northern blotting

Cells (80–90% confluency) were pretreated for 1 h with the indicated kinase inhibitors, before a 4 h incubation in basal medium with or without ZnSO₄ (100 µM) or CdCl₂ (20 µM). Total RNA was isolated using TRIzol[®] reagent (Invitrogen). Poly(A)⁺ (polyadenylated) RNA was purified from 100–250 µg of total RNA using Oligotex mRNA mini kits (Qiagen, Valencia, CA, U.S.A.). Total RNA (2 µg) or poly(A)⁺ RNA (from 20 µg of total RNA), as indicated in the legend to Figure 2, was separated by denaturing agarose–formaldehyde gel electrophoresis, transferred and UV cross-linked to Nytran Supercharge nylon membranes (Schleicher and Schuell, Keene, NH, U.S.A.). Northern blots were prehybridized, hybridized with an MT-I or ZnT-1 cRNA probe and washed as described previously [28]. Hybrids were detected by autoradiography at –70 °C with intensifying screens. Acridine Orange staining of rRNA or Northern blotting with a β-actin cRNA probe was used as a control to normalize for RNA amount and integrity.

Metabolic labelling and immunoprecipitation of MTF-1

Hepa cells (75% confluent in 10 cm plates) were washed twice with PBS, three times with prewarmed phosphate-deficient medium and incubated for 3 h in 2 ml of prewarmed phosphate-deficient medium containing 2% serum and 2 mCi of [³²P]P_i (150 mCi/ml). The cells were then treated for 30 min with zinc (100 µM) or cadmium (10 µM), washed three times with cold PBS containing 0.1 mM Na₃VO₄, and scraped off the plates in 800 µl of lysis buffer (50 mM Tris, pH 7.4, 500 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM Na₃VO₄, 20 mM β-glycerol phosphate, 5 mM benzamidine, 10 µg/ml leupeptin, 10 units/ml aprotinin, 1 mM PMSF and 1% Brij 98). The scraped cells were incubated on ice for 30 min, and cell lysates were clarified by centrifugation for 10 min at 500 g at 4 °C. MTF-1 was collected by immunoprecipitation using a rabbit antiserum against recombinant mouse MTF-1 described previously [19]. The supernatants (800 µg of protein), diluted in lysis buffer containing 150 mM NaCl, were incubated overnight with Protein A–Sepharose beads (60 µl of 1:1 slurry) at 4 °C to remove the proteins that bind to the Sepharose beads non-specifically, and the beads were removed by centrifugation. The precleared supernatants were then incubated with 1 µl of anti-MTF-1 antibody or preabsorbed anti-MTF-1 antibody (as a negative control) at room temperature (23 °C) for 1 h, followed by incubation with Protein A–Sepharose beads (60 µl of 1:1 slurry) for an additional 2 h. The preabsorbed anti-MTF-1 antibody was prepared by incubating the anti-MTF-1 antibody with recombinant MTF-1 purified from *Escherichia coli* at 4 °C for 2 h. The beads were washed four times with lysis

buffer containing 150 mM NaCl. MTF-1, eluted in $1.5 \times$ SDS sample buffer, was then subjected to SDS/PAGE. The gel was dried and exposed to a Kodak film at -70°C .

Isolation of recombinant mouse MTF-1_{flag} from *E. coli*

MTF-1_{flag} cDNA [15] was cloned into the bacterial expression vector pET-21a (Novagen, Madison, WI, U.S.A.) and transformed into *E. coli* strain BL21(DE3). Cells were grown in Luria–Bertani broth containing ampicillin and induced with isopropyl β -D-thiogalactoside for 2 h before harvesting. Cells were lysed by sonication in 25 mM Mops (pH 7.0), 150 mM NaCl, 0.5 % Tween 20 and $1 \times$ protease inhibitor cocktail without EDTA (Roche, Mannheim, Germany), and soluble proteins were bound to anti-FLAG M2 affinity resin (Sigma, St. Louis, MO, U.S.A.). MTF-1_{flag} was eluted from the affinity resin using $3 \times$ FLAG peptide (Sigma). The amino acid sequence of full-length MTF-1_{flag} was confirmed by two-dimensional electrophoresis–MS ‘fingerprinting’ (Kendrick Labs, Madison, WI, U.S.A.). The isolated protein was used as an *in vitro* substrate for protein kinases and as a mobility standard for unmodified MTF-1_{flag} in two-dimensional electrophoresis.

