Metallothionein Induction by Hypoxia Involves Cooperative Interactions between Metal-Responsive Transcription Factor-1 and Hypoxia-Inducible Transcription Factor-1α

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Abstract

Mammalian *metallothionein* (*MT*) genes are transcriptionally activated by the essential metal zinc as well as by environmental stresses, including toxic metal overload and redox fluctuations. In addition to playing a key role in zinc homeostasis, MT proteins can protect against metal- and oxidant-induced cellular damage, and may participate in other fundamental physiologic and pathologic processes such as cell survival, proliferation, and neoplasia. Previously, our group reported a requirement for metal-responsive transcription factor-1 (MTF-1) in hypoxia-induced transcription of mouse *MT-I* and human *MT-IIA* genes. Here, we provide evidence that the protumorigenic hypoxia-inducible transcription factor-1α (HIF-1α) is essential for induction of *MT-1* by hypoxia, but not zinc. Chromatin immunoprecipitation assays revealed that MTF-1 and HIF-1α are both recruited to the mouse *MT-I* promoter in response to hypoxia, but not zinc. In the absence of HIF-1α, MTF-1 is recruited to the *MT-I* promoter but fails to activate *MT-I* gene expression in response to hypoxia. Thus, HIF-1α seems to function as a coactivator of *MT-I* gene transcription by interacting with MTF-1 during hypoxia. Coimmunoprecipitation studies suggest interaction between MTF-1 and HIF-1α, either directly or as mediated by other factors. It is proposed that association of these important transcription factors in a multiprotein complex represents a common strategy to control unique sets of hypoxia-inducible genes in both normal and diseased tissue. (Mol Cancer Res 2008;6(3):483–90)

Introduction

The cysteine-rich metallothioneins (MT) bind metals, including zinc, copper, and cadmium, with high affinity and capacity (1, 2). In mice, MT-I and MT-II are the most prevalent of the four known MT isoforms, and they are recognized to regulate heavy metal metabolism and detoxification and to participate in oxidant scavenging (1, 2). Other studies suggest that these proteins are pluripotent, contributing to a number of other fundamental processes, including proliferation, survival, metabolism, inflammation, and extracellular remodeling (3-18). It is therefore not surprising that aberrant expression patterns of MTs can correlate with a number of pathologic conditions, including malignant progression. For example, high levels of MT-I and MT-IIA have been detected in many (but not all) human tumors, including those of the breast, prostate, cervix, testes, kidney, bladder, brain, and oral epithelium (19-25). Underlying molecular mechanisms are believed to include the ability of MT to activate survival pathways and confer resistance against drug and radiation therapy (5, 26-28). Collectively, these data suggest that an understanding of the control mechanisms regulating MT isoform expression will provide unique therapeutic insights.

The metal-responsive transcription factor-1 (MTF-1) is a central regulator of metal-inducible expression of *MT-I* and *MT-II*. Current models of MTF-1 activation suggest that zinc directly and reversibly modulates highly specific, linker-mediated zinc finger interactions in MTF-1, resulting in translocation of this transcription factor to the nucleus and rate-limiting binding to metal response elements [MRE; TGC(G/C)CNC(G)] within proximal *MT* promoters (29-34). In addition to zinc, other heavy metals (e.g., cadmium), hypoxia, oxidative stress, nitric oxide, and high temperature induce the transcriptional activity of MTF-1 (1, 20, 30, 35-43). Although the precise mechanisms of action of these other MTF-1 activators remain to be determined, a common underlying theme seems to involve a displacement of labile zinc, perhaps from the MT protein, zinc-sensitive activation of MTF-1, and altered expression of MTF-1 target genes (20, 40, 44, 45). Studies by our group, and collaborators, suggest that MTF-1 can contribute to tumorigenic processes, in part, through its transcriptional action as either a positive or a negative regulator of numerous genes, including *zinc transporter 1* (*ZnT1), PIGF, transforming growth factor-β1* (*TGF-β1*), *tissue transglutaminase-2* (*TG2*), and *Zip10* (20, 46-49).

A number of studies designed to characterize transcription factor interactions at the *MT-I* promoter have been reported (e.g., refs. 31, 32, 43, 50-54). However, a limited number of these studies have focused on the chromatin-packaged *MT-I* promoter, and those only in the context of activation by metal

