Two major branches of anti-cadmium defense in the mouse: MTF-1/metallothioneins and glutathione

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ABSTRACT

Metal-responsive transcription factor 1 (MTF-1) regulates expression of its target genes in response to various stress conditions, notably heavy metal load, via binding to metal response elements (MREs) in the respective enhancer/promoter regions. Furthermore, it serves a vital function in embryonic liver development. However, targeted deletion of Mtf1 in the liver after birth is no longer lethal. For this study, Mtf1 conditional knockout mice and control littermates were both mock- or cadmium-treated and liver-specific transcription was analyzed. Besides the well-characterized metallothionein genes, several new MTF-1 target genes with MRE motifs in the promoter region emerged. MTF-1 is required for the basal expression of selenoprotein W, muscle 1 gene (Sepw1) that encodes a glutathione-binding and putative antioxidant protein, supporting a role of MTF-1 in the oxidative stress response. Furthermore, MTF-1 mediates the cadmium-induced expression of N-myc downstream regulated gene 1 (Ndrg1), which is induced by several stress conditions and is overexpressed in many cancers. MTF-1 is also involved in the cadmium response of cysteine- and glycine-rich protein 1 gene (Csrp1), which is implicated in cytoskeletal organization. In contrast, MTF-1 represses the basal expression of S1ci39a10, a putative zinc transporter. In a pathway independent of MTF-1, cadmium also induced the transcription of genes involved in the synthesis and regeneration of glutathione, a cadmium-binding antioxidant. These data provide strong evidence for two major branches of cellular anti-cadmium defense, one via MTF-1 and its target genes, notably metallothioneins, the other via glutathione, with an apparent overlap in selenoprotein W.

INTRODUCTION

All organisms have evolved mechanisms to cope with a variety of stress situations. One type of stress response is triggered by heavy metals, such as zinc, copper and cadmium (for convenience, the terms zinc, copper and cadmium are also used here to denote Zn$^{2+}$, Cu$^{2+}$ and Cd$^{2+}$, respectively). Metallothioneins (MTs), small, cysteine-rich proteins, play an important role in metal homeostasis and detoxification due to their ability to bind different heavy metal ions (1–3). In the mouse, there are four metallothionein genes, designated as Mt1 to Mt4. Basal, as well as heavy metal-induced, expression of Mt1 and Mt2 is mediated by metal-responsive transcription factor 1 (MTF-1) (4–7). This zinc finger protein recognizes short cis-acting DNA sequences, termed metal response elements (MREs; core consensus sequence TGCCRCNC), which are present in the promoters of target genes (8,9). MTF-1 is conserved in evolution, and homologs have been characterized in the mouse (10), humans (11), Drosophila (12–14) and fish (15,16).

The role of MTF-1 has been studied most extensively in the mouse. Besides coping with heavy metal load, MTF-1 can also mediate the induction of Mt genes in response to other stress situations, such as oxidative stress (5,17) and hypoxia (18). In addition, it is required for the metalloregulation of Znt1, encoding the major plasma membrane-localized zinc efflux transporter (19), the hypoxic/anoxic induction of the gene encoding the major plasma membrane-localized zinc efflux transporter (19), the hypoxic/anoxic induction of the gene for placental growth factor (Plgf), an angiogenic protein of the vascular endothelial growth factor (VEGF) family (20), and has recently been invoked in tumor development (21,22). Furthermore, MTF-1 has an essential function during embryogenesis: targeted disruption of Mtf1 results in embryonic lethality around 14 days post coitum due to hepatocyte necrosis (23). In contrast, mice with null mutations for the stress-inducible metallothionein genes (Mt1 and Mt2) are viable, though sensitive to cadmium (24,25), indicating that additional important MTF-1 target genes are involved in the lethal phenotype. With the Cre-loxP conditional knockout technique, it is possible to circumvent the embryonic lethal phenotype of conventional Mtf1 knockout mice. Previous experiments with this technique revealed that deletion of...
Mtf1 from the liver after birth is no longer lethal under non-stress conditions (26).

For this study, an inducible, liver-specific Mtf1 knockout mouse line was generated to perform a search for MTF-1 target genes and cadmium-inducible genes in the adult liver. A number of target gene candidates emerged upon a transcriptome analysis of mock- and cadmium-treated Mtf1 conditional knockout mice and control littermates and several of these were confirmed by semiquantitative RT–PCR. Besides the stress-inducible metallothionein genes that were already known as target genes of MTF-1, we find that MTF-1 is important for basal liver expression of the gene for selenoprotein W, muscle 1 (Sepw1) as well as for cadmium-induced expression of N-myc downstream regulated gene 1 (Ndr1) and the gene encoding cysteine- and glycine-rich protein 1 (Csrp1). In addition, MTF-1 appears to repress the expression of solute carrier family 39, member 10 gene (Scl39a10), which encodes a putative metal ion transporter. In an MTF-1-independent transcriptome response, several genes involved in glutathione metabolism are induced. Further studies confirmed a dual anti-cadmium defense, one via glutathione and another one via MTF-1 and its target genes, including metallothioneins.