Two-dimensional electrophoresis and Western blotting

Recombinant mouse MTF-1_{flag}, isolated from *E. coli* by anti-FLAG affinity chromatography as described above, was dissolved 1:1 in urea sample buffer [9.5 M ultrapure urea, 2 % (w/v) Nonidet P40, 5 % (v/v) 2-mercaptoethanol, 2 % (w/v) ampholines consisting of 1.6 % (pH 5–7) and 0.4 % (pH 3.5–10) ampholines], heated at 50°C for 1 min and used for two-dimensional electrophoresis (Kendrick Labs). Whole cell lysates were prepared from MTF-KO (MTF-1 knockout) MEFs and MTF-1_{flag}-expressing cells (line 42) cultured in 10 % basal medium with or without ZnSO_4 (100 μM) or CdCl_2 (20 μM) for 1 h. Cells were collected in cold PBS, lysed at 4°C for 15 min in osmotic lysis buffer (10 mM Tris, pH 7.4, 0.3 % SDS, 1 mM Na_3VO_4 and 20 mM NaF) and incubated at 4°C for another 1 h in this buffer containing $1 \times$ nuclease stock [5 mM MgCl_2 , 10 mM Tris, pH 7.0, 50 $\mu\text{g}/\text{ml}$ RNase A (Sigma), 100 $\mu\text{g}/\text{ml}$ DNase I (Sigma) and $1 \times$ protease inhibitors [0.2 mM 4-(2-aminoethyl)benzenesulphonyl fluoride (Calbiochem, La Jolla, CA, U.S.A.), 10 $\mu\text{g}/\text{ml}$ leupeptin (Sigma), 3.6 $\mu\text{g}/\text{ml}$ *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (Sigma), 5 mM EDTA (Calbiochem) and 56 $\mu\text{g}/\text{ml}$ benzamide (Sigma)]. Total protein content was determined using the BCA (bicinchoninic acid) protein assay (Pierce, Rockford, IL, U.S.A.) with BSA (Sigma) as a standard. An equal volume of urea sample buffer was then added to the lysate. The resulting mixture was heated at 50°C for 1 min, spun briefly and the supernatant (150 μg of protein) was used for two-dimensional electrophoresis (Kendrick Labs). IEF (isoelectric focusing) was performed using 2 % (pH 3.5–10) ampholines (LKB–Pharmacia, Piscataway, NJ, U.S.A.). An IEF internal standard, tropomyosin (pI 5.2), was included in each sample. Recombinant MTF-1_{flag} isolated from *E. coli* was detected by Coomassie Blue staining, and the identity of the protein was confirmed by MS fingerprinting (Kendrick Labs). For these mammalian cell lysates, the SDS slab gel was transblotted on to PVDF membranes (Kendrick Labs) and MTF-1_{flag} was detected using an anti-FLAG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) in conjunction with a goat anti-rabbit IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology), as described previously [19], and visualized using ECL[®] reagent (Amersham Biosciences).

As another approach to resolve the constellation of MTF-1_{flag} spots, two-dimensional electrophoresis with samples prepared in SDS and a pH gradient of 4–7 was employed. SDS affects

the acidic end of the pH gradient and may alter the apparent pI of MTF-1. MTF-1_{flag}-expressing cells (lines 9 and 42; 5×10^6 cells each), cultured in 10 % basal medium with or without ZnSO_4 (100 μM , 1 h), CdCl_2 (20 μM , 1 h), staurosporine (0.5 μM , 4 h), dicoumarol (150 μM , 4 h) or DRB (5,6-dichloro-1- β -D-ribofuranosylbenzimidazole; 50 μM , 4 h) before harvesting. Cells were collected in cold PBS, homogenized in 3 vol. of whole cell extraction buffer [20 mM Hepes, pH 7.9, 1.5 mM MgCl_2 , 420 mM KCl, 0.5 mM dithiothreitol, 20 mM NaF, 1 mM Na_3VO_4 , 25 % (v/v) glycerol and $1 \times$ protease inhibitor cocktail without EDTA (Roche)], stirred on ice for 30 min and cleared by centrifugation at 4°C at 80000 *g* for 30 min. The extracts, diluted by 2-fold with whole cell extraction buffer without KCl, were then incubated with anti-FLAG M2–agarose affinity resin (Sigma) at 4°C for 2 h, and the resin was collected by centrifugation. After three washes with wash buffer [20 mM Hepes, pH 7.9, 150 mM KCl, 30 μM ZnSO_4 , 20 mM NaF, 1 mM Na_3VO_4 and $1 \times$ protease inhibitor cocktail without EDTA (Roche)], MTF-1_{flag} was eluted from the beads by heating at 95°C for 10 min in SDS sample buffer without 2-mercaptoethanol. The supernatant was collected, adjusted to 5 % 2-mercaptoethanol and heated at 95°C for another 5 min. These samples were then separated by two-dimensional electrophoresis (Kendrick Labs), subjected to Western blotting and detected using an anti-FLAG antibody (Santa Cruz Biotechnology) as described above, except that IEF was performed using 1 % (pH 4–6) ampholines (LKB–Pharmacia) and 1 % (pH 4–8) ampholines (Gallard–Schlesinger Industries, Garden City, NY, U.S.A.).