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and oxidative stresses (31, 32). The proximal *MT-I* promoter is occupied by several transcription factors on at least 10 sites in response to zinc overload. In addition to MTF-1, upstream stimulatory factor (USF)-1, USF-2, Sp1, c-Fos, and c-Jun are bound to the proximal promoter (32). USF-1 and USF-2 strongly interact with the *MT-I* promoter regardless of the zinc concentration or the presence of MTF-1. The heterodimers of USF-1 and USF-2 most likely facilitate the functions of MTF-1 through the maintenance of an open promoter configuration, allowing direct binding of MTF-1 to specific MREs within the proximal promoter in response to metals (32). MTF-1 seems to be only weakly associated with the promoter under normal zinc conditions, whereas increased zinc levels result in binding of MTF-1 to all five MREs within the proximal −250 bp promoter (relative to the transcription start site). C-Fos is also associated with the *MT-I* promoter during zinc exposure, and this MTF-1–dependent recruitment coincides with robust activation of the gene (32). Our recent studies[4] show that metals cause the formation of a protein complex containing (at least) MTF-1, Sp1, and the histone acetyltransferase p300. In this study, the acidic transactivation domain of MTF-1 was found to be essential for *MT-I* gene activation and the binding of p300. MTF-1–containing transcription factor complexes involved in regulation by stresses other than metals remain to be identified.

The cellular response to hypoxia is regulated, in part, by the hypoxia-inducible factor-1 (HIF-1), a basic helix-loop-helix transcription factor composed of two subunits, HIF-1α and HIF-1β (55). Whereas the HIF-1β subunit is constitutively expressed, HIF-1α expression is regulated by oxygen tension through a protein stabilization process involving inhibition of posttranslational ubiquitination-triggered proteolysis (56). Our previous studies have placed MTF-1 in as yet unidentified pathway required for stabilization/accumulation of HIF-1α protein levels in response to hypoxia (57). Under low oxygen tensions, HIF-1α forms heterodimers with HIF-1β that mediate nuclear translocation and binding to hypoxic response elements (core consensus sequence RCGTG) within the proximal promoters of target genes. HIF-1 regulates a wide range of genes, including many involved in metabolism, angiogenesis, cell survival, and invasion. Specific gene expression patterns depend on the degree and duration of hypoxic insult, cell type, and tissue background (58, 59). Other transcription factors (e.g., c-Jun, nuclear factor-κB, signal transducer and activator of transcription 3, Sp1, and early growth response-1) have been shown to interact with HIF-1α and cooperate in regulating target gene expression. Some of these proteins can also act independently of HIF-1α and control unique sets of hypoxia-responsive target genes (e.g., see refs. 20, 60, 61). In addition, the general transcriptional coactivator p300/cyclic AMP-responsive element binding protein–binding protein directly binds to the COOH terminus transcriptional activation domain of HIF-1α and synergistically enhances its transcriptional activity in response to hypoxia (60, 62).

We previously found MTF-1 to be transactivated by hypoxia and required for the hypoxia-inducible transcription of the *MT-I* and *MT-IIa* genes (43). Transfection and gel shift analyses identified hypoxic-responsive MREs within these promoters (43). In addition, our group identified the angiogenic *placenta growth factor* (*Plgf*) as another MTF-1–dependent hypoxic target gene (47). Subsequent collaborative studies found a corequirement for the redox-sensitive nuclear factor-κB in transcriptional control of the *Plgf* (63). Herein, we show that HIF-1α is also required for the hypoxia-inducible transcription of *MT-I*. Moderate hypoxia caused the recruitment of MTF-1, HIF-1α, and p300 to the mouse *MT-I* proximal promoter. Furthermore, MTF-1 and HIF-1α physically interact under these conditions in a mechanism yet to be elucidated.

Results

HIF-1α and MTF-1 Regulate Hypoxia Induction of MT-I mRNA

MT-I transcription is increased during hypoxia in a MTF-1 (and MRE)–dependent manner; increases in *MT-I* mRNA levels occur within 4 hours after exposure, and maximum induction occurs within 8 to 12 hours (43, 64). MTF-1 is also required in the hypoxia accumulation of HIF-1α, a central transcriptional regulator of the cellular response to hypoxia (57). Herein, we assessed a possible role of HIF-1α in the hypoxia-inducible induction of *MT-I*. Northern blot analyses revealed that loss of *HIF-1α* in both TAg and TAg/*ras* mouse embryonic fibroblasts [MEF; *HIF-1α* knockout (HIF-KO)] attenuated the induction of *MT-I* mRNA levels during hypoxia but had no effect on zinc induction (Fig. 1A and B, respectively). To confirm this finding, we used RNA interference to silence HIF-1α expression in *ras*-NIH 3T3 cells. Figure 1C shows a real-time reverse transcription-PCR (RT-PCR) analysis of *MT-I* mRNA levels in aerobic and hypoxic *ras*-NIH 3T3 cells that were transiently transfected with either mouse *HIF-1α* or control, noncoding, small interfering RNA (siRNA) oligomers. Although the inductions by both hypoxia and zinc were modest, compared with the MEF model, the induction of *MT-I* mRNA levels by hypoxia was nevertheless completely reversed by *HIF-1α* RNA interference. The ability of the siRNA sequence to inhibit HIF-1α expression, and thus accumulation, was confirmed by Western analysis (Fig. 1C). Our data also showed that, in contrast to MTF-1 control over HIF-1α accumulation, loss of *HIF-1α* had no effect on either whole-cell or nuclear MTF-1 protein levels from aerobic or hypoxic cells (Fig. 1D). This Western analysis also indicated that hypoxia had no apparent inducing effect on MTF-1 nuclear translocation, compared with significant translocation in response to zinc exposure (e.g., see Fig. 3; ref. 31). These data suggest that MTF-1 and HIF-1α are both essential for induction of *MT-I* gene expression in response to hypoxia and that HIF-1α probably directly acts at the *MT-I* promoter. Furthermore, hypoxia may affect that transactivation capacity of MTF-1 that resides in the nucleus.

