Heat and Heavy Metal Stress Synergize to Mediate Transcriptional Hyperactivation by Metal-responsive Transcription Factor MTF-1*

Mammalian cells react to heavy metal stress by transcribing a number of genes that contain metal-response elements (MREs) in their promoter/enhancer region; this activation is mediated by metal-responsive transcription factor-1 (MTF-1). Well-known target genes of MTF-1 are those encoding metallothioneins, small, cysteine-rich proteins with a high affinity for heavy metals. The response to heat shock, another cell stress, is mediated by heat shock transcription factor 1 (HSF1), which activates a battery of heat shock genes. Little is known about the cross-talk between the different anti-stress systems of the cell. Here we report a synergistic activation of metal-responsive promoters by heavy metal load (zinc or cadmium) and heat shock. An obvious explanation, cooperativity between MTF-1 and HSF1, seems unlikely: transfected HSF1 boosts the activity of an Hsp70 promoter but hardly affects an MRE-containing promoter upon exposure to metal and heat shock. A clue to the mechanism is given by our finding that heat shock leads to intracellular accumulation of heavy metals. We propose that the known anti-apoptotic effect of heat shock proteins allows for cell survival despite heavy metal accumulation and, consequently, results in a hyperactivation of the metal response pathway.

The mammalian metal-responsive transcription factor-1 (MTF-1)1 is a zinc finger transcription factor that regulates the transcription of target genes in response to heavy metals (1–3). The best characterized target genes of MTF-1 are those encoding metallothioneins (MTs), a family of small, cysteine-rich metal-binding proteins with roles in heavy metal detoxification and homeostasis of heavy metals, radical scavenging, and maintenance of cellular redox state (4–7). The expression of metallothioneins can be induced by a variety of physiological and environmental stresses such as heavy metals, oxidizing agents, hypoxia, phosphor esters, ultraviolet and ionizing radiation, glucocorticoid hormones, and infectious agents.

MTF-1 activates metallothionein gene expression through MRE (metal response element) sequences of core consensus TGCRCNC; multiple MREs are present in the promoter regions of MT-1 and MT-II genes (1, 8). In resting cells, MTF-1 resides in the cytoplasm and translocates to the nucleus in response to heavy metal exposure (9–11). Recently, we have shown that this translocation occurs not only after heavy metal stress but also after heat shock (11).

Heat is another important stress condition that elevates, through HSF1, transcription of the genes for heat shock proteins (Hsps), which can maintain/restore cellular protein functions via chaperone activity (12–15). The expression of heat shock proteins is also associated with pathological states, including inflammation, fever, infection, ischemia, and cancer (16–18). Moreover, some Hsps, notably Hsp70, have been shown to protect cells against apoptosis (19–22).

In yeast cells, the CUP1 copper metallothionein gene is activated not only by excess copper but also by heat shock through the HSE sequences in the promoter (23, 24). Conversely, in several species, cadmium induces heat shock genes as well as metallothionein genes (25–27). In mammalian cells, heat shock-induced nuclear translocation of MTF-1 has been shown to be insufficient to activate transcription from a metallothionein gene promoter (11). Consistent with this finding, we could not find any heat shock element (HSE) consensus sequence in the mouse metallothionein gene promoter.

In this study, we report that a metallothionein promoter is hyperactivated in an MTF-1-dependent manner by a combined exposure to heat and heavy metals. This induction is associated with an accumulation of heavy metals in the cell during heat exposure. Unlike a similar hyperactivation of the heat shock 70 gene promoter by heat and cadmium where heat shock transcription factor HSF1 plays a crucial role, HSF-1 is apparently not required for metallothionein promoter activation.

MATERIALS AND METHODS

Transient Transfections and Luciferase Assays—HEK293 (adenovirus-transformed human embryonic kidney) cells and HeLa (cervix carcinoma) cells were used for transient transfections. Reporter genes consisted of the firefly luciferase coding sequence driven either by a mouse metallothionein I promoter, a synthetic 4xMREd/TATA box promoter, or by the mouse Hsp70 promoter (kindly provided by Olivier Bensaude). References were β-galactosidase (CMV-LacZ) or Renilla luciferase (pRL-CMV) genes under the control of the ubiquitously active CMV promoter. Reporter and reference genes were transfected into cells by the calcium phosphate method (28). 38 h post-transfection, cells were exposed to heat shock at 43 °C during the indicated time periods. Heavy metals, H2O2, serum, low pH (6.0), and cycloheximide were administered to the culture either during heat shock at 43 °C or at 37 °C as indicated. After heat treatments, cells were transferred to 37 °C and incubated for another 3 h to allow for the recovery of the reporter protein, luciferase (29). Cells were harvested and analyzed by measuring luciferase activities according to the instructions of the manufacturer (Promega). Firefly luciferase units were normalized to either β-galactosidase values or Renilla luciferase values (30).