In vitro phosphorylation of MTF-1_{flag}

Recombinant mouse MTF-1_{flag} (approx. 100 ng) was incubated at 30°C for 60 min (i) with JNKs (20 ng for JNK1 or 100 ng for JNK2; Upstate Cell Signaling Solutions, Lake Placid, NY, U.S.A.) in 50 μl of JNK buffer (20 mM Mops, pH 7.2, 25 mM β -glycerol phosphate, 5 mM EGTA, 1 mM Na_3VO_4 , 1 mM dithiothreitol and 15 mM MgCl_2 , supplemented with 100 μM ATP/10 μCi of [γ - ^{32}P]ATP) or (ii) with CKII (50 units; Calbiochem) in 50 μl of CKII buffer {20 mM Tris/HCl (pH 7.5), 50 mM KCl and 10 mM MgCl_2 , supplemented with 200 μM ATP/10 μCi of [γ - ^{32}P]ATP} or (iii) with PKC (70 ng; Calbiochem) in 50 μl of PKC buffer (20 mM Tris/HCl, pH 7.5, 10 mM MgCl_2 , 0.5 mM CaCl_2 , 0.3 mg/ml phosphatidylserine, 0.03 mg/ml diacylglycerol and 0.3 % Triton X-100, supplemented with 15 μM ATP/5 μCi of [γ - ^{32}P]ATP). Reactions were then boiled for 5 min in SDS sample buffer and subjected to SDS/PAGE. The phosphorylated MTF-1_{flag} was detected by autoradiography at -70°C with intensifying screens.

ChIP (chromatin immunoprecipitation)

ChIP assays were performed as described previously [15] with modifications. Cells were cultured for 1 h in basal medium with or without kinase inhibitors and then for 1 h in the same medium with or without ZnSO_4 (100 μM) or CdCl_2 (20 μM) 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 [16], the nuclei were lysed in sonication buffer [15], the chromatin was sonicated to an average length of 600–1000 bp and clarified by centrifugation at 12000 *g* for 10 min at 4°C . The chromatin was diluted by 10-fold in ChIP dilution buffer [29], precleared with Protein A–Sepharose (Sigma) and immunoprecipitated with anti-FLAG M2–agarose affinity gel (Sigma) or an anti-MTF-1 antibody [19] or an anti-RNA polymerase II antibody (Santa Cruz Biotechnology) plus Protein A–Sepharose. The beads were

Figure 2 Effects of CKII, JNK and PKC inhibitors on metal-induced *MT-I* and *ZnT-1* gene expression in Hepa and MEF cells

Hepa cells (**A–C**) or MEF cells expressing MTF-1_{flag} (line 42) (**D, E**) were pretreated for 1 h with the indicated kinase inhibitors and then treated for 4 h with zinc (Zn; 100 μ M) or cadmium (Cd; 20 μ M) as indicated. Total RNA (**A, D, E**) or poly(A)⁺ RNA (**B, C**) was extracted and analysed for MT-I or ZnT-1 mRNA levels by Northern blotting. Acridine Orange staining (**D**) or Northern blotting with a β -actin cRNA probe (**A–C, E**) was used as controls to normalize for RNA amount and integrity. Unless otherwise indicated, the kinase inhibitors used were DRB (50 μ M for MEF cell line 42; 400 μ M for Hepa cells), dicoumarol (Dicu; 150 μ M), H7 (100 μ M for MEF cell line 42; 800 μ M for Hepa cells), BIM (10 μ M for MEF cell line 42; 30 μ M for Hepa cells) and staurosporine (Stau; 0.5 μ M for MEF cell line 42; 1 μ M for Hepa cells).

washed, the immunocomplexes were eluted and the cross-links were reversed as described previously [29]. The DNA was phenol/chloroform-extracted, reconstituted in TE buffer (10 mM Tris/1 mM EDTA, pH 8.0) and analysed by PCR using primers that span –230 to +123 bp (relative to the transcription start site) of the mouse *MT-I* gene. PCR products (31 cycles) were separated by electrophoresis on TAE-agarose gels (composition of TAE: 40 mM Tris/40 mM acetate/1 mM EDTA, pH 8.3) and then stained with SYBR Green (Sigma), quantified using the ChemiImager System (Alpha Innotech, San Leandro, CA, U.S.A.) and normalized to input products. For initial experiments, input and immunoprecipitated DNA samples were analysed at various cycles (27–33 cycles) to ensure that the PCR products were obtained in the linear range of amplification.

RESULTS

Effects of inhibitors of PKC, CKII and JNK on metal-induced *MT-I* and *ZnT-1* gene expression

To examine the roles of signalling cascades in the metal-induced gene expression regulated by MTF-1, cell-permeant inhibitors of

various kinases were used to treat Hepa and MEF cells before metal treatment, and MT-I and ZnT-1 mRNA levels in these cells were determined by Northern blotting. The MEF cells used in the present study were MTF-KO MEFs, stably transfected with an expression vector for a full-length mouse MTF-1 tagged with a FLAG epitope (MTF-1_{flag}), which facilitates the detection and purification of the protein in subsequent studies. Similar to the native protein, MTF-1_{flag} translocates into the nucleus and shows increased *in vitro* DNA binding after exposure of the cells to zinc, forms a stable complex with the *MT-I* promoter chromatin *in vivo* in response to zinc and cadmium, and fully restores metal-induced *MT-I* gene expression [15].