Hypoxia Induces Recruitment of MTF-1 and HIF-1α to the MT-I Promoter

Chromatin immunoprecipitation (ChIP) assays were done to more fully address the mechanisms by which MTF-1 and HIF-1α regulate hypoxia-inducible transcription of the *MT-I* gene *in vivo*. TAg/*ras*-transformed wild-type (WT), MTF-KO,

[4] Y. Li et al., in preparation.

and HIF-KO MEFs were exposed to hypoxia (1% O_2; 6 hours) or $ZnSO_4$ (100 μmol/L; 1 hour), or left untreated before ChIP assays. Cross-linked chromatin was isolated from each culture, sheared, and immunoprecipitated with antibodies against MTF-1, HIF-1α, and p300. The precipitated DNA was analyzed by PCR using primers specific for the proximal region (−264 to +43 bp) of the *MT-I* gene (Fig. 2). The results show that MTF-1 is recruited to the *MT-I* promoter in response to hypoxia or zinc. The magnitude of recruitment in response to hypoxia was evident but clearly less than that observed after zinc exposure (Fig. 2A and B, *top*), and was not dependent on HIF-1α (Fig. 2B). Hypoxia also caused the recruitment of HIF-1α and p300 to the *MT-I* promoter in WT MEFs (Fig. 2A and B).

The *in vivo* kinetics of binding of MTF-1 and HIF-1α to the *MT-I* promoter in response to hypoxia was examined (Fig. 2C). MTF-1 was recruited within the first hour of hypoxia and remained associated with this promoter for at least 8 hours. In contrast, HIF-1α binding to the *MT-I* promoter was not detected until 4 hours of hypoxia. The intensity of the HIF-1α signal was always modest relative to that of MTF-1; this pattern may be due to a number of possibilities, including

