Metallothionein Is Part of a Zinc-scavenging Mechanism for Cell Survival under Conditions of Extreme Zinc Deprivation*

(Received for publication, October 14, 1998, and in revised form, December 24, 1998)

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Metallothionein (MT) is a small cysteine-rich protein thought to play a critical role in cellular detoxification of inorganic species by sequestering metal ions that are present in elevated concentrations. We demonstrate here that metallothionein can play an important role at the other end of the homeostatic spectrum by scavenging an essential metal in a mouse fibroblast cell line that has been cultured under conditions of extreme zinc deprivation (LZA-LTK). These cells unexpectedly produce constitutively high levels of metallothionein mRNA; however, the MT protein accumulates only when high concentrations of zinc are provided in the media. Until this MT pool is saturated, no measurable zinc remains in the external media. In this case, zinc deprivation leads to amplification of the MT gene locus in the LZA-LTK cell line. Furthermore, the intracellular zinc levels in the fully adapted cells remain at the normal level of 0.4 fmol zinc/cell, even when extracellular zinc concentration is decreased by 2 orders of magnitude relative to normal media.

Zinc is an ubiquitous and essential component in biological systems. Iron is the only other transition metal that is more abundant in humans; however, if one subtracts the amount of iron in hemoglobin, zinc becomes the most abundant transition metal (1). Zinc has been identified as a central component of over 300 enzymes and plays an essential structural function in an entire class of transcription factors. The biological essentiality of zinc implies the existence of homeostatic mechanisms that regulate its absorption, distribution, cellular uptake, and excretion (2). However, until recently little was known about how these processes occur within the cell or about the molecules that mediate their action (3).

Metallothionein (MT)¹ is an abundant zinc-binding protein and one of the few eukaryotic proteins identified as having an essential role in heavy metal detoxification. Copious levels of MT protein and mRNA are found in organisms and tissues exposed to high levels of zinc or cadmium (4). Transcriptional activation of the MT genes in response to high concentrations of these potentially toxic metals is mediated through trans-acting proteins that bind to DNA regulatory elements located upstream of the MT gene coding sequences (5–8). These metal-responsive elements (MREs) are necessary and sufficient to confer zinc- and cadmium-responsive activation of MT genes (9). Each MT protein molecule may bind up to seven atoms of either zinc or cadmium, where each metal is tetrahedrally coordinated to cysteine residues. Because newly synthesized MT proteins sequester the inducing metals, elevated tissue concentrations of the ions are often detected upon metal exposure (10–12). Conversely, MT protein and mRNA levels generally decrease in a dose-dependent manner in tissues of animals fed zinc-restrictive diets (13–15). Additional studies showed that moderate maternal zinc deficiency in rats during pregnancy and lactation results in the reduced expression of MT in the livers of their pups (12, 16–18). Such results have led to suggestions that the MT protein may serve to buffer intracellular zinc levels (12).

We have described the methods used to generate a cell line derived from mouse fibroblasts (L-M(TK⁻)), herein designated as LTK⁻, which survives conditions of extreme zinc deprivation (19). Previous attempts at establishing similar zinc-deficient cell lines by other laboratories were unsuccessful in producing a system that would survive beyond a few passages of growth in medium containing less than 0.1 μM zinc (20–24). This cell line, designated as low zinc-adapted (LZA-LTK⁻) survives continuous passage in media that contain less than 60 nm zinc and thus provides a useful system to study fundamental components of the cellular machinery responsible for zinc homeostasis. Furthermore, despite continuous culture in these conditions of extreme zinc deprivation, these cells remarkably maintained intracellular zinc at levels comparable with the parental LTK⁻ cell line grown in normal media containing 1–5 μM zinc (19).

In this paper, we present evidence that MT plays a role in the maintenance of intracellular zinc at the standard 0.4 fmol zinc/cell level in the LZA-LTK⁻ cells, thus identifying a zinc-scavenging role for the protein. Unlike the studies in which animals were fed zinc-restrictive diets (12, 16–18), MT mRNA is not degraded in the LZA-LTK⁻ cell line; instead, it is constitutively present at high levels. Our results suggest that the overexpression of MT is one step that enhances the survival of the LZA-LTK⁻ cells in conditions of extreme zinc deprivation.

EXPERIMENTAL PROCEDURES

Cell Culture—BALB/c 3T3 and LTK⁻ cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum, 5 mM glutamine, and 30,000 units/liter of penicillin/streptomycin (all manufactured by Life Technologies, Inc.). Batch treatments of Chelex (Bio-Rad) were used to strip the media and other solutions of divalent...
cations. The Chelex resin was prepared according to the manufacturer's instructions with neutralization of the resin to physiological pH and multiple washings with 0.25 M HEPES, pH 7.4, before its addition to media (at 40 g/liter) and other solutions (at 20 g/liter) with constant stirring at 4 °C for 20–24 h. Aliquots of the solutions were removed prior to or after Chelex treatment, and the concentrations of divalent cations in the samples were determined using an inductively coupled plasma-atomic emission spectrophotometer (ICP-AES) on an Atomscan 25 (Thermo Jarrell Ash) with Thermospec software (version 5.02). Divalent cations other than zinc were restored to their normal concentrations by the addition of appropriate metal chloride salts. Sterilization of the growth media and following Chelex treatment was accomplished by filtering through a 0.2 μm Nyl na Plus filter unit (Nalgene).