MATERIALS AND METHODS

Generation of Mtf1 conditional knockout mice and liver-specific deletion

Mtf1 conditional knockout mice were generated in collaboration with Dr Michael Leviten (San Carlos, CA). Two genomic clones containing exons 3 to 6 of Mtf1 were used to construct a gene targeting vector for homologous recombination (Supplementary Data). A neomycin resistance cassette (PGK-neo) flanked by two loxP sites was cloned into the SacI site 5′ of exon 3 of Mtf1, the third loxP site was cloned into the Scal site 3′ of exon 4. A thymidine kinase (TK) cassette was inserted in the HpaI site 3′ of exon 6. 129 ES cells were electroporated with the linearized targeting vector, selected in the presence of G418 and FIAU, and screened for correct integration events by PCR and Southern blot analysis. Transient expression of Cre recombinase led to removal of the PGK-neo cassette, and mice carrying the modified Mtf1loxP allele were generated by injection of positive clones into C57Bl/6 blastocysts and subsequent crosses. Homozygous conditional knockout animals (Mtf1loxP/loxP) were crossed with the Cre recombinase transgenic line Mx-cre (a gift from Prof. Michel Aguet) to obtain an inducible, liver-specific Mtf1 knockout line. The mice were genotyped by PCR using the following primers (Microsynth): Cre recombinase: 5′-CTATCCAGCAACATTGGGCCAGC-3′; 5′-CCAGGT-TACCGATATAGTTCATGAC-3′; Mtf1loxP or wild-type allele: 5′-CACACCCCCAGTTGTTAGTGTCTTC-3′; 5′-CATGATAAGGCAAAACTTGGC-3′.

Animal treatment

At 8 weeks of age, male Mtf1loxP/loxP mice harboring the Mx-cre transgene (Mtf1loxP/loxP Mx-cre, abbr.: Mtf1loxP/Mx-cre) and control littermates without transgene (Mtf1loxP/loxP, abbr.: Mtf1loxP) received four intraperitoneal injections each of 300 μg synthetic double-stranded RNA polyinosinic-polycytidylic acid [pI–pC; Sigma; in a volume of 60 μl phosphate-buffered saline (PBS)] at 3 day intervals. Only in the case of DNA-binding studies with MRE sequences from MTF-1 target gene candidates, the control mice received no pI–pC injections. For experiments with metal treatment, mice received 2 days after the last pI–pC treatment a subcutaneous (s.c.) injection of either 20 μmol/kg body weight CdSO4 (2 mM CdSO4 in H2O; cadmium treatment) or 10 ml/kg body weight H2O (mock treatment) 6 h before sacrificing them.

Microarray analysis and data processing

Total RNA was isolated from liver tissue of pI–pC-induced, mock- or cadmium-treated Mtf1loxP/Mx-cre and Mtf1loxP mice (n = 3 per genotype and respective treatment; all male) essentially as described by Chomczynski and Sacchi (27).

Gene expression analysis was performed in the Functional Genomics Center Zurich using GeneChip® Mouse Genome 430 2.0 Arrays (Affymetrix) according to the manufacturer’s instructions and the following reagents and conditions. cDNA was synthesized with SuperScript™ Double-Stranded cDNA Synthesis Kit (Invitrogen), using 15 μg total RNA. In vitro transcription was performed with BioArray™ High Yield™ RNA Transcript Labeling Kit (Enzo) and 3.5 to 6 μg of each cDNA. Clean-up of both cDNA and cRNA samples was done using GeneChip® Sample Clean-up Module (Affymetrix). For the automated washing and staining in the Affymetrix fluidics station 450, the protocol EukGE-Ws2v4.450 was used. The probe arrays were scanned with the Affymetrix GS 3000 scanner. Raw data are available at ArrayExpress (http://www.ebi.ac.uk/arrayexpress, accession number E-MEXP-438).

Data analysis was performed with GeneSpring 6.1 software (Silicon Genetics), applying a significance level P ≤ 0.05. Furthermore, multiple testing correction was used in addition to obtain cadmium-responsive, MTF-1-independent genes. Genes were considered to be differentially expressed if there was at least a 2-fold difference in expression levels of the compared experimental groups (genotype and respective treatment). The result was considered reliable if signal values for the respective gene were scored ‘present’ at least for all mice in one experimental group or for two mice in each of two groups.

To screen for the presence of the MRE core consensus sequence TGCRNC in the promoter region, the upstream sequences of the respective genes were obtained from the University of California Santa Cruz (UCSC) Genome Browser Database (http://genome.ucsc.edu; October/November 2004) (28).

RT–PCR

All RT–PCRs were performed with QIAGEN® OneStep RT–PCR Kit (QIAGEN) according to the manufacturer’s instructions, using 150–200 ng DNase I-digested total RNA (RNA isolation see microarray analysis). The reactions were carried out using the following primers: Csrp1: 5′-TTCGG-ATGTGCCAAGATGGCCAGC-3′; 5′-AGTAGAGGATGGACATTACG-3′; hypoxanthin-guanin-phosphoribosyltransferase (Hprt): 5′-GCTGGTGGAAGGACCTCTCG-3′; 5′-CCAGGACTAGTACACCTGC-3′, Mtf1: 5′-GTCATTTT-GAGACTGTACTGAGTG-3′; 5′-CATGCAAGAGAACAT-
S1 nuclease mapping of transcripts (S1 analysis)

S1 analysis was performed as previously described (29), using 100 μg DNase I-digested total RNA (RNA isolation see microarray analysis). The gels were developed using PhosphorImager (Molecular Dynamics). S1 analysis was done with the following 32P-labeled oligonucleotides: Hprt S1: 5′-CTCTCATCTGATAAAATCTACAGTCATAAAGGAATCAGTGATTACATTAAAC-3′; S1: 5′-CTTCTGACTGAAAAATCTACAGTCATAAAGGAATCAGTGATTACATTAAAC-3′; Sepw1 S1: 5′-TTCAACCGGGAACACCTCGGAACATCCTGCTGTCTTATTCTGGAGTGCAAGTTAGTC-3′.