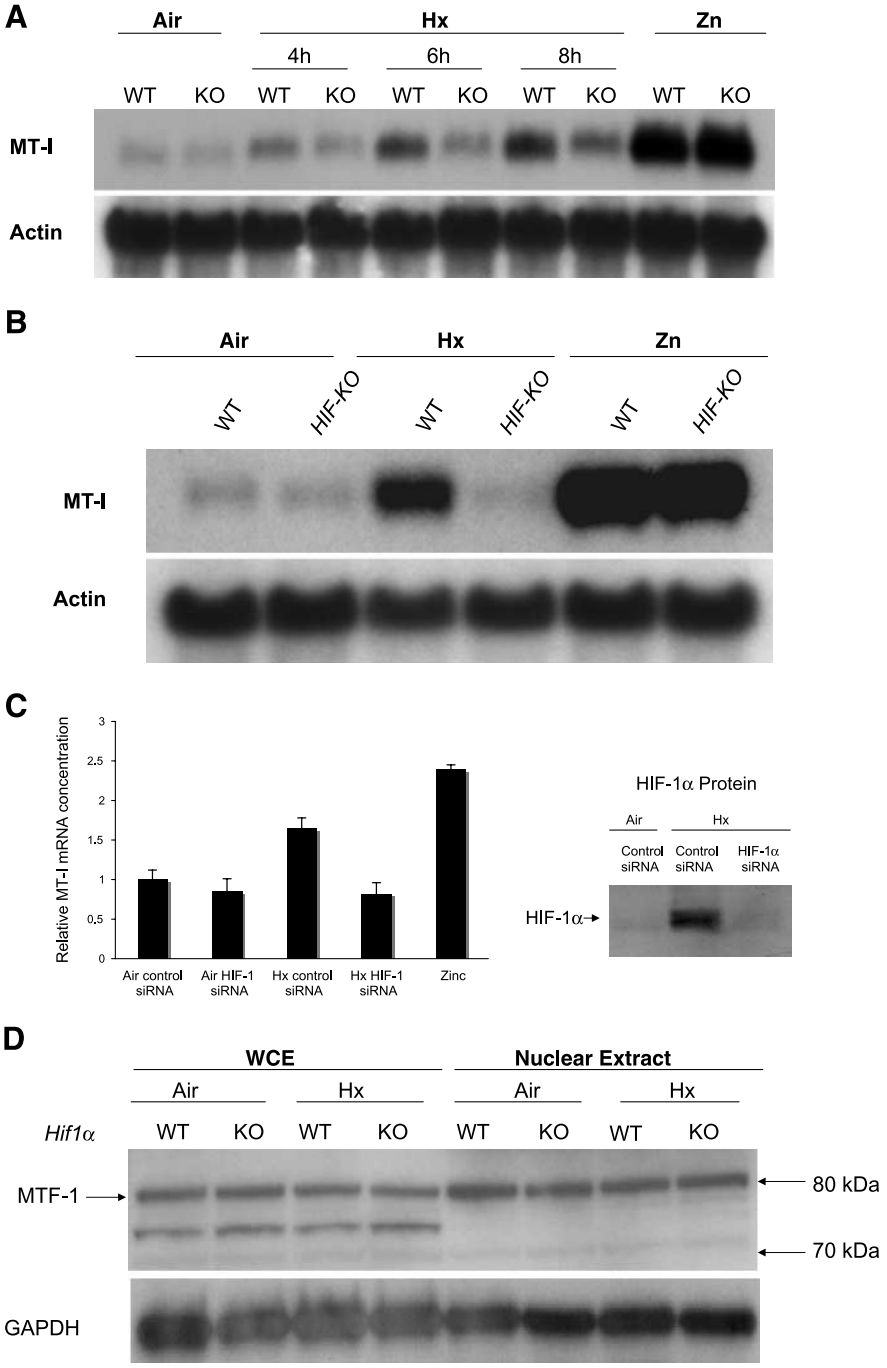

FIGURE 1. HIF-1α contributes to hypoxia-induced *MT-I* transcription. **A** and **B.** Northern blot analysis of *MT-I* mRNA levels as a function HIF-1α expression. TAg- and TAg/ras-WT and HIF-KO MEFs were examined for *MT-I* mRNA levels as functions of hypoxia (*Hx*; 1% O_2; 2-8 h) or $ZnSO_4$ (*Zn*; 100 μmol/L; 1 h) exposures. **C.** Real-time RT-PCR analysis of *MT-I* mRNA levels as a function of HIF-1α silencing in hypoxic *ras*-NIH 3T3 cells. Near-confluent cultures were transfected with HIF-1α siRNA oligonucleotides (see Materials and Methods) and allowed to recover for 16 h before exposure to hypoxia (8 h). Relative *MT-I* mRNA levels were determined by real-time RT-PCR. Western blotting of nuclear HIF-1α protein was also done to confirm HIF-1α silencing. **D.** Western analysis of whole-cell extract (*WCE*) and nuclear MTF-1 protein levels as a function of HIF-1α loss. KO in **D** denotes HIF-KO. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. A full-length Western blot is presented in Supplementary Data. The position of the MTF-1 protein band, under these electrophoresis conditions, was previously established through the use of WT and *MTF-1* KO MEFs (57) and through reintroduction of *MTF-1* cDNA into KO cells (ref. 31; also see Fig. 3, *bottom*, and Supplementary Data).