DRB is an inhibitor of CKII. Pretreatment of Hepa cells and MEF cells expressing MTF-1_{flag} (line 42) with DRB severely attenuated the metal-induced expression of both *MT-I* and *ZnT-1* genes (Figures 2A, 2B and 2D). Both MT-I and ZnT-1 mRNA levels were greatly reduced in cells treated with metals compared with cells treated with metals only. Similarly, pretreatment of Hepa and MEF cells (line 42) with dicoumarol, an inhibitor of JNK signalling, significantly attenuated metal-induced *MT-I* and *ZnT-1* gene expression (Figures 2A, 2B and 2D). These results are consistent with roles for CKII and JNK

Figure 3 Metal treatment does not cause increased incorporation of labelled-phosphate into MTF-1 *in vivo*

Hepa cells were incubated in phosphate-deficient medium containing [32 P] P_i (1 mCi/ml) for 3 h and then treated with Zn (100 μ M) or Cd (10 μ M) for 30 min. Whole cell lysates were immunoprecipitated with an anti-MTF-1 antibody or a preabsorbed anti-MTF-1 antibody. The immunoprecipitates were separated by denaturing PAGE and detected by autoradiography. Equal loading of each lane was verified by silver staining of the slab gel (results not shown).

in metal regulation of gene expression, as suggested previously [25–27].

H7 and staurosporine are broad-spectrum inhibitors of protein kinases including PKC, whereas BIM I (bisindolylmaleimide I) is a selective inhibitor of PKC. To our surprise, these inhibitors exerted differential effects on basal as well as metal-induced *MT-I* and *ZnT-1* gene expression. In mouse Hepa cells, H7 had no effect on the metal-induction of the *MT-I* gene, but completely abolished *ZnT-1* induction even when present at low concentrations (Figure 2C). In contrast, H7 blocked both *MT-I* and *ZnT-1* induction in MEF cells (line 42; Figures 2D and 2E). BIM I caused a large increase in the basal levels of *ZnT-1* mRNA in Hepa cells, but the magnitude of this effect on the basal levels of *MT-I* mRNA was variable. The metal-induced *MT-I* and *ZnT-1* mRNA levels in BIM I- and metal-treated Hepa cells were similar to those in cells treated with metals only (Figures 2A and 2B). Similarly, staurosporine had little effect on the metal induction of the *MT-I* gene, and had variable effects on *ZnT-1* gene expression in Hepa cells (Figures 2A and 2B). In contrast, BIM I and staurosporine severely attenuated metal induction of the *MT-I* gene in MEF cells, but had little effect on the basal levels of *MT-I* mRNA (Figure 2D). These results suggest cell-type- and gene-specific roles for PKC in the metal regulation of gene expression.

MTF-1 is phosphorylated *in vivo*, but the extent of incorporation of phosphate is not altered after metal treatment

MTF-1 is phosphorylated *in vivo* in transfected COS and HEK-293 cells [25–27]. In a recent study, a 2–4-fold increase in MTF-1 phosphorylation was detected after 20 min of zinc treatment [26]. However, MTF-1 is overexpressed significantly in transfected cells, which may affect these results. In other studies, phosphorylated MTF-1 increased in the nucleus after metal treatment [25,27], consistent with metal-induced MTF-1 nuclear translocation [19,20]. In the present study, we examined the phosphorylation state of native MTF-1 in response to zinc and cadmium, and we focused on the time period of induction of gene transcription by metals (within 1 h). Mouse Hepa cells were incubated in phosphate-deficient medium containing [32 P] P_i and treated for 30 min with zinc or cadmium. MTF-1 was immunoprecipitated, separated on a denaturing polyacrylamide gel and detected by autoradiography. Equal loading of each lane was confirmed by silver staining of the slab gel (results not shown). A radioactive protein with an apparent molecular mass of 100 kDa was detected in control as well as metal-treated cells (Figure 3). This protein co-migrated with immunoreactive MTF-1 and was absent from the control reactions that were immunoprecipitated with preabsorbed antiserum (Figure 3). These results confirmed that MTF-1 is phosphorylated *in vivo*. Although this experiment may not have detected small changes in MTF-1 phosphorylation,

Figure 4 Metal treatment does not cause a significant change in the overall modification pattern of MTF-1

