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Divalent metal-dependent regulation of hepcidin expression by MTF-1

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ABSTRACT

Hepcidin is a small acute phase peptide that regulates iron absorption. It is induced by inflammation and infection, but is repressed by anaemia and hypoxia. Here we further reveal that hepcidin transcription also involves interactions between functional metal response elements (MREs) in its promoter, and the MRE-binding transcription factor-1. Analysis of hepcidin mRNA and protein levels in hepatoma cells suggests that its expression may be regulated by divalent metal ions, with zinc inducing maximal effects on hepcidin levels. These data suggest that this peptide may be a pleiotropic sensor of divalent metals, some of which are xenobiotic environmental toxins.

1. Introduction

Iron and zinc are two of the most abundant metals found in the body, and appear to reciprocally affect each other's bioavailability and absorption. In humans, non-heme iron has been shown to reduce zinc uptake in the intestine [1,2]. Conversely, zinc reduces intestinal iron uptake in rats and mice [3]; its inhibitory effects on liver and splenic iron stores have also been observed in rats fed a high zinc diet [4]. In addition, in vitro studies have demonstrated that iron and zinc, as well as other divalent metals such as copper inhibit each other's uptake [5–7]. The interactions and plasma concentrations of both metals are also influenced by infection and inflammation [8]. Their absorption is tightly regulated at the molecular level by transporters at the apical and basolateral surfaces of epithelial cells in the intestine. These transport proteins include zinc–iron regulated transporter-like proteins (ZIPs) or Slc39a family members which function in intracellular uptake of zinc uptake and other metals. A second group of zinc transporters consists of members of the solute carrier 30A (Slc30a) family or ZnTs whose role is in zinc efflux and compartmentalization [9,10]. In plants, the ZIP proteins have broad substrate specificity and have also been shown to transport iron [11–13]. Both mice and humans have 14 ZIP isoforms [14,15]; ZIP1 and ZIP2 have been identified as zinc transporters [16–18].

Iron uptake into enterocytes occurs via divalent metal transporter-1 (DMT-1), in a proton-dependent manner [19]. DMT1 is found on the apical surface of enterocytes but is localized in intracellular vesicles in other cell types, from where it transports iron into the cytoplasm [20–22]. While DMT1 has broad substrate specificity and was initially thought to transport zinc, studies have shown that although DMT1 mRNA and protein are up-regulated by zinc, a distinct uptake pathway exists for both iron and zinc [23–26]. On the other hand, iron export from cells occurs via the basolateral transporter, ferroportin [27–29]. Hepcidin, a liver-derived peptide regulates body iron stores by inhibiting dietary iron absorption, and by inducing the internalisation and degradation of ferroportin [30,31].

The control of hepcidin expression is increasingly complex because of the large number of effectors of its expression [32]; however, the mechanism for its transcriptional regulation by iron remains elusive. We proposed that in addition to interaction between iron and zinc at the cellular level, zinc might regulate iron uptake and efflux by regulating expression of hepcidin. In support of this we show here that MTF-1 a divalent metal ion sensitive transcription factor, regulates hepcidin transcription by binding to its cognate response elements within the hepcidin promoter.

Abbreviations: MRE, metal-response element; MTF-1, MRE-binding transcription factor-1; EMSA, electrophoretic mobility shift assay; βgal, beta-galactosidase; BHK, baby hamster kidney; MT, metallothionein

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2. Materials and methods

2.1. Promoter construct

Approximately 1.8 kb of the human hepcidin promoter was amplified from placental genomic DNA with primers CAT GGT ACC AAC ATC CCC GGG CTC TGG TGA CT (sense) and CAT CTC GAG CGA GGA GGA GGA GGA GCA (antisense); primers were designed based Acc # AD000684 (MWG Biotech, Ebersberg, Germany). The PCR products were digested with KpnI–XhoI (New England Biolabs, Hitchin, UK) (sites underlined), purified with Geneclean (BIO101) and ligated into KpnI–XhoI-restricted pGL3 Basic vector (Promega) using the Quick ligation kit (NEB) to generate HepcP1.8-luc.