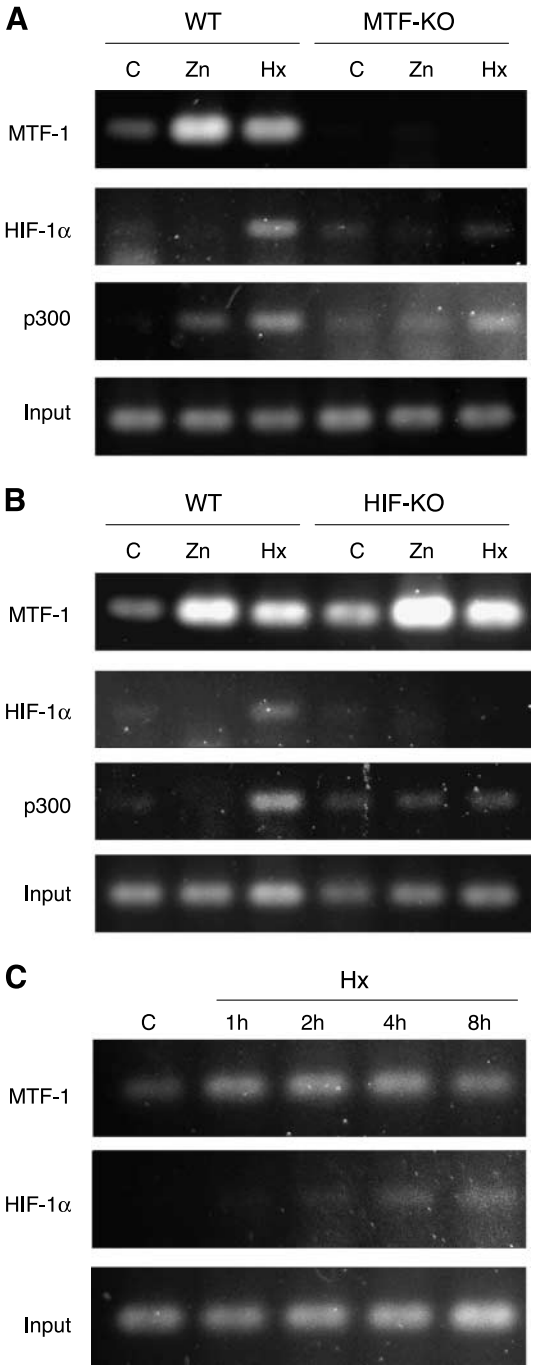

FIGURE 2. ChIP analysis of HIF-1α, p300, and MTF-1 with the *MT-I* promoter. WT and MTF-KO (**A**) or HIF-KO MEFs (**B**) were exposed to 100 μmol/L $ZnSO_4$ (*Zn*) for 1 h or hypoxia (*Hx*; 1% O_2; 8 h). C, control. **C.** A time course analysis of MTF-1 and HIF-1α recruitment to the *MT-I* promoter using WT MEFs. Chromatin was fixed, sonicated, and then precipitated using a polyclonal against Flag or MTF-1. The relative amounts of *MT-I* promoter DNA precipitated were determined by PCR and normalized to amplification of input DNA (*bottom blot* in **A** and **B**).

antibody affinity and efficiency of recovery in the immunoprecipitation steps, the inability to cross-link HIF-1α efficiently, or the lower number of HIF-1α molecules bound to the *MT-I* promoter.

MTF-1 Associates with HIF-1α in Hypoxia-Treated Cells

Ras-MTF-KO cells stably transformed with a FLAG-tagged *MTF-1* cDNA (MTF-1_{flag} MEFs) and MTF-KO MEFs (as a control) were used to assess a possible association between MTF-1 and HIF-1α. Earlier studies showed that stable introduction of a FLAG-tagged *MTF-1* cDNA vector into MTF-KO cells rescued metal responsiveness of the *MT-I* gene (31). Immunoprecipitation of FLAG-MTF protein was done on nuclear extracts from aerobic and hypoxic (1% O_2; 7 hours) MTF-KO and MTF-1_{flag} cells. The immunoprecipitates were analyzed by Western blot detection of the HIF-1α protein (Fig. 3). HIF-1α protein does coprecipitate with FLAG-MTF protein in hypoxic MTF-1_{flag} cells, but not under zinc stress, indicating a specific association of MTF-1 with HIF-1α under hypoxic conditions (Fig. 3). Similar patterns were obtained with TAg-immortalized MEFs (data not shown). Figure 3 also includes Western analysis of FLAG-MTF from the coimmunoprecipitated extracts. As expected, the nuclear level of MTF-1 protein in the MTF-1_{flag} cells was greatly enhanced in response to zinc treatment (31). However, we detected no discernible increase in nuclear MTF-1 protein from hypoxic MTF-1_{flag} MEFs. These data are consistent with our analysis of total MTF-1 protein in whole-cell and nuclear extracts (Fig. 1B). However, immunofluorescence analysis of vesicular stomatitis virus–tagged MTF-1 protein indicated that at least a fraction of cytoplasmic MTF-1 stores is transported into the nucleus under hypoxic conditions (63).[5] Regardless, these new findings show for the first time that MTF-1 forms a complex that includes HIF-1α and that these proteins are both recruited to the promoter *in vivo* under hypoxia exposure.

Discussion

The data presented herein confirm and extend our original findings regarding the involvement of MTF-1 in cellular responses to hypoxic stress, and specifically in its interactions with HIF-1α? Our group had previously reported that hypoxia induces the expression of MT-I and MT-IIA in a MTF-1/MRE–dependent manner (43). In subsequent studies, we found that MTF-1 participates in a more general hypoxia-inducible mechanism, contributing to the stabilization and nuclear accumulation of the HIF-1α protein, a central regulator of the cellular response to hypoxia (57). This new study provides evidence for recruitment of MTF-1, HIF-1α, and p300 to the *MT-I* promoter and indicates that MTF-1 and HIF-1α are components of a transcriptional complex in response to hypoxia.

Cellular hypoxia is a common stress characterizing a number of physiologic and pathogenic conditions, including development, wound healing, ischemia, inflammation, and tumorigenesis (20, 65-67). For example, the establishment of hypoxia microenvironments within many solid tumor types correlates with the phenotypes of increased angiogenesis, survival, and metastasis (see ref. 20 for a review). HIF-1α, which is stabilized and highly expressed in many solid tumors, is considered a master transcriptional regulator of many of the genes that contribute to these malignant phenotypes and also to normal tissue responses to hypoxia (65, 68).