Transcript Analysis by S1 Mapping—S1 nuclease mapping of transcripts was performed according to the standard procedure (31, 32). OVEC-4xMREd promoter or OVEC-mMT-I promoter (10 μg/100-mm plate) and OVEC-reference driven by the CMV promoter (1 μg/100-mm plate) were transfected into 293 cells. Cells were treated with zinc,
cadmium, heat, or heavy metal/heat shock combinations for the indicated times. Cells were then harvested and analyzed for the transcriptions.

**Western Blot—**HEK293 cells were transfected with a VSV-tagged MTF-1 expression vector (11), and 36 h post-transfection cells were treated with either zinc (100 \( \mu \)M), cadmium (60 \( \mu \)M), and/or heat (42 °C, 1.5 h). After a 3-h recovery period of cells at 37 °C, nuclear extracts were prepared as described previously (33). 30 \( \mu \)L of nuclear extracts was loaded onto an SDS-polyacrylamide gel (7.5%) and transferred to polyvinylidene difluoride membranes (Amersham Biosciences) using Trans-blot-SD Semi-dry Transfer Cell (Bio-Rad). Before blocking, the membrane was stained with Ponceau to verify the amount of proteins loaded into each well. Before incubation with the primary antibody, the membrane was incubated in blocking buffer (5% nonfat milk, 0.5% Tween 20 in 1× PBS) for 1 h at room temperature. The membrane was then incubated with mouse anti-VSV antibody (1:10,000, Sigma) for 1 h at room temperature. After washing three times for 10 min each with washing buffer (0.5% Tween 20 in 1× PBS), the membrane was incubated for 1 h at room temperature with the secondary antibody, antimouse horseradish peroxidase-labeled (1:10,000, Vector Laboratories) in a buffer containing 2.5% nonfat milk, 0.5% Tween 20 in 1× PBS. After three washes of 15 min each, the horseradish peroxidase signal was detected using ECL Plus (Amersham Biosciences) following the manufacturer’s instructions.

**Heavy Metal Determination in Cell Extracts—**HEK293 cells were treated with 100 \( \mu \)M zinc or 60 \( \mu \)M cadmium (final concentration in DMEM culture medium containing 5% fetal calf serum), and left either at 37 °C or 42 °C for 1 h. The cells were scraped off with 5 mL of PBS ultra pure (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4). A total of 1 mL of cell suspension was diluted with 1 mL of 65% HNO3. All samples were microwave-digested (MLS Ethos 900, MLS, Leutkirch, Germany) at 210 °C and subsequently diluted with Milli Q water up to 10 mL before inductively coupled plasma mass spectrometry (ICP-MS) analysis. For analysis of metal concentrations, ICP-MS was performed using a HP4500 Series 300 ShieldTorch System instrument (Agilent, Waldbronn, Germany) in peak-hopping mode with a spacing of 0.05 atomic mass unit, 3 points/peak, 3 scans/sample, and an integration time of 300 ms/point. The rate of plasma flow was 15.5 liters/min with an auxiliary flow of 1.0 liter/min. The radio frequency power was 1.18 kilowatts. The samples were introduced using a cross-flow nebulizer at a flow rate of 1.02 liter/min. The apparatus was calibrated using a 6.5% zinc or 60 \( \mu \)M cadmium; Fig. 2B). This indeed appears to be the case, because determination of metals irrespective of heat shock (Fig. 2B).

Next we considered the possibility that the heat shock factor 1 (HSF1), the major transcription factor involved in the heat shock response, was directly or indirectly participating in this synergy effect. HSF1 is conserved from yeast to humans and binds to so-called heat shock elements (HSEs) of consensus sequence nGAAnnTTCn (35–38). Unlike the situation in yeast, where heat shock activates metallothionein (CUP1) expression via a heat shock response element, there are no obvious binding sites for HSF-1 in the mouse metallothionein-1 promoter, let alone the synthetic 4xMREd promoter; however, an indirect effect seemed also possible, e.g., with a cofactor-like binding of HSF-1 to MTF-1, rather than to DNA. To test this possibility, we cotransfected an expression plasmid for HSF1 together with the 4xMREd promoter. The effect of HSF1 on this promoter was at most marginal (Fig. 3). By contrast, activity of an Hsp70 promoter was strongly boosted by the combination of HSF1 and heavy metal (Fig. 4). (The poor inducibility by heat without transfected HSF1 can be explained by the relatively high basal activity of the Hsp70 promoter in HEK293 cells (39).