Electrophoretic mobility shift assay (EMSA)

Protein was extracted from liver tissue with T-PER Protein Extraction Reagent (Pierce) according to the manufacturer's instructions, 5 pmol of unlabeled competitor oligonucleotide was added to the binding reaction before addition of the extract. All EMSA gels were developed using PhosphorImager (Molecular Dynamics). The following oligonucleotides were annealed and used for the reactions: Csrp1 MRE1: 5′-GAGGAGAGGAGATGCACACGGCACTCG-3′; 5′-ACACCTGCTCCTCGGTGTGCATCTCCTCT-3′; S1: 5′-GCTGCTGCTCCTCGGTGTGCATCTCCTCT-3′; Slc39a10 MRE2: 5′-CGACAGACCCCCTG-3′; 5′-CCAGGGAGCTCTGCACACGGCACTCG-3′; 5′-ACACCTGCTCCTCGGTGTGCATCTCCTCT-3′; Slc39a10 MRE3: 5′-GGTAC-3′; 5′-CGAGGGAGCTCTGCACACGGCACTCG-3′; 5′-ACACCTGCTCCTCGGTGTGCATCTCCTCT-3′; Slc39a10 MRE4: 5′-GGGTTGAGGTGGAA-3′; 5′-CCAGGGAGCTCTGCACACGGCACTCG-3′; 5′-ACACCTGCTCCTCGGTGTGCATCTCCTCT-3′.

Cell culture

Primary embryonic fibroblasts were isolated from a 12.5 day old Mtf1loxP mouse embryo and grown in DMEM supplemented with 10% fetal bovine serum (ICN), 100 U/ml penicillin-streptomycin ( Gibco BRL) and 2 mM L-glutamine ( Gibco BRL). 100 mm plates with primary cells were transfected with 10 μg of an expression plasmid coding for simian virus 40 (SV40) large T antigen driven by the cytomegalovirus (CMV) promoter, using lipofectamine™ reagent (Invitrogen) according to the manufacturer's instructions. Cell foci were isolated and the immortalized mouse embryonic fibroblast cell line ckoC was derived from one of them. The Mtf1loxP genotype of this line as well as the genomic integration of the T antigen were confirmed by PCR. 100 mm plates with these cells were further transfected by the calcium phosphate method (30) with 19.6 μg of an expression plasmid for Cre recombinase driven by the CMV promoter and 0.4 μg of an expression plasmid for the neomycin resistance gene under the control of the TK promoter. Stably transfected cells were selected in the presence of 0.4 μg/μl G418 (Calbiochem), isolated clones of resistant cells were harvested and grown independently, and the expression of Cre recombinase and excision of exons 3 and 4 of Mtf1 were analyzed by RT-PCR. The cell lines delC19, delC21 and delC23 with a deletion of Mtf1 were chosen for further experiments.

Cytotoxicity assay

Samples of 1×10^5 cells/well were plated in 96-well tissue culture plates and allowed to adhere for 24 h. The cells were then pre-incubated for 24 h in medium containing 0, 5, 10, 25 or 50 μM L-buthionine-[S,R]-sulfoximine (BSO) (Sigma), a drug that inhibits glutathione synthesis (31). Later, cells were exposed to 0, 5, 10 or 20 μM CdCl2 in the specified pre-incubation medium for an additional 24 h. Cytotoxicity was determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromid)-based Cell Proliferation Kit I (Roche) according to the manufacturer's instructions.

RESULTS

Generation of an inducible, liver specific Mtf1 knockout mouse line

Using a homologous recombination strategy, mice were obtained with a modified Mtf1 allele Mtf1loxP where exons 3 and 4, encoding four of the six zinc fingers of the DNA-binding domain, are flanked by loxp sites (Figure 1a). Mice homozygous for the Mtf1loxP allele were further crossed with animals of the Cre recombinase transgenic line Mx-cre. Cre recombinase is expressed in this line under the control of the mouse Mx1 gene promoter, which is inducible by administration of interferon alpha or beta, or synthetic double-stranded RNA pl–pC (32). Cre-mediated deletion was reported to be
complete in the liver, while varying in other tissues, ranging from 94% in spleen to 8% in brain (32). After Cre-mediated deletion of exons 3 and 4 (Mtf1<sup>loxP</sup>) results in loss of function via loss of an essential part of the DNA-binding domain and the generation of a new stop codon right after exon 2. Exons 3 to 7 of Mtf1 are indicated by grey boxes, <i>loxP</i> sites by black triangles. TK, thymidine kinase cassette. Restriction enzymes: San, SanDI; B, BbvCI; S, SrfI; H, HpaI. The HpaI site indicated by the crossed H was lost during the cloning procedure for the targeting vector. (b) RT–PCRs with total liver RNA from pl–pC-induced male Mtf1<sup>Mx-cre</sup> or Mtf1<sup>loxP</sup> mice. The used primer pair results in products of 589 bp and 218 bp with full-length mRNA and mRNA without exons 3 and 4, respectively. (c) EMSA with liver protein extract of a pl–pC-induced male Mtf1<sup>Mx-cre</sup> or Mtf1<sup>loxP</sup> mouse. MTF-1 protein–DNA complex formation was tested with <sup>32</sup>P-labeled MRE consensus oligonucleotide MRE-s. Specificity of binding was verified with excess of unlabeled competitor MREd or unrelated Gal4 oligonucleotide; Sp1 bandshifts with <sup>32</sup>P-labeled Sp1 consensus oligonucleotide were included as a loading control.