[5] B. Sato and B. Murphy, unpublished results.

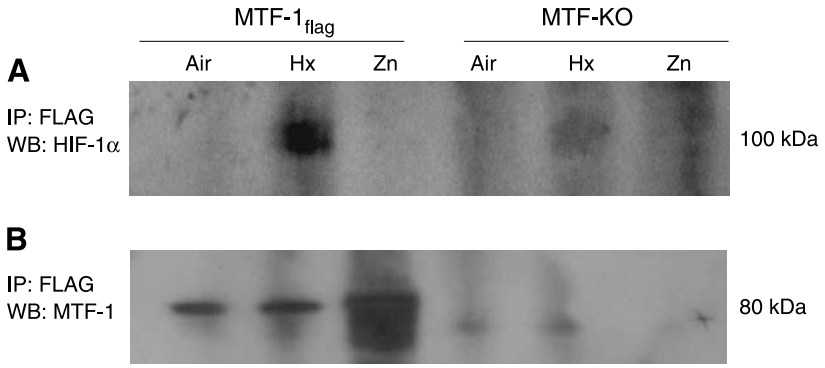

FIGURE 3. Coimmunoprecipitation of mouse MTF-1 and HIF-1α. MTF-KO and MTF-1_{flag} MEFs were incubated under hypoxia (*Hx*; 1% O_2; 8 h) or $ZnSO_4$ (*Zn*; 100 μmol/L; 1 h). Nuclear extracts were immunoprecipitated (*IP*) with an antibody against FLAG followed by Western blot (*WB*) analysis on the immunoprecipitates for HIF-1α (**A**) and MTF-1 (**B**) proteins. Full-length blots are presented in Supplementary Data.

Our preliminary Northern analysis of TAg-immortalized and TAg/*ras*-transformed fibroblasts showed that HIF-1α, in addition to MTF-1, is essential for hypoxia-associated induction of *MT-I* mRNA levels. Importantly, alternative knockdown of HIF-1α expression (by siRNA), in *ras*-NIH 3T3 cells, confirmed the validity of the data generated by genetic knockout of the gene. A search of the proximal mouse *MT-I* promoter indicates at least two putative HIF-1α binding sites, hypoxia response elements, that flank the USF element/antioxidant response element fragment (see ref. 32 for description of the complex USF/antioxidant response element site). ChIP analysis was used to examine the occupancy of the *MT-I* promoter under hypoxia. The recruitment of MTF-1 was suggested in our earlier studies (using Northern blot, promoter/reporter, and gel shift analyses), linking MTF-1 activation to the increased transcription of *MT-I* and *MT-IIA* by hypoxia (43). Hypoxic stress resulted in markedly increased MTF-1 binding to the *MT-I* promoter, and this intensity was comparable with that seen for zinc induction. Although recruitment of MTF-1 occurred within the first hour of hypoxic exposure, detection of HIF-1α binding was apparently delayed (up to 4 hours of hypoxia) and the binding was consistently less intense than that seen for MTF-1 binding. We previously reported the appearance of detectable increases in *MT-I* mRNA levels within this time frame (4-6 hours) of HIF-1α binding (43). The weaker binding of HIF-1α to the *MT-I* promoter may simply reflect a relatively lower HIF-1α antibody affinity and efficiency, compared with the FLAG antibody. The weak HIF-1α signal may also reflect a difficulty in stably cross-linking the MTF-1/HIF-1 complex. However, it is also possible that lower numbers of HIF-1 molecules actually bind the *MT-I* promoter under hypoxia or that HIF-1α must associate with other proteins for recruitment to the promoter rather than directly to the DNA itself. MTF-1 is considered the master regulator of MT genes and a requirement of MTF-1 for recruitment of other transcription factors to the *MT-I* promoter is most likely a common mechanism. For example, the presence of MTF-1 is necessary for zinc-induced c-Fos recruitment to the *MT-I* promoter (32). In sum, the available data imply that recruitment of both MTF-1 and HIF-1α to the *MT-I* promoter is required for hypoxia-inducible transcriptional activation of *MT-I*.

On stabilization, HIF-1α forms a heterodimer with HIF-1β, resulting in functional HIF-1 (69). In addition, the HIF-1α protein subunit physically interacts with a number of other transcription-related proteins, resulting in tissue-specific expression patterns. HIF-1 complexes are usually composed of a number of other transcription factors and coactivators such as p300. Under hypoxia, p300/cyclic AMP–responsive element binding protein–binding protein acetylates H3 histone, through its intrinsic histone acetyltransferase activity, and synergistically enhances HIF-1α transactivation, in part through direct physical binding (60). Our ChIP data clearly show that p300 is recruited to the HIF-1 complex at the *MT-I* promoter in response to hypoxia. Although loss of HIF-1α expression markedly attenuates p300 recruitment, MTF-1 does not affect p300 binding to the *MT-I* promoter. More detailed studies will be required to define the complex interactions between MTF-1, p300, and HIF-1α.