2.2. Site-directed mutagenesis of MREs

HepcP1.8-luc was subjected to site-directed mutagenesis using the QuikChange Multi Site-Directed Mutagenesis system (Strategene, Amsterdam, The Netherlands) as instructed by the manufacturer. Mutagenic primers (mutations in lowercase) were as follows: MRE1/2 (incorporating a XhoI site), TTG ATC CCC TGG GCC aTc Tcg AgC gTG AGC TGG GCC TGG T; MRE3 (incorporating a BglII site), CTG TCA CCC AGG CTG cAG atC tGT CAC ACA ATC ATA GCc c; MRE4 (incorporating a Xba*I* site), GCT GCT GGC CAT GCC CCa TcT agA TGT AGG CGA TGG GGA. After initial denaturation for 1 min at 95 °C, PCR cycling parameters were 95 °C (1 min), 55 °C (1 min), and 65 °C (12 min), for a total of 30 cycles. Following DpnI digestion of wild-type HepcP1.8-luc, transformation of XL10 Gold cells with the mutagenesis reaction and selection on Luria-Bertani agar/ampicillin plates, plasmid DNA was purified from overnight cultures of single colonies and restricted with XhoI, BglII and XbaI (Promega, UK) to identify colonies containing the relevant mutation. The resulting mutant promoter constructs were designated Δ1/2, Δ3, Δ4, and Δ1/2, 3, 4 for mutants of MRE1/2, MRE3, MRE4 and MRE1/2, 3, 4, respectively.

2.3. Cell culture, transfection, and reporter assays

All cell culture reagents were obtained from Invitrogen. Huh7 hepatoma cells were obtained from the UCL Institute of Hepatology (United Kingdom) and baby hamster kidney (BHK) cells were kindly provided by Jill Norman, University College London. The cells were cultured in Dulbecco's Minimal Essential Medium (DMEM) with Earle salts and supplemented with non-essential amino acids, 10% fetal bovine serum, and antibiotics; cells were grown under standard cell-culture conditions. Transfection assays were performed with BHK cells due to their attenuated MT-1 (metallothionein-1) expression, whereas Huh7 cells were used for mRNA analysis of hepcidin because they constitutively express MT-1. Hence for transfections, 10^4 BHK cells were seeded in each well of Costar 24-well plates (Corning, Cambridge, MA). Approximately 100 ng of MTF-1 expression plasmid (kindly provided by Glen Andrews, University of Kansas Medical Center), were diluted in OptiMEM 1 and co-transfected with 100 ng HepcP1.8-luc or its derivatives (site-directed mutants). All transfections were performed and analysed as previously described [32].

2.4. Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Huh7 cells were harvested at log phase of growth by trypsinization, washed twice with phosphate-buffered saline, and resuspended in lysis buffer (10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, and 1 mM DTT) supplemented with Complete protease inhibitor cocktail (Roche, Lewes, United Kingdom). Nuclear extracts were prepared and EMSA was performed as previously described [33]. We synthesized two complementary oligonucleotides encompassing MRE1/2 (underlined) as follows: GATCCCTGGGCCGTGTGCACCCTGAGCTGGGC and GCCCAGCTCAGGGTGCACACGGCCCAGGGATC; a mutant MRE oligonucleotide (CCCTGGATTAGCCCGGGGGCAAGC) was also synthesized. For competitive inhibition, a 100-fold molar excess of the cold MRE oligonucleotide was added to the binding reaction 10 min before adding the labeled probe.

2.5. Huh7 cell treatment, RNA extraction and RT-PCR

Huh7 cells were seeded onto 6-well plates (Nunc) at a density of 10^5 cells/well. At ~90% confluence, complete medium was replaced with serum-free medium supplemented with the appropriate concentrations of metal. Cadmium was used at a much lower concentration than the other metals due to its highly toxic nature. Cells were washed with PBS, and RNA was extracted with TRIzol (Invitrogen) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed using the Verso cDNA kit (Thermofisher Scientific), according to the manufacturer's instructions. All reactions were performed in the Lightcycler (Roche) using GAPDH as internal standard. Each reaction was performed in duplicate and contained 10 pmoles of specific primers (Table 1), 1× SYBR Green Mastermix (Qiagen), and 1 μl of cDNA in a 20 μl reaction. Samples without cDNA were included as negative controls. Cycle threshold (Ct) values were obtained for each gene of interest and the GAPDH internal standard. Gene expression was normalized to GAPDH and represented as ΔCt values. For each sample the mean of the ΔCt values was calculated. Relative gene expression was normalized to 1.0 (100%) of controls.