(A) Two-dimensional electrophoresis (pH 3.5–10)–Western blotting of MTF-1_{flag} was performed using lysates prepared in urea. MTF-KO MEF cells that lack endogenous MTF-1 and those cells expressing MTF-1_{flag} (line 42) were cultured for 1 h in 10% basal medium (ctrl) or 10% basal medium plus 100 μ M Zn or 20 μ M Cd. For comparison, recombinant mouse MTF-1_{flag} isolated from *E. coli* was prepared in urea, subjected to two-dimensional electrophoresis and detected by Coomassie Blue staining. MTF-1_{flag} from mammalian cells was detected by using an anti-FLAG antibody. (B) Two-dimensional electrophoresis (pH 3.5–10)–Western blotting of MTF-1_{flag} was performed using lysates prepared in SDS. The effects of metal treatment on the modification pattern of MTF-1_{flag} were examined. MTF-1_{flag}-expressing MEF cells (lines 9 and 42) were cultured for 1 h in 10% basal medium (ctrl) or 10% basal medium plus 100 μ M Zn or 20 μ M Cd before harvesting. MTF-1_{flag} was isolated from whole cell extracts using anti-FLAG M2–agarose affinity chromatography, dissolved in SDS sample buffer, fractionated by two-dimensional electrophoresis (pH 4–7) and detected by Western blotting with an anti-FLAG antibody. (A, B) The asterisk indicates the position of an internal standard with a pI of 5.2.

the results suggest that the level of MTF-1 phosphorylation is not significantly altered after metal treatment (Figure 3).

MTF-1_{flag} is highly modified *in vivo*, but metal treatment does not cause a significant change in its modification pattern

To determine the extent of MTF-1 modification and to assess the effects of metal treatment on the modification pattern of MTF-1, we employed two-dimensional electrophoresis coupled with Western blotting. To facilitate detection of the MTF-1 protein, two stably transfected MEF cell lines expressing MTF-1_{flag} (lines 9 and 42) were examined [15].

In initial experiments, recombinant MTF-1_{flag} isolated from *E. coli* by anti-FLAG affinity chromatography and whole cell lysates from MTF-KO cells and MTF-1_{flag}-expressing cells (line 42) before or after metal treatment were prepared in urea and subjected to two-dimensional electrophoresis (pH 3.5–10). The resulting SDS slab gel was stained with Coomassie Blue or transblotted on to PVDF membranes and Western blotted using an anti-FLAG antibody. A constellation of 6–8 spots with pI values ranging from 4.8 to 5.7 was detected in MTF-1_{flag}-expressing cells (line 42; Figure 4A). This constellation represented MTF-1_{flag}, since it was absent from the cell lysate from MTF-KO cells that lack endogenous MTF-1 (Figure 4A). Recombinant mouse MTF-1_{flag} isolated from *E. coli* migrated as a single spot with a pI of approx. 5.7 (Figure 4A). These results indicate that MTF-1_{flag} expressed in mammalian cells is highly modified. The fact that these modifications reduced the pI of the protein is consistent with MTF-1 being phosphorylated. However, the electrophoretic migration pattern of MTF-1_{flag} appeared to be essentially the same in untreated and metal-treated cells (Figure 4A). Thus metal treatment did not cause a significant change in the modification pattern of MTF-1, although we cannot exclude the possibility of subtle changes that are not revealed using this method.

To extend the above observations and to resolve better the constellation of MTF-1, MTF-1_{flag} was isolated by anti-FLAG

Figure 5 MTF-1_{flag} can serve as an *in vitro* substrate for CKII, PKC and JNK

Recombinant mouse MTF-1_{flag} was isolated from *E. coli* and used as a substrate in *in vitro* kinase assays. Purified CKII (A) or PKC (B) or JNKs (JNK1 or JNK2) (C) were incubated with (+) or without (–) MTF-1_{flag} in the appropriate buffers containing [γ -³²P]ATP. The reactions were then separated by SDS/PAGE and the radiolabelled products were detected by autoradiography. Two experiments were performed for each kinase and similar results were obtained in each experiment.

M2-agarose affinity chromatography from whole cell extracts prepared from MTF-1_{flag}-expressing cells (lines 9 and 42) before or after a 1 h treatment with metals and then dissolved in SDS sample buffer and subjected to two-dimensional electrophoresis–Western blotting using a narrower pH gradient (pH 4–7). A constellation of MTF-1_{flag} with pI values ranging from 5.1 to 5.6 was detected in both cell lines (Figure 4B), indicating that MTF-1_{flag} in both cell lines is highly modified. However, consistent with the above observations, the electrophoretic migration pattern of MTF-1_{flag} appeared to be essentially the same in untreated and metal-treated cells in both cell lines (Figure 4B), confirming that metal treatment did not cause a significant change in the modification pattern of MTF-1.

MTF-1 can serve as an *in vitro* substrate for CKII, PKC and JNK, but inhibition of these protein kinases does not cause a significant change in its modification pattern *in vivo*

To determine whether MTF-1_{flag} can serve as a substrate for CKII, PKC and JNK, *in vitro* assays were performed using purified kinases and recombinant mouse MTF-1_{flag} isolated from *E. coli* by anti-FLAG M2-agarose affinity chromatography.