Figure 1. Deletion of Mtf1 in adult mouse liver. (a) Generation of Mtf1 conditional knockout mice. The targeted allele was obtained by homologous recombination of wild-type (wt) allele and targeting vector in ES cells. Removal of the neomycin cassette (NEO) by Cre recombinase led to the conditional knockout allele Mtf1<sup>loxP</sup>. Conditional Cre-mediated deletion of exons 3 and 4 (Mtf1<sup>Mx-cre</sup>) results in loss of function via loss of an essential part of the DNA-binding domain and the generation of a new stop codon right after exon 2. Exons 3 to 7 of Mtf1 are indicated by grey boxes, <i>loxP</i> sites by black triangles. TK, thymidine kinase cassette. Restriction enzymes: San, SanDI; B, BbvCI; S, SrfI; H, HpaI. The HpaI site indicated by the crossed H was lost during the cloning procedure for the targeting vector. (b) RT–PCRs with total liver RNA from pl–pC-induced male Mtf1<sup>Mx-cre</sup> or Mtf1<sup>loxP</sup> mice. The used primer pair results in products of 589 bp and 218 bp with full-length mRNA and mRNA without exons 3 and 4, respectively. (c) EMSA with liver protein extract of a pl–pC-induced male Mtf1<sup>Mx-cre</sup> or Mtf1<sup>loxP</sup> mouse. MTF-1 protein–DNA complex formation was tested with <sup>32</sup>P-labeled MRE consensus oligonucleotide MRE-s. Specificity of binding was verified with excess of unlabeled competitor MREd or unrelated Gal4 oligonucleotide; Sp1 bandshifts with <sup>32</sup>P-labeled Sp1 consensus oligonucleotide were included as a loading control.

livers, indicating a successful excision of exons 3 and 4 of Mtf1 in these animals. On close examination, a very faint band similar in size to full-length signal was also observed in those mice, probably due to a low amount of residual full-length Mtf1 mRNA. The level of functional MTF-1 protein was examined by EMSA (Figure 1c): MTF-1 protein–DNA complex was detectable with liver protein extract from an Mtf1<sup>loxP</sup> control mouse, but no functional MRE-binding protein was observed with an Mtf1<sup>Mx-cre</sup> sample. Thus, deletion of exons 3 and 4 of Mtf1 in the liver of Mtf1<sup>Mx-cre</sup> mice was virtually complete. All examined liver-specific knockout
mice were viable under laboratory conditions and appeared normal.

MTF-1 target gene search

For the identification of MTF-1 target genes, we compared the liver transcript profiles of mice with and without functional Mtf1 gene that had been mock-treated or exposed to cadmium (n = 3 per genotype and respective treatment).

In a first screen, the transcripts were analyzed with a differential display-based method, called amplification of double-stranded cDNA end restriction fragments (ADDER) (33). Thereby an overwhelming number of signals was obtained for the two stress-inducible metallothioneins (Mt1 and Mt2), due to the abundance of their transcripts both in mock-treated and especially in cadmium-treated livers that harbored a functional MTF-1 gene (data not shown). This result confirmed the importance of MTF-1 for both basal and metal-induced expression of metallothionein genes.

In a second approach, the gene expression profile in livers of the above mentioned mice was compared by Affymetrix GeneChip® Mouse Genome 430 2.0 Arrays (Table 1). When analyzing the probe array data of livers from mock-treated Mtf1loxP and Mtf1Mx-cre mice, an at least 2-fold, reliable down-regulation of expression was detected in Mtf1loxP livers for 13 Affymetrix GeneChip® probe sets corresponding to 11 characterized genes (Table 1, a). Seven of these genes contain one or more MRE core consensus sequence TGCCRCNC within a segment of 1000 bp upstream of the transcription start. For 26 probe sets corresponding to 24 different characterized genes, a 2-fold or higher, reliable up-regulation was detected in Mtf1Mx-cre livers (Table 1, b); 17 of these 24 genes contain MRE core consensus sequences in the upstream region. The data set for livers of cadmium-treated Mtf1loxP and Mtf1Mx-cre mice revealed an at least 2-fold, reliable down-regulation in Mtf1loxP livers for 21 probe sets corresponding to 16 different characterized genes (Table 1, c); 10 of these contain MRE core consensus sequences in their upstream region. For 9 probe sets corresponding to 9 different characterized genes, an at least 2-fold, reliable up-regulation was detected (Table 1, d); five of them contain MRE motifs. In addition to characterized genes, ESTs and RIKEN cDNA sequences were also found in the comparison of Mtf1loxP and Mtf1Mx-cre livers to be differentially expressed (Supplementary Table 1). Down-regulation of Mt1 and Mt2 was detected in Mtf1loxP livers for both conditions (though the level of significance for the downregulation of Mt1 in mock-treated animals was above 0.05; data not shown).