2.6. Hepcidin synthesis, refolding and antibody production

Human hepcidin (25-amino acids: DTHFPICIFCCGCCHRSKCGMCCKT) was synthesized. The lyophilised crude reduced hepcidin (25 mg) peptide was dissolved in neat trifluoroacetic acid (30 ml) and dried to a thin film under vacuum for 24 h in a quick-fit flask, using a rotary evaporator. To this dried film, 50 ml of 0.1 M of deaerated ammonium bicarbonate was added and stirred for 48 h open to the atmosphere for air oxidation; complete oxidation of the peptide was ascertained with the Ellman reagent. The fully-oxidised mixture was then purified by HPLC on Vydac columns, and monitored by UV detection at 220 nm. Purity was checked by MALDI-TOF; the molecular weight of the peptide was measured as 2789 Da (*M*r 2789.40). The peptide was next conjugated to keyhole limpet hemocyanin by standard glutaldehyde reaction, mixed with complete Freunds adjuvant and used to immunize rabbits. Following three booster injections of the peptide in incomplete Freunds adjuvant, antibody titre was monitored by ELISA.

2.7. SDS–PAGE and Western blotting

Huh7 cells either untreated or treated with zinc or cadmium were lysed in RIPA buffer containing protease inhibitors (Sigma). Cellular protein content was determined with the Pierce protein assay system as instructed by the manufacturer; 50 μg aliquots of homogenates were diluted with an equal volume of 2× Laemmli sample buffer, heated at 40 °C for 30 min and resolved on a 12% polyacrylamide gel (Bio-Rad) at 40 mA. Proteins were transferred onto 0.2 μm PVDF membranes (Bio-Rad), using a Bio-Rad Trans-Blot SD semi-dry blotter. After transfer, the blots were washed in distilled water (3 × 1 min) and incubated in 10% glutaraldehyde overnight. After washing 3 × 5 min with distilled water, the membrane was incubated with 5% non-fat dry milk in PBS-T (PBS/0.1% Tween 20) overnight at 4 °C, with gentle agitation. A 1:2000 dilu-

Table 1
Forward (FWD) and reverse (RVS) primers for human hepcidin, MT-1 and GAPDH real-time RT-PCR.

Target	Primer sequence (5′ → 3′)
Human hepcidin	FWD: CTGCAACCCCAGGACAGAG RVS: GGAATAAATAAGGAAGGGAGGGG
Human MT- I	FWD: ATGGACCCCAACTGCTCCTGC RVS: GGCACAGCAGCTGCACTTCTC
Human GAPDH	FWD: TGGTATCGTGGAAGGACTC RVS: AGTAGAGGCAGGGATGATG

tion of affinity-purified anti-hepcidin antibody was added and again incubated overnight at 4 °C. It was then washed 1 × 15 min and then a further 3 × 5 min with PBS-T, and incubated with HRP-conjugated donkey anti-rabbit IgG antibody (Amersham) for 1 h; signal detection employed the SuperSignal West Pico Western blotting detection system (Pierce), and a Fluor-S MultiImager (Bio-Rad). The membrane was also probed with a 1:20 000 dilution of HRP-conjugated anti-actin antibody AC-15 (Abcam) to control for equivalent sample loading.