The coprecipitation experiments show an association between the MTF-1 and HIF-1α proteins during hypoxic stress. These findings are consistent with the formation of a MTF-1/HIF-1α transcriptional complex that forms at the *MT-I* promoter in response to hypoxia. The precise interaction between these two hypoxia-inducible transcription factors and their interactions with p300 remain to be elucidated.

Another potentially important finding involves the lack of detectable MTF-1 protein translocation into the nucleus under hypoxia. More sensitive immunofluorescence studies previously detected some nuclear translocation of the MTF-1 protein under hypoxia in at least two human cancer lines (63).[5] This apparent lack of nuclear translocation in the MEFs is in direct opposition to the robust nuclear accumulation of MTF-1 seen under zinc treatment. However, our ChIP data showed relatively similar binding of MTF-1 to the *MT-I* promoter in response to both hypoxia and zinc. It is certainly possible that the level of MTF-1 translocation detected by immunofluorescence is sufficient for this hypoxia-inducible recruitment. On the other hand, hypoxia may induce other signaling pathways that activate preexisting pools of nuclear MTF-1, perhaps through posttranslational modification of the transcription factor as described elsewhere (70, 71).

These new studies have uncovered the existence of a potentially central hypoxia-inducible complex that includes MTF-1 and HIF-1α (and p300). It is likely that MTF-1 is involved in other hypoxia-inducible transcriptional complexes. For example, MTF-1 cooperates with nuclear factor-κB to control the expression of the proangiogenic and prosurvival

PlGF (63). We therefore propose that MTF-1 and HIF-1α are components of a transcriptional complex that along with other hypoxia-inducible transcription factors and coactivators form combinatorial transcriptional complexes that are activated in a cell- and gene-specific manner. The cooperative interaction, and potential for gene therapy, between MTF-1 and HIF-1α has been independently verified by at least one other report showing that a chimeric promoter consisting of multiple copies of MREs and hypoxia response elements (and early growth response-1 binding elements) greatly enhanced expression of a reporter in response to hypoxia compared with that seen for any one element (72). Taken together, these studies offer a completely unique insight into the cooperative roles of MTF-1 and HIF-1α as transcriptional regulators of a subset of hypoxia-sensitive genes. The potential for the development of combined MTF-1/HIF-1α therapeutic strategies against diseases such as cancer is promising but remains to be tested.

Materials and Methods

Materials

Blasticidin S hydrochloride was obtained from Calbiochem; Polybrene (hexadimethrine bromide) was from Sigma; protein G and A/G Plus-agarose beads were from Santa Cruz Biotechnology; and Phoenix-Eco cells were from Orbigen. The gWzl-Blast H-*ras* expression vector was a generous gift from Dr. David Dankort (University of California, San Francisco, CA). The polyclonal antibody against recombinant mouse MTF-1 was generated (by G.K.A.) as previously described (73). The antibody against Flag peptide was purchased from Sigma, and the polyclonal antibody against p300 was from Santa Cruz Biotechnology. Lipofectamine 2000 was purchased from Invitrogen Life Technologies.

Cell Culture, Transfection, and Hypoxia Treatments

Ras-transformed NIH 3T3 cells were purchased from the American Type Culture Collection. The SV40 large T antigen TAg/*ras*-transformed WT and *HIF-1α* null MEFs (HIF-KO) were a generous gift of Dr. Keith Laderoute (SRI International, Menlo Park, CA). The HIF-KO cells were derived from the immortalized WT MEFs and there was no selection for individual clones (74). The TAg immortalized/Harvey (H)-*ras*–transformed WT and *MTF-1* null (MTF-KO) MEFs used in the Northern and ChIP analyses were a generous gift from Dr. Walter Schaffner (University of Zürich, Zurich, Switzerland). For detailed descriptions of these cell lines, see refs. (47, 57, 75). All cell lines (and new thaws) were genotyped by PCR analysis to determine the presence of the *MTF-1* WT and null alleles, as well as for genomic integration of *TAg* and oncogenic H-*ras*. The MEFs were cultured in DMEM with 10% fetal bovine serum. For coimmunoprecipitation studies, MTF-KO cells stably transfected with a mouse FLAG-tagged *MTF-1* expression vector were used. These cells are designated as MTF-1_{flag}. The generation of these immortalized SV40-TAg stably transfected cell lines is described in ref. (31). The MTF-KO and MTF-1_{flag} MEFs were then transformed with H-*ras*. Briefly, retrovirus-containing gWzl-Blast H-*ras* was prepared in Phoenix-Eco cells. Approximately 5×10^5 cells were plated in 100-mm dishes and infected the next day with retrovirus containing supernatant (1 mL) that also contained 5 mg/mL Polybrene (3 μL) in 2 mL of DMEM + 10% fetal bovine serum. After 5 h incubation at 37°C, an additional 7 mL of DMEM + 10% fetal bovine serum were added. The cells were incubated for 36 h, and then transferred into medium containing 2 μg/mL blasticidin. Selection was continued until all the mock-infected cells died. Frozen stocks of the surviving cells were prepared. *Ras* transformation was verified by soft agar colony formation. Large colonies were observed in the plates of transformed cells and not in the plates of the mock-infected cells.