From this data it still seems possible that, if not HSF-1 itself, at least some heat shock proteins might be involved in the hyperactivation of the 4xMREd promoter. Chaperones are known to facilitate formation of some transcriptional regulatory complexes, which in turn activate or repress transcription from certain promoters (40–45). First, to test if Hsp90 is involved in the transcriptional activation of MTF-1, we treated HepG2 cells with geldanamycin, which specifically blocks the ATP binding cassette of Hsp90 and thereby abolishes its chaperone activity (46). In our case, however, neither the transcriptional activity nor the nuclear translocation of MTF-1 was affected by geldanamycin (data not shown).

In contrast to the role of chaperones in assisting the formation of transcription complexes, recent studies by Freeman and Yamamoto indicate that some chaperones, notably p23 and Hsp90, can interfere with transcription by disassembling regulatory complexes; this was revealed by tethering chaperone-Gal4 fusion proteins to a reporter promoter (47). Along this vein of thought, it could be argued that, upon heat shock, such interfering hsps are titrated by unfolded proteins, which would boost the transcriptional response of some promoters. To test whether the molecular chaperones p23, Hsp90, and Hsp70 mediate disassembly of the MTF-1-containing transcriptional complexes, each of these proteins was fused to the DNA binding domain of Gal4 and tested on a luciferase gene driven by three Gal4 binding sites followed by four metal response elements (MREd). However, neither Gal4-p23, Gal4-Hsp90, nor Gal4-Hsp70 significantly affected basal or heat/heavy metal-stimulated levels of transcription from this reporter gene (data not shown).

Because we could not observe any involvement of the key proteins of the heat shock response in the synergistic activation of MTF-1, we considered the possibility that MTF-1 was the principal effector of transcriptional activation, whereas heat shock somehow altered the cellular handling of heavy metals. This indeed appears to be the case, because determination of zinc and cadmium concentrations in HEK293 cells shows that heat stimulates the intracellular accumulation of these metals (Fig. 5).
DISCUSSION

Recent work from our laboratory pointed to a relationship between MTF-1 and heat shock stress: not only heavy metals but also heat causes the translocation of MTF-1 from the cytoplasm to the nucleus. Other conditions tested, such as low pH (6.0), H$_2$O$_2$, and high serum concentration, were also found to induce, at least to some extent, this translocation. However, none of these other conditions was able per se to activate MTF-1-dependent transcription under our assay conditions (11). Here we show that heat shock is ineffective by itself, but heat and heavy metals can synergize to hyperactivate metal-inducible promoters.

A straightforward explanation for this effect, cooperative action of MTF-1 and heat shock transcription factor (HSF1), was ruled out. Unlike the mouse Hsp70 promoter, which is also synergistically activated by heat and cadmium and where transfected HSF1 potentiates the effect, the presence or absence of HSF1 had no influence on the 4xMREd promoter.

Because zinc is the main physiological inducer of MTF-1 in mammalian cells, one could simply postulate that heat shock triggers a zinc release from cellular zinc stores, which in turn binds to MTF-1 allowing it to activate its target genes. However, zinc liberated by heat shock alone would be expected to activate MTF-1, which is not the case: transcription is only induced when exogenous zinc or cadmium is provided during heat shock. Interestingly, relatively low concentrations of these heavy metals, which by themselves hardly induce transcription, are sufficient for synergistic activation.

We find that the amount of MTF-1 associated with nuclear structures is not increased by heat/metal treatment versus metal alone (Fig. 2B). Alterations in the phosphorylation state of MTF-1 have been invoked in the process of transcriptional activation.
induction (48, 49). It is unknown at present whether heat treatment results in additional modifications of MTF-1 and/or of a putative cofactor that remains to be identified.

Taken together, the hyperactivation of the metallothionein promoter by heat and metal is best explained by the propensity of cells to accumulate heavy metal under heat shock. Indeed we find that at a given heavy metal concentration in the culture medium, heat shock resulted in 2.5- and 2.3-fold higher intracellular concentrations of zinc and cadmium, respectively. However, our attempts to achieve the same high level of activation by merely increasing extracellular heavy metal concentration at 37°C were unsuccessful, invariably resulting in massive cell death (not shown). This finding of course raises the question why the cells did not die in the combination of metal and heat shock. It is well-documented that heat shock proteins, notably Hsp70, exert a strong anti-apoptotic effect (13). Therefore, we propose that heat treatment not only results in heavy metal accumulation but also allows for survival at intracellular metal concentrations that could not be tolerated otherwise. Although cadmium is not a physiological trace element, zinc is
essential for proper functioning of the immune system (50), and it is tempting to speculate that fever, a natural heat shock condition (51), contributes to the defense against infectious agents by promoting zinc uptake, among other effects. Furthermore, upon infection stress, an increased production of MT agents by promoting zinc uptake, among other effects. Further-
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by Metal-responsive Transcription Factor MTF-1
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J. Biol. Chem. 2003, 278:31879-31883.
doi: 10.1074/jbc.M302138200 originally published online June 12, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M302138200

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