Incubation of recombinant MTF-1_{flag} with CKII (a promiscuous kinase that is constitutively active [30]), PKC (affinity-purified from rat brain, contains predominantly the conventional PKC isoforms α , β_1 , β_2 and γ) or JNKs (JNK1 or JNK2, the two main isoforms of JNKs that are ubiquitously expressed [31]) in the presence of [γ -³²P]ATP resulted in a radiolabelled product with an apparent molecular mass of 100 kDa, which was not present in control reactions in which no MTF-1_{flag} was added (Figure 5). Incorporation of ³²P into MTF-1_{flag} was kinase-dependent, since no radiolabelled MTF-1_{flag} was detected when the kinases were omitted from the reaction (results not shown). Although the extent of incorporation of ³²P into MTF-1_{flag} by PKC was very low when compared with that by CKII or JNKs under comparable reaction conditions, these results demonstrated that MTF-1_{flag} can serve as a substrate for each of these kinases *in vitro*.

As another approach for revealing the roles of PKC, CKII and JNK in MTF-1 modification, we investigated whether inhibition of these kinases affects the modification pattern of MTF-1, using two-dimensional electrophoresis–Western blotting. MTF-1_{flag} was affinity-purified from MTF-1_{flag}-expressing cells (line 42) [15] treated with kinase inhibitors for 4 h. The results demonstrated that the modification pattern of MTF-1_{flag} from DRB-treated cells was essentially indistinguishable from that of control cells,

Figure 6 Treatment with protein kinase inhibitors does not cause a significant change in the overall modification pattern of MTF-1_{flag}

MTF-1_{flag}-expressing MEF cells (line 42) were cultured for 4 h in 10% basal medium containing no inhibitor (ctrl), staurosporine (0.5 μ M), dicoumarol (Dicu; 150 μ M) or DRB (50 μ M) before harvesting. MTF-1_{flag} was isolated, fractionated and detected as described in the legend to Figure 4(B). The asterisk indicates the position of an internal standard with a pI of 5.2.

whereas treatment with staurosporine or dicoumarol appeared to reduce slightly, but clearly did not eliminate, the complex modification pattern of MTF-1_{flag} (Figure 6). Overall, inhibition of these protein kinases had little effect on the modification pattern of MTF-1_{flag}. These results suggest that the protein kinases examined are not solely responsible for maintaining the complex modification pattern of MTF-1 and/or that these modifications are relatively stable and do not turn over within the 4 h inhibition period.

Inhibitors of PKC, CKII and JNK have no effect on the metal-dependent recruitment of MTF-1_{flag} to the endogenous *MT-I* promoter

A ChIP assay was employed to assess whether PKC, CKII and JNK exert their effects on metal-induced *MT-I* gene expression by modulating metal-dependent recruitment of MTF-1 to the *MT-I* promoter *in vivo*. Soluble cross-linked chromatin was prepared from Hepa cells or MTF-1_{flag}-expressing cells (line 42) that have been pretreated with kinase inhibitors before metal treatment. The chromatin was immunoprecipitated using anti-FLAG M2-agarose affinity resin, anti-MTF-1 antibody plus Protein A–Sepharose or Sepharose beads alone. The amount of *MT-I* proximal promoter DNA present in the precipitates was quantified by PCR and normalized to input DNA. Consistent with our previous results [15], the amount of MTF-1 bound to the chromosomal *MT-I* promoter was rapidly (1 h) and significantly increased (by approx. 5-fold in Hepa cells and approx. 2.5-fold in line 42) after exposure of the cells to zinc or cadmium (Figure 7). However, the increased binding of MTF-1 to the *MT-I* promoter in response to metals was not attenuated by pretreatment with any of the kinase inhibitors examined, including DRB and dicoumarol in either MEF cells (line 42; Figure 7A) or Hepa cells (Figure 7B). Interestingly, in Hepa cells, the basal chromosome-binding activity of MTF-1 was apparently increased (2.1–2.7-fold) in cells that were pretreated with kinase inhibitors alone compared with the untreated control. However, the metal-induced recruitment of MTF-1 to the promoter was still measurable (Figure 7A). Thus CKII, PKC and JNK exert their role in metal-induced *MT-I* gene expression at a step subsequent to the metal-induced recruitment of MTF-1 to the *MT-I* promoter.