3. Results

3.1. The hepcidin gene contains three functional consensus MRE motifs

Sequence analysis of HepcP1.8-luc revealed four putative consensus MRE motifs (Fig. 1A). The most distal of these comprises two overlapping MREs in reverse orientations. To test whether these elements were functional, HepcP1.8-luc was co-transfected with MTF-1 into BHK cells in which MT-1 genes are quiescent; these cells therefore have a lower capacity to buffer intracellular zinc, cadmium and copper, thus enabling the effects of extracellular zinc on hepcidin transcription to be assessed. We found a concentration-dependent increase in luciferase activity in transfected BHK cells when zinc was added to the medium (Fig. 1B). This metal induced over a 3-fold increase in luciferase activity compared to non-treated control. Other metals such as cadmium and copper have been shown to induce the transcription of genes that contain MREs [34]. To address this, transfected BHK cells were treated with both cadmium and copper. Luciferase activity in BHK cells transfected with the hepcidin promoter construct and subsequently treated with cadmium or copper showed a significant increase in luciferase expression (Fig. 2). However, this increase was minimal at 1.5-fold and 1.3-fold for cadmium and copper, respectively. As hepcidin is an iron regulatory gene and is also regulated by hypoxia, both iron and the hypoxia mimetic $CoCl_2$ were added to the medium of BHK cells co-transfected with HepcP1.8-luc and MTF-1. Luciferase activity in those cells did not alter significantly from the non-treated controls.

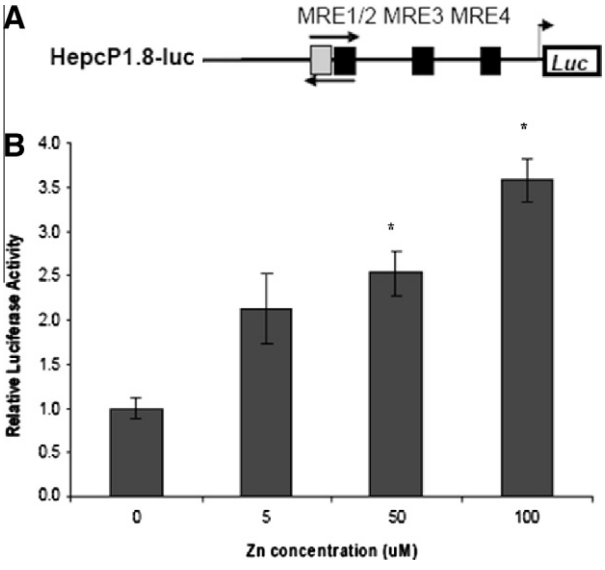

Fig. 1. Promoter region of the human hepcidin gene. (A) Spatial arrangement and nucleotide sequences of the MREs (boxes) within the cloned human hepcidin gene promoter. (B) Zinc transactivates the hepcidin promoter. HepcP1.8-luc (100 ng) was transfected into BHK cells, along with 100 ng MTF-1. Transfected cells were subsequently treated with various amounts of zinc (5, 50, 100 μM $ZnSO_4$). Fold activation was calculated with respect to the activity of the hepcidin promoter alone. All transfections were performed in triplicate and included pSVβgal as internal control; luciferase levels were normalized to βgal activity. Data are representative of two independent experiments and are presented as means ± S.E.M. $^*P < 0.05$.

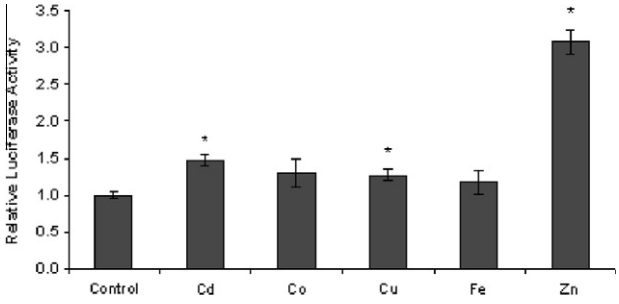

Fig. 2. Differential transactivation of the hepcidin promoter by divalent metals. HepcP1.8-luc (100 ng) was transfected into BHK cells, along with 100 ng MTF-1. Transfected cells were subsequently treated with various amounts of cadmium (10 μM $CdCl_2$); cobalt (100 μM $CoCl_2$); copper (50 μM $CuSO_4$); iron (50 μM $FeSO_4$) and zinc (100 μM $ZnSO_4$). Fold activation was calculated with respect to the activity of the hepcidin promoter alone. All transfections were performed in triplicate and included pSVβgal as internal control; luciferase levels were normalized to βgal activity. Two independent experiments were performed. Data are presented as means ± S.E.M. $^*P < 0.05$.