For all MTF-1 target genes characterized so far, such as Mt1, Mt2 and Znt1, MTF-1 exerts its transcriptional activation activity via standard MRE sequences located proximal to the transcription start (4,5,8,18,19). Even a specific search for MTF-1 binding sites by selection from a pool of double-stranded oligonucleotides with random sequences yielded no new binding motif for MTF-1 in addition to the known MREs (34). Thus, an MRE sequence is to date the only indication for a direct MTF-1 target gene, and four MRE-containing target gene candidates were further analyzed.

Basal expression of Sepw1 depends on MTF-1

Sepw1 was found in microarray analysis to be significantly downregulated in livers from cadmium- and mock-treated Mtf1loxP mice (Table 1, a and c). SEPW1 is a selenocysteine-containing protein that binds glutathione (35) and is thought to act as an antioxidant in vivo (36).

Sepw1 expression in livers of pl-pC-induced, mock- or cadmium-treated Mtf1loxP and Mtf1loxP mice was further analyzed by semiquantitative RT–PCRs and S1 analysis (Figure 2a and b). In accordance with microarray data a slight, if any, upregulation of Sepw1 transcription was observed in livers from Mtf1loxP mice upon cadmium treatment. The basal level was reduced in livers from mock- and cadmium-treated Mtf1loxP mice, indicating that MTF-1 is important for the basal expression of Sepw1.

Three MRE core consensus sequences were found in the region upstream of the mouse Sepw1 transcription start (Figure 2c). Two of them in opposite orientation overlap almost completely proximal to the transcription start (MRE1, −40 bp), the third one is located further upstream (MRE2, −527 bp). Specific binding of MTF-1 to Sepw1 MRE1 but not MRE2 oligonucleotide was observed in EMSA with liver protein extract from an Mtf1loxP control mouse (Figure 2d). As a control, no binding to MRE1 was detected with extract from a pl–pC-induced Mtf1loxP mouse, confirming that the bandshift was indeed dependent on the presence of MTF-1.

Cadmium response of Ndrg1 depends on MTF-1

Ndrg1 was significantly downregulated in microarrays of liver transcripts from cadmium-treated Mtf1loxP mice compared to similarly treated Mtf1loxP control mice (Table 1, c). Ndrg1 probably has some role in stress response since various stimuli, including hypoxia and nickel compounds, activate expression of rodent Ndrg1 and/or its human ortholog (37–40).

The Ndrg1 microarray results were confirmed with semi-quantitative RT–PCRs (Figure 3a): for Mtf1loxP control livers, a clear increase of Ndrg1 expression was observed after cadmium exposure; in livers from Mtf1loxP mice, this cadmium response was not detectable, while basal expression was similar to controls. This indicates that cadmium-induced expression of Ndrg1 depends on MTF-1.

Five MRE core consensus sequences are located upstream of the mouse Ndrg1 transcription start (Figure 3b). Four of them are clustered (MRE1 to MRE4, −138 to −332 bp), the fifth one is located farther upstream (MRE5, −883 bp). EMSA was performed to test whether MTF-1 is interacting with some or all of the four proximal MRE sequences (Figure 3c). Separate oligonucleotides were tested for MRE1 and MRE2, whereas one oligonucleotide spanning both sequences was used for MRE3 and MRE4 (MRE3,4). No complex was seen with MRE1, but specific MTF-1 complexes were observed for both the MRE2 and MRE3,4 oligonucleotides with liver protein extract from an Mtf1loxP mouse. As expected, no bandshift was observed with protein extract from a mouse lacking MTF-1 (Mtf1loxP).

Cadmium response of Csrp1 depends on MTF-1

Csrp1 was found in microarray analyses to be significantly downregulated in cadmium-treated Mtf1loxP mice compared to Mtf1loxP mice (Table 1, c). CSRPR1 is a member of the evolutionary conserved CRP family of proteins that have
The animals had obtained either mock s.c. injections (−Cd) or s.c. injections with 20 μmol/kg body weight CdSO4 (+Cd) 6 h before sacrificing them. The expression values for each gene are given as mean value of three animals per group, normalized to the mean value of group Mtf1Mx-cre/C0 (relative activity). Grey shading indicates MRE-s (<1000 bp) of at least 0.05.

*Only incomplete region up to −1000 bp from transcription start is available in database.

†Mean values of two independent Affymetrix probe sets.

‡Mean value of four independent Affymetrix probe sets.
been implicated in myogenesis and cytoskeletal remodeling (41,42).

Semiquantitative RT–PCRs confirmed the microarray results, namely, that Csrp1 expression is elevated in Mtf1loxP livers upon cadmium exposure (Figure 4a). In contrast, no cadmium response was detectable in livers from Mtf1Mx-cre mice, suggesting that MTF-1 is required for cadmium induction of Csrp1.

Three MRE core consensus sequences were found upstream of the Csrp1 transcription start (MRE2 to MRE4, –56 to –366 bp), one was found immediately downstream (MRE1, +7 bp; Figure 4b). Specific binding of MTF-1 was observed with EMSA for MRE2 oligonucleotide and protein extract from an Mtf1loxP liver, but not an Mtf1Mx-cre liver extract lacking MTF-1, confirming the participation of MTF-1 in the complex (Figure 4c).