Similarly, none of these kinase inhibitors reduced the amount of RNA Pol II (RNA polymerase II) bound to the *MT-I* promoter (Figure 7). In cells cultured in basal medium, there was a significant amount of RNA Pol II bound to the *MT-I* promoter (Figure 7). This reflects the fact that this gene is actively transcribed even in the absence of exogenous metals, probably due to the presence of zinc in the medium. In the absence of kinase inhibitors,

Figure 7 Effects of kinase inhibitors on metal-induced recruitment of MTF-1_{flag} and RNA Pol II to the chromosomal *MT-I* promoter

Chromatin was isolated from MTF-1_{flag}-expressing MEF cells (line 42) (A) or Hepa cells (B) pretreated for 1 h with the indicated kinase inhibitors before a 1 h incubation in basal medium alone (–) or basal medium plus 100 μ M Zn or 20 μ M Cd. The kinase inhibitors used were as described in the legend to Figure 2. ChIP was performed with an anti-FLAG M2-agarose affinity resin (anti-FLAG, for MEF cell line 42), an anti-MTF-1 antibody plus Protein A-Sepharose (anti-MTF-1 for Hepa cells), an anti-RNA Pol II antibody plus Protein A-Sepharose (anti-RNA Pol II) or beads alone (no Ab). The precipitated DNA was analysed by PCR of the mouse *MT-I* proximal promoter, and the PCR products were quantified by SYBR Green staining of the gel. Fold induction was calculated after normalizing antibody-specific PCR products to input products and was expressed relative to the amount of binding in control cells (lane 1). Two experiments, with duplicate PCRs for each experiment, were performed, yielding similar results.

the amount of RNA Pol II bound to the *MT-I* promoter was evidently increased (by approx. 2-fold) after exposure of the cells to metals. In the presence of kinase inhibitors, the basal levels of RNA Pol II bound to the promoter were slightly increased and did not change significantly after metal treatment (Figure 7). Thus inhibition of metal-induced *MT-I* gene expression does not result from a loss of RNA Pol II at the promoter.

DISCUSSION

It has been shown previously that protein kinase signalling cascades play a role in metal-induced *MT-I* gene expression [24–27], and this role was suggested to involve changes in the phosphorylation of MTF-1 [24–27]. Our results extend our understanding of the involvement of protein kinase signalling in metal-induced gene expression, but suggest that changes in MTF-1 phosphorylation may not play a paramount role in this regulation, at least during the initial phase of gene activation. A novel finding that inhibitors of PKC exert gene- and cell-type-specific effects on the expression of these two genes is consistent with the suggestion that MTF-1 is not the direct target(s) of signalling in these cells. Instead, a cofactor or a group of cofactors specific to each gene may be the real targets of PKC modification. The finding of cell-type-specific effects of inhibitors of PKC on metal regulation of these genes is surprising, but supports the concept that this regulation may involve multiple kinase cascades and gene-specific co-activators that may be uniquely represented in each of these cell types. The involvement of multiple signal transduction cascades, possible cross-talk between them, and their remarkable interdependent effects on the metal-dependent regulation of these genes are consistent with this concept. It should also be kept in mind that the inhibitors used in these studies are by no means

specific to only one kinase, and unidentified kinases may also be inhibited by these compounds. Furthermore, there are multiple isoenzymes of PKC, which further complicates the interpretation of these results.

Consistent with the idea that MTF-1 may not be the real target of these signalling events, our results demonstrate that endogenous MTF-1 is constitutively phosphorylated *in vivo* in Hepa cells, and provide evidence for the first time that metal treatment does not cause a significant change in the extensive modification pattern of MTF-1. This result contrasts with a previously reported increase in the incorporation of phosphate into MTF-1 after metal treatment [26]. The reason for this difference is not clear. However, LaRochelle et al. [26] analysed the phosphorylation of the MTF-1 overexpressed in transiently transfected COS cells, whereas we examined the phosphorylation of the endogenous native MTF-1 in Hepa cells. Also, our experiment would not reveal small changes (< 2-fold) in MTF-1 phosphorylation.

For direct phosphorylation of MTF-1 to play a role in the metal-oregulatory functions of MTF-1, the responsible kinase(s) would be predicted to act quickly in response to metals, which would require the critical phosphate group(s) to be labile. The subtle changes in the modification pattern of MTF-1 after a prolonged inhibition of PKC, CKII and JNK, which attenuates metal induction of MTF-1 target genes, suggest that most of the modifications of MTF-1 are quite stable. Although previous findings that inhibition of these protein kinases impaired the metal-induced expression of MREd-driven reporter genes [25–27] suggest that MTF-1 is involved in the regulation of metal-induced gene expression by these kinases, the differential effects of protein kinase inhibitors on metal-induced expression of the *MT-I* and *ZnT-I* genes, both MTF-1 target genes, argues against a common mechanism of signalling cascades directly modulating MTF-1 activity. Given that our results also demonstrated that kinase inhibitors did not block the metal-induced formation of the MTF-1–chromatin complex, we suggest that phosphorylation events may indirectly modulate the metal-oregulatory functions of MTF-1, perhaps by acting on cofactors that interact with MTF-1. The idea that modifications of cofactors can alter their interactions with transcription factors and/or their activity has been well documented. Phosphorylation of NDD1p, a co-activator of the fork-head transcription factor Fkh2p, is required for its interaction with and normal transcriptional regulation by the transcription factor [32]. C/EBPs (CCAAT/enhancer-binding proteins) transactivate gene expression by recruiting the co-activators CBP (CREB-binding protein, where CREB stands for cAMP-response-element-binding protein) and p300, which, in turn, promotes transcription by acetylating histones and recruiting general transcription factors. Recruitment of CBP and p300 by C/EBP triggers the phosphorylation of CBP and p300, which is required for their co-activator functions [33,34].