3.2. Functional MREs are required for the MTF-1-dependent hepcidin transcriptional response to zinc

Is the transcription of hepcidin by zinc and cadmium MTF-1-dependent? To address this question, MTF-1 was omitted from the transfection cocktail and luciferase activity assessed after metal treatment. Omission of MTF-1 from the transfections abrogated luciferase activity in response to zinc (Fig. 3). This effect was also seen after the treatment of cells with cadmium. In order to determine whether the MREs present in the hepcidin promoter were functional, point mutations were introduced into each MRE. Mutating all four MREs diminished the zinc-specific luciferase activity seen when all the MREs are intact (Fig. 4). This decrease was approximately 3-fold compared to control. Mutation of each individual MRE decreased zinc-mediated activity by approximately 2-fold. Mutation of MRE1/2 (see Fig. 1A) had a deleterious effect on basal luciferase activity, reducing it approximately 2-fold compared to wild-type promoter activity. These results suggest that the MREs are functional and are critical to both the basal and inducible expression of hepcidin by zinc.

3.3. Binding of MTF-1 to the hepcidin MREs

EMSA was performed to determine whether zinc- or cadmium-treated cell extracts could bind to MREs in vitro (Fig. 5). Binding to MRE1/2 was observed with extracts from both non-treated and zinc-treated Huh7 cells (Fig. 5, lanes 2–4). Binding to MREs 3 and

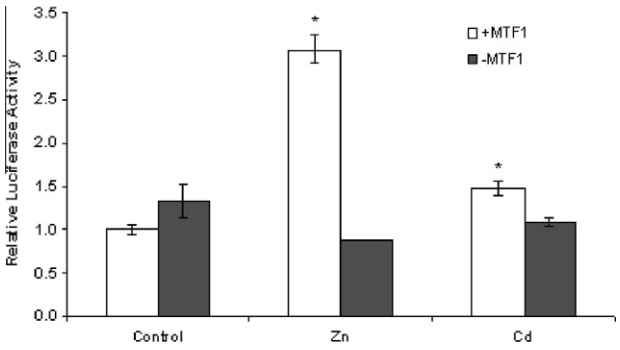

Fig. 3. MTF-1 is essential for zinc- and cadmium-mediated activation of the human hepcidin promoter. The cloned human hepcidin promoter (100 ng) was transfected into BHK cells, with or without 100 ng MTF-1. Transfected cells were subsequently treated with various amounts of cadmium (10 μM $CdCl_2$) or zinc (100 μM $ZnSO_4$). Fold activation was calculated with respect to the activity of the hepcidin promoter alone. All transfections were performed in triplicate and included pSVβgal as internal control; luciferase levels were normalized to βgal activity. Data are representative of two independent experiments and are presented as means ± S.E.M.

4 was also observed with zinc- and cadmium-treated extracts (data not shown). This binding was specifically competed out when an excess of cold probe was added (Fig. 5, lanes 5–7). These data showed that the MREs in the hepcidin promoter are integral to hepcidin transcriptional regulation by MTF-1.

3.4. Differential induction of hepcidin expression in hepatoma cells by divalent metals

The addition of zinc, cadmium and copper to the media of Huh7 cells showed a marked increase in hepcidin mRNA levels relative to control (Fig. 6A). This increase was highest with cadmium at approximately 3.5-fold, although zinc and copper elicited a similar response, increasing hepcidin mRNA levels by approximately 3-fold each. As a positive control, we also assessed MT-1 levels and found a concomitant increase in its mRNA in Huh7 cells treated with the same metals. Zinc elicited the largest response with an approximate 17-fold increase, with cadmium increasing MT-1 mRNA by approximately 5-fold and copper with a 3-fold increase. Cobalt also elicited a significant increase in MT-1 mRNA, although this increase was only slightly above basal levels (Fig. 6B). This is