**MTF-1 inhibits expression of Slc39a10**

Slc39a10 was detected in microarray analysis to be significantly upregulated in livers from both mock- and cadmium-treated Mtf1Mx-cre mice compared to control animals (Table 1, b and d). SLC39 proteins are members of the Zrt- and Irt-like protein (ZIP) family of metal ion transporters that transport, with no known exception, metal ion substrates across cellular membranes into the cytoplasm (43,44).

In accordance with microarray data, semiquantitative RT–PCRs showed a downregulation of Slc39a10 expression in livers of Mtf1loxP mice upon cadmium exposure. In samples from Mtf1Mx-cre mice, the basal expression was significantly increased; cadmium treatment still resulted in a decrease of Slc39a10 expression (Figure 5a). It cannot be judged by this experiment whether the degree of cadmium-induced reduction of Slc39a10 transcription was identical for Mtf1Mx-cre and Mtf1loxP mice or lower in the absence of MTF-1. In microarray analysis, the degree of the downregulation was either comparable to the one in control livers or lower, depending on the considered Affymetrix GeneChip probe set (data not shown). The results indicate that MTF-1 is involved in repression of the basal expression of Slc39a10. It might also participate in
the cadmium response of this gene, but it is apparently not exclusively responsible.

One MRE core consensus sequence was found just upstream of the mouse Slc39a10 transcription start (MRE1, −21 bp), another one directly downstream (MRE2, +17 bp; Figure 5b). Specific binding of MTF-1 was observed in EMSA analysis for MRE2 with liver protein extract from an Mtf1loxP mouse but not from an Mtf1Mx-cre mouse, while no binding was detected with MRE1 (Figure 5c).

Cadmium-responsive, MTF-1-independent genes

Finally, we also identified a number of cadmium-responsive genes that were independent of MTF-1 presence, by comparing the probe array data of all cadmium-treated mice with the data of all mock-treated mice, irrespective of the genotype (Table 2). An at least 2-fold, reliable upregulation was observed after cadmium exposure for 31 probe sets corresponding to 21 different characterized genes (Table 2, a). For 2 probe sets corresponding to 2 characterized genes, an at least 2-fold downregulation was detected (Table 2, b).

Several genes involved in the metabolism of the antioxidant glutathione were found to be upregulated by cadmium exposure, namely the genes encoding the catalytic subunit of glutamate-cysteine ligase (Gclc) that is the rate limiting enzyme in de novo synthesis of glutathione (45); glutathione reductase 1 (Gsr), the reducing enzyme for oxidized glutathione (45); and glutathione-S-transferase supergene family of detoxification enzymes (45). In all of these cases, induction was confirmed by semiquantitative RT–PCRs (data not shown). Gclc, also referred to as heavy chain subunit of gamma-glutamylcysteine synthetase (Ggcs-hc), had been discussed previously as a target gene of MTF-1 (6). Our expression data indicate that Gclc is induced by cadmium but, at least in the adult mouse liver, not dependent on MTF-1.

To analyze the role of the glutathione system in the cellular cadmium response, mouse embryonic fibroblasts with and without functional Mtf1 were treated with cadmium in combination with BSO, a specific inhibitor of glutamate-cysteine ligase (31), and cell viability was assessed by a colorimetric assay based on the tetrazolium salt MTT (Figure 6). Increasing concentrations of BSO or cadmium alone were to some extent synergistic, as observed after the combination of BSO with cadmium (Figure 6).
The animals had obtained either mock s.c. injections (−Cd) or s.c. injections with 20 μmol/kg body weight CdSO₄ (+Cd) 6 h before sacrificing them. The expression values for each gene are given as mean value of three animals per group, normalized to the mean value of group Mtf1loxP−Cd (relative activity).

*Mean value of six independent Affymetrix probe sets.

a Mean value of two independent Affymetrix probe sets.

### DISCUSSION

In this study, a virtually complete deletion of Mtf1 in the liver of adult, pl–pC-induced Mtf1loxP mice did not detectably affect the phenotype of the respective mice under non-stress conditions, confirming that MTF-1 is dispensable in the adult liver (26), in contrast to its essential role in embryonic liver development (23).

The comparison of gene expression in livers of mock- or cadmium-treated Mtf1loxP and Mtf1loxP mice revealed several MTF-1 target gene candidates. Transcripts of the two stress-responsive metallothionein genes Mt1 and Mt2 were severely reduced, in support of a crucial role of MTF-1 for both basal and metal-induced expression of metallothioneins (4,6).

One of the newly found target genes is Sepw1. The exact molecular function of SEPW1 protein is unknown to date, but a role as antioxidant has been proposed due to its ability to bind glutathione (35). In accordance with this, ectopic expression of mouse Sepw1 renders cells resistant to hydrogen peroxide, and this resistance is dependent on it binding glutathione (36). Furthermore, Amantana et al. (49) showed that expression of a reporter gene fused to a rat Sepw1 promoter fragment can be induced in rat glial cells by copper and zinc, but not cadmium. This response was dependent on an overlapping-inverted MRE sequence located proximal to the rat Sepw1 transcription start (49), even though initial studies failed to demonstrate MTF-1 binding to that sequence (50). Our expression and DNA-binding studies strongly suggest that MTF-1 is important for the basal expression of mouse Sepw1 by binding to the corresponding overlapping-inverted MRE sequence.