Monolayer cultures were incubated in a hypoxic chamber held at a constant oxygen tension: 1% O_2; 5% CO_2 (In$Vivo_2$ Hypoxic Workstation, Ruskinn, Inc.). All media and solutions, such as lysis buffer, were incubated in the chamber before addition to cells.

Northern Blotting, RNA Interference, and Quantitative Real-time RT-PCR Analysis

Total RNA was extracted from cells using an RNeasy Mini kit (Qiagen) and Northern blot analysis was done as previously described (64). Briefly, RNA was separated by denaturing agarose-formaldehyde gel electrophoresis and transferred (and UV cross-linked) to nylon membranes. Blots were probed with an *Eco*RI/*Hin*dIII fragment of the mouse MT-I genomic DNA labeled by the random primer method. Signals were scanned on an AlphaImager 3400 (Alpha Innotech).

For the RNA interference studies, cells were seeded into 60-mm^3 plates with fresh medium, without antibiotics, 24 h before transfection. The high-performance liquid chromatography–purified siRNAs (nonsilencing control and mouse *HIF-1α* siRNAs; >97% pure) were purchased from Qiagen-Xeragon. The control siRNA sequence does not target any known mammalian gene (sequence 5′-AATTCTCCGAACGTGTCACGT-3′). The siRNA specific for mouse *HIF-1α* was derived from the mouse *HIF-1α* mRNA sequence (5′-TAGCCACAATTGCACAATATA-3′). Lipofectamine 2000 (Invitrogen) was used for all transfections. Cells were subjected to 8 h hypoxia (or normoxia) following treatment with 200 pmol control or mouse *HIF-1α* siRNA. Zinc exposure ($ZnSO_4$; 100 μmol/L for 2 h) was used as a positive control of *MT-I* induction. Total RNA from cells was isolated using the RNeasy method and DNA from the RNA preparations was removed by treatment with DNase (TURBO DNA-free Kit). First-strand cDNA was synthesized from total RNA using iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. Transcript analysis was done by real-time RT-PCR using the Taqman assay. Hybridization probes and primers (Inventoried, chosen from the online catalog) were purchased from Applied Biosystems. All samples were amplified simultaneously in triplicate and amplifications were run in a 7300 Real-time PCR System (Applied Biosystems). Each value was normalized to the corresponding *GAPDH* mRNA levels.

Chromatin Immunoprecipitation

ChIP assays of the proximal *MT-I* promoter were carried out as previously described (31, 32, 34, 76). MEFs exposed to hypoxia or aerobic controls were fixed in 1% formaldehyde and sheared, chromatin prepared, precleared with protein

G-agarose, and immunoprecipitated with specific antibody overnight at 4°C. The flag antibody (F3165) was purchased from Sigma and the HIF-1α antibody (NB100-105) was from Novus. Protein G–agarose was used to capture the immune complexes, and the formaldehyde cross-links were reversed. The DNA was analyzed by PCR using primers that span the −264 to +43 bp segment of the mouse *MT-I* promoter. The PCR products (after 28 cycles) were separated by agarose electrophoresis and stained with SYBR green I, quantified using the ChemiImager System (Alpha Innotech), and normalized to input products. All PCR products were obtained in the linear range of amplification as determined from analysis at various cycles (27-33 cycles).

Preparation of Nuclear Extract, Coimmunoprecipation, and Western Analysis

Nuclei from either aerobic or hypoxic MEF cultures (MTF-KO and MTF-1_{flag}) were isolated using the method of Ryan et al. (77) with modifications described elsewhere (57). Cells were washed with ice-cold PBS before addition of lysis buffer that also contained protease inhibitor cocktail. Nuclei from hypoxic MEFs were harvested in the $InVivo_2$ Hypoxic Workstation.

Protein concentrations were determined using the BCA reagent (Pierce Biotechnology). Equal nuclear protein extracts (200 μg) from hypoxia-treated and aerobic MEFs (MTF-KO and MTF-1_{flag}) were incubated with 2 μg of anti-Flag antibody and 20 μL of protein A/G Plus-agarose beads (constant mixing at 4°C overnight). The beads were centrifuged (2,500 rpm × 5 min) and washed in NP40 lysis buffer five times before Western blotting for HIF-1α (Novus) and Flag (Sigma).

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