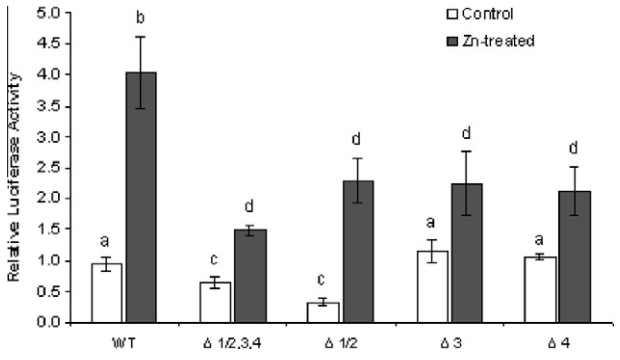

Fig. 4. Site-directed mutagenesis of MREs in the hepcidin promoter. Promoter constructs were produced with mutations in MRE1/2 (Δ1/2), MRE3 (Δ3), MRE4 (Δ4), and in all MREs (Δ1/2, 3, 4). Basal activities of both mutant and wild-type (WT) promoters were compared with zinc treated after transfection in BHK cells with MTF-1 (100 ng). All transfections were performed in triplicate and included pSVβgal as internal control; luciferase levels were normalized to βgal activity. Two independent experiments were performed. Data are presented as means ± S.E.M. A statistically significant difference ($P < 0.05$) is indicated by a different letter above the bar.

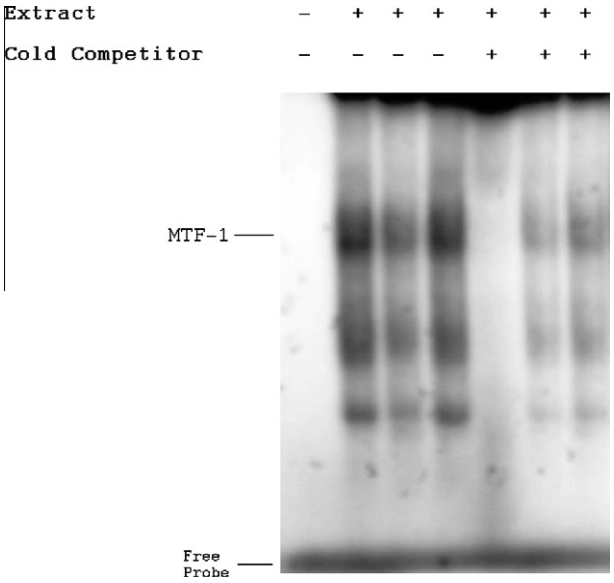

Fig. 5. Promoter occupancy by MTF-1 in vitro. Huh7 cell nuclear extracts (10 μg) treated with zinc (100 μM $ZnSO_4$; lanes 3 and 6) or cadmium (10 μM $CdCl_2$; lanes 4 and 7) for 6 h were incubated with radiolabelled MREs. Excess unlabeled or cold competitor oligonucleotide (100 pmol) was added to the binding reactions where indicated.

not surprising as MT-1 has been shown to bind many divalent metals, including cobalt [35,36]. Notably, cobalt chloride (a hypoxia mimetic) decreased hepcidin mRNA levels 5-fold supporting observations that hypoxia down-regulates hepcidin expression. Also consistent with previous observations, iron caused a marked de-