### Table 2. Comparison of liver gene expression for cadmium- and mock-treated mice (up- or downregulation at least 2-fold, P ≤ 0.05)

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<th>Gene symbol</th>
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<td>(a) Genes upregulated in cadmium-treated mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chr3</td>
<td>Carbonyl reductase 3</td>
<td>1</td>
<td>19.600</td>
</tr>
<tr>
<td>Npsa</td>
<td>Neoplastic progression 3</td>
<td>1</td>
<td>12.907</td>
</tr>
<tr>
<td>Ddc</td>
<td>Dopa decarboxylase</td>
<td>1</td>
<td>7.166</td>
</tr>
<tr>
<td>Serpin9</td>
<td>Serine (or cysteine) proteinase inhibitor, clade A, member 9</td>
<td>1</td>
<td>6.345</td>
</tr>
<tr>
<td>Ppffh2</td>
<td>Protein tyrosine phosphatase, receptor-type, F interacting, binding protein 2</td>
<td>1</td>
<td>3.771</td>
</tr>
<tr>
<td>Gclc</td>
<td>Glutamate-cysteine ligase, catalytic subunit</td>
<td>1</td>
<td>3.678</td>
</tr>
<tr>
<td>Pgd</td>
<td>Phosphogluconate dehydrogenase</td>
<td>1</td>
<td>3.550</td>
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<tr>
<td>Il6Rg</td>
<td>Inhibitor of kappalF kinase gamma</td>
<td>1</td>
<td>3.319</td>
</tr>
<tr>
<td>Tnndr1</td>
<td>Thioredoxin reductase 1</td>
<td>1</td>
<td>3.273</td>
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<tr>
<td>Kdelr2</td>
<td>KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2</td>
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<tr>
<td>Ddi42</td>
<td>DNA-damage-inducible transcript 4-like</td>
<td>1</td>
<td>2.906</td>
</tr>
<tr>
<td>Aqp8</td>
<td>Aquaporin 8</td>
<td>1</td>
<td>2.810</td>
</tr>
<tr>
<td>Gstm4</td>
<td>Glutathione-S-transferase, mu 4</td>
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<tr>
<td>Bag3</td>
<td>Bcl2-associated athanogene 3</td>
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<tr>
<td>Gsr</td>
<td>Glutathione reductase 1</td>
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<td>2.376</td>
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<td>Pir</td>
<td>Pirin</td>
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<td>2.235</td>
</tr>
<tr>
<td>Huetp2</td>
<td>HIV-1 tat interactive protein 2, homolog (human)</td>
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<td>Mocos</td>
<td>Molybdenum cofactor sulfurase</td>
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<td>2.114</td>
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<tr>
<td>Abc4</td>
<td>ATP-binding cassette, subfamily C, member 4</td>
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<td>2.095</td>
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<tr>
<td>Russf6</td>
<td>Ras associated (RafGDS/AF-6) domain family 6</td>
<td>1</td>
<td>2.056</td>
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<tr>
<td>Entpsd</td>
<td>Ectonucleoside triphosphate diphosphohydrolase 5</td>
<td>1</td>
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<tr>
<td>(b) Genes downregulated in cadmium-treated mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sntg2</td>
<td>Syntrophin, gamma 2</td>
<td>1</td>
<td>0.400</td>
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<tr>
<td>G6pc</td>
<td>Glucose-6-phosphatase, catalytic</td>
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<td>0.152</td>
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extent cytotoxic for the examined cell lines. Treatment with both BSO and cadmium resulted in an enhanced lethality particularly for the cells without functional Mtf1, indicating that a depletion of glutathione together with a lack of Mtf1 impair an efficient anti-cadmium defense. Thus, adequate glutathione supply as well as MTF-1 and its target genes are essential for the survival of the cell under cadmium stress.

Besides genes related to the glutathione pathway, several other stress-related genes were upregulated upon cadmium exposure, including genes for thioredoxin reductase 1 (Txnrd1), one of the reducing enzymes of the antioxidant thioredoxin (46); KDEL endoplasmic reticulum protein retention receptor 2 (Kdelr2) participating in ER stress response (47); and the anti-apoptotic Bcl2-associated athanogene 3 (Bag3) involved in stress-induced apoptosis (48).
induction by stimuli like nickel and hypoxia suggests an involvement in the cell stress response. Such a role is strongly endorsed by our finding that \textit{Ndrg1} gene expression is also induced by cadmium, and that MTF-1 plays a crucial role in this induction.

In the case of \textit{Csrp1}, expression analyses and DNA-binding studies indicate that MTF-1 is required for cadmium induction by binding to an MRE upstream of the transcription start. Studies with human, avian and chicken CSRP1 have shown that this protein is localized at adhesion plaques and in association with filamentous actin, and interacts with the adhesion plaque protein zyxin, as well as the actin-cross-linking protein alpha-actinin (54–57). The ability to bind these partners suggests a role in cytoskeletal organization (58). Exposure of cultured cells to cadmium causes a decrease in, and destruction of, cellular contact proteins and the actin cytoskeleton (59). In the proximal tubule cells of the rat kidney, a partial loss of actin and the actin-bundling protein villin is observed upon cadmium treatment, as well as the derangement and depolymerization of microtubules (60). Assuming that CSRP1 is important for the organization of cytoskeletal elements in the mouse, its upregulation by cadmium might protect the organism from damage of the cytoskeleton. Such a mechanism would expand the role of MTF-1 in stress response.