To date, no co-activators have been identified to interact with MTF-1. Upstream stimulatory factors have been shown to participate in cadmium-induced *MT-I* gene expression [35]. However, a direct interaction of upstream stimulatory factor 1 with MTF-1 has not been observed. Although a role for a metal-sensing inhibitor of MTF-1 has been suggested in metal regulation of *MT-I* gene expression [36], the isolation of such an inhibitor has not been reported. If protein kinases act on this inhibitor to modulate metal-induced gene expression, it is unlikely that this inhibitor is involved in the chromatin-binding activity of MTF-1. The increased basal expression of the *MT-I* and *ZnT-I* genes in BIM I- or staurosporine-treated Hepa cells suggest that dephosphorylation events possibly facilitate the release of this inhibitor from the chromatin-bound MTF-1, allowing the transcription factor to recruit a co-activator and/or the general transcription

machinery. In addition to potentially acting on putative MTF-1-interacting cofactors, some of these kinase inhibitors may affect the general transcription machinery. DRB is known to inhibit kinases that phosphorylate the C-terminal domain of the largest subunit of RNA Pol II and efficiently blocks RNA Pol II in the early elongation stage [37,38]. Therefore the effects of DRB treatment on metal-induced gene expression may reflect inhibition of transcriptional elongation.

Although we consider it unlikely, our results cannot formally exclude the possibility that metal ions may cause a subtle change in the phosphorylation state of MTF-1 or a change in a subpopulation of MTF-1 molecules, which directly affect its metalloregulatory functions. Interestingly, there is a highly conserved PKC consensus site (S/T-X-R/K) and a JNK consensus site (S/T-P) within a conserved cysteine-rich region near the C-terminus of MTF-1. This cysteine-rich region has been suggested to play a role in the metal-regulated transactivation of gene expression by MTF-1 [21]. It is possible that metal-activated signal transduction cascades may specifically affect the ability of this region to interact with metals and/or other transcription factors or co-activators.

Another point worth noting is that most of the MTF-1 resides in the cytoplasm in resting cells, but moves into the nucleus after exposure to zinc [19,20]. The similar two-dimensional migration pattern of MTF-1 in both untreated and zinc-treated cells suggests that cytoplasmic and nuclear MTF-1s share similar modifications. This, in turn, suggests that the majority of phosphorylation events occur in the cytoplasm and have no effect on zinc-induced nuclear translocation of MTF-1.

It has been proposed that cadmium utilizes a distinct mechanism, relative to zinc, to induce gene expression. However, so far, studies have not been able to differentiate these two metals in terms of gene induction. The findings that the modification pattern of MTF-1 appears to be the same after zinc or cadmium treatment, and that kinase inhibitors block *MT-I* and *ZnT-I* gene induction by both zinc and cadmium to the same extent, all point to a common mechanism utilized by these two metals. As suggested previously [23,36], cadmium may cause redistribution of a small fraction of cellular zinc, probably from the zinc pool bound by MT, which is sufficient to activate nuclear MTF-1 to form a stable complex with the promoter and then activate gene transcription. This, in turn, suggests that a subpopulation of MTF-1 is sufficient to form a stable chromatin complex and to carry out its metalloregulatory functions. However, inhibition of PKC, CKII or JNK does not affect cadmium-induced MTF-1 binding to the *MT-I* promoter chromatin.

In summary, our studies confirm the involvement of PKC, CKII and JNK signalling in metal-induced *MT-I* and *ZnT-I* gene expression. However, the effects of PKC signalling are gene- and cell-type-specific. We also demonstrate that, although MTF-1 is phosphorylated *in vivo* and can serve as a substrate for these kinases *in vitro*, neither metal treatment nor inhibition of these kinases changes the MTF-1 modification pattern significantly. Furthermore, all three kinases seem to act subsequent to the metal-dependent recruitment of MTF-1 to the *MT-I* promoter. Taken together, our results suggest that inhibitors of PKC, CKII and JNK modulate metal-induced gene expression by acting on putative MTF-1-interacting cofactors.

G. K. A. was supported by NIH grant no. ES05704. We are indebted to J. Geiser and G. Lin for excellent technical assistance.

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Received 29 March 2004/11 May 2004; accepted 14 May 2004

Published as BJ Immediate Publication 14 May 2004, DOI 10.1042/BJ20040504