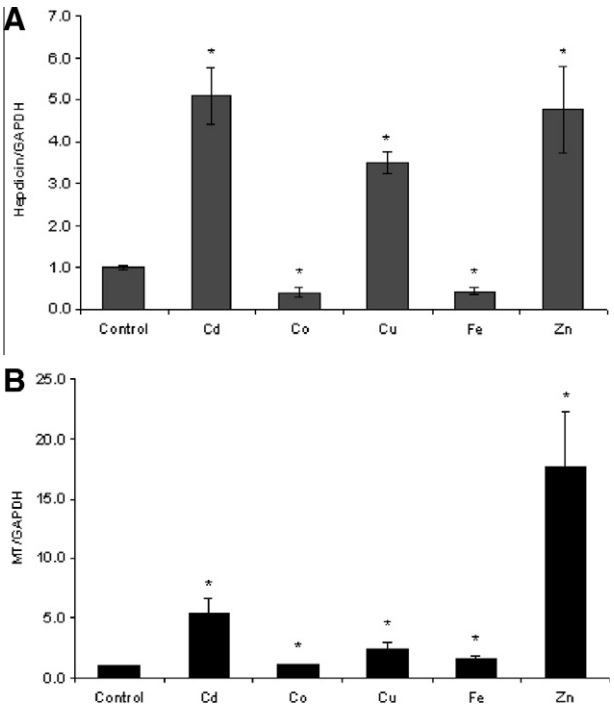

Fig. 6. Hepcidin and MT-1 mRNA expression in Huh7 cells. Q-PCR analysis of (A) hepcidin and (B) MT-1 mRNA expression in Huh7 cells. Cells were treated with cadmium (10 μM $CdCl_2$); cobalt (100 μM $CoCl_2$); copper (50 μM $CuSO_4$); iron (50 μM $FeSO_4$) and zinc (100 μM $ZnSO_4$) for 6 h. All treatments were performed in duplicate with two independent experiments being performed. Data are presented as means ± S.E.M.

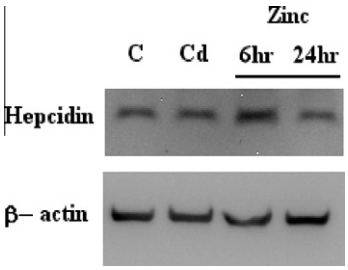

Fig. 7. Western blotting. Hepcidin expression at the protein level was determined with Huh7 cells either untreated (control) or treated with cadmium (10 μM $CdCl_2$) for 6 h or with zinc (100 μM $ZnSO_4$) for 6 and 24 h.

crease in hepcidin mRNA. Surprisingly, iron induced a residual increase in MT-1 mRNA levels.

To ascertain whether hepcidin mRNA was in fact translated following induction with metals, we performed Western blotting with extracts of Huh7 cells treated with either cadmium or zinc. After 6 h, we found a 2-fold increase in hepcidin protein induction with zinc treatment compared with untreated controls or with cadmium. Protein expression however returned to basal levels after 24 h of zinc treatment (Fig. 7).

4. Discussion

Hepcidin has been shown to be regulated by several factors including iron, inflammation and hypoxia. The transcriptional mechanisms underlying its induction in inflammation are only now beginning to be unravelled; however, its transcriptional induction by iron remains elusive. Findings reported in this study that treating cultured hepatocyte cell lines with iron do not have an effect on transcription of hepcidin is in agreement with previous reported studies. One possible explanation for this non-response of hepcidin expression to iron in vitro is that other hepatic or extrahepatic cell types may be involved in this process. In pursuit of transcriptional regulation of hepcidin by other metals, we identified metal response elements within the promoter; these are responsive to zinc, and to a lesser extent, cadmium and copper. Induction of luciferase activity in BHK cells transfected with the hepcidin promoter fused to a luciferase reporter gene showed that optimal activity was zinc-dependent and occurred in a dose-dependent manner. Other divalent metals, notably cadmium and copper also caused an increase in luciferase activity. In BHK cells, a cell line with attenuated MT-1 expression, it appeared that only zinc could induce metal sensitivity fully, with minimal activity seen after cadmium and copper exposure. The loss of functional MT-I in this case highlights the direct effect of zinc on MTF-1 function and demonstrates the inability of other metals to activate MTF-1 directly. It has been shown that although cadmium can induce MT-1 expression by MTF-1, only zinc can activate the DNA-binding activity of this transcription factor by its reversible interactions with specific zinc fingers [37–42]. This may explain why hepcidin mRNA expression in Huh7 cells, a cell line with normal MT-1 expression, was increased to almost the same degree after cadmium and copper exposure as after zinc treatment. It has previously been demonstrated in vivo that hepcidin mRNA was up-regulated in the livers of mice fed a high copper diet [43]. The mechanism of induction of hepcidin mRNA by both cadmium and copper could be an indirect one, where MTF-1 is activated to bind to MREs in the hepcidin promoter by the displacement of zinc from MT-1 and other zinc-binding proteins. MT-1 isolated from mouse tissues has been shown to be normally bound to zinc [35,36]; however the affinity of MT-1 for cadmium and copper is higher [44].