Our expression studies also suggest that MTF-1 represses basal transcription of \textit{Slc39a10}, in contrast to its role as activator for the expression of other target genes like \textit{Mt1}, \textit{Mt2}, and \textit{Znt1} (4,19). SLC39A10 is one of 14 mouse SLC39 members, which belong to the ZIP family of metal ion transporters (43,44). All members of the ZIP family characterized so far increase intracellular cytoplasmic metal ion concentrations by promoting extracellular and vesicular ion transport into the cytoplasm. ZIP proteins have been reported to be transporters of zinc, iron, manganese and/or cadmium (44,61–63). Although SLC39A10 is largely uncharacterized (44), it is referred to in several databases as putative zinc transporter. It has been previously shown that MTF-1 is important for both basal expression and metal induction of the mouse \textit{Znt1} gene (19). ZnT proteins represent a different family of transporters that reduce intracellular cytoplasmic zinc by promoting zinc efflux from cells or into intracellular vesicles. Thus, members of the ZnT and ZIP family with zinc as predominant substrate have opposite roles in cellular zinc homeostasis (43). Assuming that SLC39A10 is indeed a zinc transporter, MTF-1 would control expression of two zinc transporters with antagonistic functions, namely, \textit{Znt1} and \textit{Slc39a10}. Specific binding of MTF-1 was observed for an MRE located just downstream of the \textit{Slc39a10} transcription start. In a simple model, such a binding could interfere with the accessibility of the

![Figure 6](https://academic.oup.com/nar/article-abstract/33/18/5715/2401207)
transcriptional start site for RNA polymerase II and/or general transcription factors, thus preventing transcription initiation of the gene. Indeed, such a mechanism has been described in yeast for the zinc-responsive activator protein 1 (Zap1) and its target gene, zinc-regulated transporter 2 (ZRT2) (64). However, the inhibition of Slc39a10 expression by MTF-1 may well be more complex than a competition for promoter binding. Independent of MTF-1, cadmium treatment also leads to downregulation of Slc39a10 transcripts, suggesting that some other factor is mediating this response.

A previous target gene search for MTF-1 with mouse embryos of conventional Mtf1 knockout phenotype revealed, besides metallothionein genes, the multifunctional alpha-fetoprotein (Afp) and the liver-enriched transcription factor CCAAT/enhancer binding protein alpha (Cebpa) as prime candidates (65). After an early onset during hepatogenesis, Afp expression is repressed postnatally and replaced by albumin (66). Thus, our adult Mtf1<sup>loxP</sup> mice lacking MTF-1 were not suitable to analyze Afp expression. Cebpa is expressed in the adult liver as well as in other tissues (67), but the present microarray data revealed no significant expression differences in livers from adult Mtf1<sup>loxP</sup> and Mtf1<sup>Mx-cre</sup> mice (data not shown). Therefore, MTF-1 may affect Cebpa expression only during embryonal development, perhaps in combination with as yet unidentified factors.

The present study confirms and extends the role of MTF-1 as an important stress response regulator. We have identified and preliminarily characterized four target genes of MTF-1 in the adult mouse liver: in the case of Sepw1, MTF-1 is required to maintain basal expression, supporting a role of mouse MTF-1 in oxidative stress response. In addition, MTF-1 contributes to the cadmium-induced expression of Ndrg1 and Csrl1. Furthermore, MTF-1 helps to repress the basal expression of Slc39a10, in contrast to its role as transcriptional activator for genes like Mtl, Mt2 or Znt1. Thus the same transcription factor apparently serves as an activator or repressor, depending on the target gene.

The comparison of liver gene expression of cadmium- and mock-treated mice also revealed a number of genes that were responsive to cadmium exposure, independent of the presence or absence of MTF-1. Evidence suggests that the production of reactive oxygen species is a major effect of acute cadmium toxicity (68,69), and exposure of cultured cells or animals to cadmium is associated with depletion of reduced glutathione, lipid peroxidation and DNA damage (70–73). Oxidative stress and the subsequent restoration of cellular homeostasis have been shown to induce the expression of genes encoding acute-phase proteins and antioxidant enzymes (74). In mammals, cadmium tends to accumulate in the kidney and liver as a cadmium-metallothionein complex that has an extremely slow turnover (75,76). Furthermore, metallothioneins provide protection against oxidative stress (1.17). In addition to metallothioneins, glutathione was postulated as a first line of protection against oxidative stress (1,17). In addition to metallothionein complexes cadmium (78) and scavenges free radicals and other reactive oxygen species directly, and indirectly via enzymatic reactions (45). In such reactions, glutathione is oxidized and has to be regenerated by glutathione reductase. Also, glutathione-S-transferases mediate the conjugation of various electrophiles to glutathione. The observed cadmium-induced upregulation of Gclc, Gsr and Gstm4 supports the importance of glutathione in the cellular cadmium response. The enhanced sensitivity to cadmium toxicity that we found for mouse embryonic fibroblasts upon a combination of Mtf1 deletion and depletion of glutathione further corroborates the importance of MTF-1 and its target genes as well as reduced glutathione for an efficient anti-cadmium defense.

Our data provide strong evidence for at least two branches of cellular anti-cadmium defense, one via MTF-1 and its target genes, notably metallothioneins, the other via glutathione, with an apparent overlap in Sepw1.

SUPPLEMENTARY DATA

Supplementary data are available at NAR online.

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Conflict of interest statement. None declared.

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