Since zinc regulates many genes through MTF-1/MRE interactions [38], do our observations suggest that this metal may directly regulate iron uptake and body iron stores? From what is known about the interactions between iron and zinc (operating through MTF-1), we conjecture that this may be the case. Another possible explanation is that cadmium-responsive factors may be indirect co-regulators of hepcidin expression; these proteins have been reported by Koizumi et al. [37,45,46]. However, because MTF-1 is a zinc- rather than a cadmium-sensor and binds DNA only in the presence of zinc, this would suggest that cadmium-dependent induction of hepcidin or MT-1 transcription may be indirect. In other words, MTF-1 may cooperate with other transcription factors or co-regulators in regulating hepcidin expression.

Metal response elements function synergistically and it is thought that two or more MREs are needed for maximal promoter inducibility by or sensitivity to zinc and MTF-1; neither the spacing nor the orientation appears to be critical for function. Metal responsiveness is dependent on the MRE core consensus sequence, TGCRCNC [47,48]. The metallothioneins are archetypal proteins that are regulated by intracellular zinc ion levels through MTF-1/MREs interactions [38]. They are cysteine-rich, metal-binding proteins involved in zinc homeostasis and heavy metal detoxification by binding cadmium and copper; they are also involved in protection against oxidative stress. Many genes have also been found to have MREs present in the promoter, including ZnT-1 [49], γGCS_{hc} [50] and other genes involved in detoxification and general stress response [51]. Taken together, our findings would suggest that hepcidin may belong to this family of proteins. Intriguingly, MREs in the MT-1 promoter differentially respond to different metals including copper and mercury; one MRE also bound a zinc-dependent co-regulator [46]. Therefore, it is possible that one or more of the hepcidin MREs may respond to cadmium (or other metals) depending on co-regulator type; however the overall effect of this would be smaller than that induced by zinc. Previous studies have shown that MTF-1 and USF1 cooperate during mouse development to induce optimal expression of the MT-1 gene in visceral endoderm cells [52] and that USF1 is important in the induction of mouse MT-1 by cadmium [53]. This may be pertinent as USF1 has been shown to be important in the transcriptional regulation of the hepcidin gene [33].

The involvement of MREs and MTF-1 in the metal response of hepcidin was given further credence after mutation analysis. Point mutants of each MRE showed a decrease in zinc-dependent luciferase activity. This effect appeared to be additive as mutation of all four MREs almost completely diminished the zinc response. However, mutating all four MREs did not reduce luciferase activity to basal levels after zinc treatment, supporting our view that other transcription factor(s) may synergize with MTF-1 in hepcidin responses to divalent metals. Our EMSA data also demonstrated that zinc-treated Huh7 cells could bind to MREs in vitro; this binding could be competitively inhibited by excess cold MRE probe.

Interestingly, our study also showed that MT-1 mRNA was up-regulated by iron. This has been previously demonstrated in the livers of iron-loaded rats by Brown et al. [54]; MT-1 mRNA was up-regulated with a concomitant increase in hepcidin mRNA. A similar effect of iron on MT-1 transcription has also been demonstrated in vitro [55]. Although the function of MT-1 in heavy metal detoxification has been established, the teleology of hepcidin induction by these metals is not clear at present but nonetheless intriguing. Is the effect of zinc on hepcidin transcription one of protection against oxidative damage? It has been suggested that the cytoprotective effects of MT-1 are due to its ability to release zinc to inhibit membrane lipid peroxidation by iron [56]. Whether hepcidin performs a similar function remains to be tested although our results suggest that it may also be a sensor of other metal ions aside from iron. In other words, hepcidin may mediate cross-talk

between various divalent metal ions. This study may also establish the rationale for the immunomodulatory effects of zinc supplementation [57] since this would, among others, up-regulate the expression of hepcidin which is primarily a defensin.

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