Measurement of Intracellular Zinc—LZA-LTK− cells were passaged into zinc-deficient medium supplemented with various concentrations of zinc chloride from a 5 mM stock solution. Following growth, the media were decanted and saved for zinc analysis by ICP-AES. The cells were washed three times with metal-stripped 1× phosphate-buffered saline (PBS) (136 mM NaCl, 2.6 mM KCl, 10.14 mM Na2HPO4, 1.76 mM KH2PO4, pH 7.4) to remove the contaminating traces of zinc. The cells were scraped into metal-stripped 1× PBS and were pelleted at 14,000 × g for 1 min. The cell pellet was dried overnight in a 65 °C oven, digested with 150 μl of concentrated nitric acid at 80 °C for 15 min, and diluted to 1.5 ml with distilled H2O prior to analysis on the ICP-AES.

RNA and DNA Filter Hybridization—RNA was isolated from cell cultures lysed with guanidinium thiocyanate by sedimentation through a cesium chloride cushion (25). Equivalent amounts of RNA were fractionated on formaldehyde-agarose gels (26), transferred to nitrocellulose, and hybridized with radiolabeled cDNA probes for mouse MT-1 (a gift from Dr. Richard Palmiter) or GAPDH (a gift from Dr. Richard Morimoto). Genomic DNA was purified from cells lysed with SDS/proteinase K solution by phenol/chloroform extraction (27). DNA digested with EcoRI was fractionated by agarose gel electrophoresis, transferred to nitrocellulose, and probed with radiolabeled mouse MT-1 or GAPDH cDNA. The nitrocellulose filters were washed and exposed to x-ray film at −70 °C with an intensifying screen.

Results

Subcellular Fractionation—LZA-LTK− cells that had been grown in various [Zn] media were washed with metal-stripped 1× PBS. Cells were scraped into metal-stripped 1× PBS, pelleted by centrifugation, and resuspended in 0.25× sucrose buffered with 5 mM MOPS, pH 7.4. Whole cell zinc levels were measured by ICP-AES using an aliquot of the resuspended pellet that had been digested with nitric acid. Other cell aliquots were passed three times through a stainless steel syringe filter holder that was lined with two polycarbonate 16.0-μm filters (Osmonics). β-Hexosaminidase activity assays, which test for the integrity or latency of the organelles, were conducted on aliquots of each homogenate (30). Differential velocity sedimentation resulted in four fractions: 1) P1 (pellet from homogenate spun at 1,000 × g, 10 min), which typically contains nuclei and unbroken cells; 2) P2 (pellet from the P1 supernatant spun at 10,000 × g, 10 min) containing large intracellular membranes; 3) P3 (pellet from the P2 supernatant spun at 100,000 × g, 60 min), which typically contains smaller intracellular membranes and vesicles; and 4) the soluble fraction or cytosol (the supernatant from the 100,000 × g, 60-min spin) (31). As with the total intracellular zinc measurements, the pellets from each of the fractions were dried overnight, dissolved in concentrated nitric acid, diluted in distilled H2O, and analyzed for zinc content by ICP-AES.

Gel Filtration Chromatography—Cytosols from subcellular fractionation experiments were chromatographed on a G-75 Sephadex superfine size exclusion column (Amersham Pharmacia Biotech) using 20 mM Tris, pH 7.8, as the running buffer. In the cadmium saturation analyses, a portion of the cytosol or purified horse MT (Sigma) was incubated with a 5-fold molar excess of cadmium chloride, relative to zinc concentration of the sample, in the presence of 5 mM β-mercaptoethanol at 37 °C for 1 h immediately prior to sample application to the column (32, 33). Zinc and/or cadmium content of the resultant fractions was measured via ICP-AES.

DNA Transfection and Reporter Construct Assays—RSV-CAT and MRE-βGeo (a gift of Richard Palmiter) plasmids were transiently transfected into cells via the DEAE-dextran method (34). After 3 days of growth, cells were harvested and split into samples for the reporter construct assays. The Hoechst 33258 staining assay was used to measure the amount of DNA per plate of cells (7). β-Galactosidase activity from the MRE-Geo was determined as the per cent cleavage of an O-nitrophenyl β-galactopyranoside substrate and expressed as A405, per μg of DNA from the harvested cells. CAT activity from the RSV-CAT construct was measured as the percentage of acetylation of a radiolabeled chloromphenicol substrate (35).

Results

LZA-LTK− Cells Maintain Basal Zinc Levels When Grown in Zinc-deficient Conditions and Are Efficient Scavengers of the Metal—The ability of the LTK− and LZA-LTK− cell lines to accumulate Zn(II) was examined in response to the addition of a wide range of zinc concentrations in external media ([Zn] media). LTK− cells and LZA-LTK− cells were seeded into unmanipulated media and zinc-stripped media (19), respectively, supplemented with various concentrations of ZnCl2. Following growth to near confluency, samples of the media were collected, and the cells were washed several times with PBS, which had been treated with Chelex to remove residual traces of zinc. Cells from each plate were harvested, counted, and then lysed in concentrated nitric acid. The amount of zinc per cell was determined at each [Zn] media by ICP-AES. Basal levels of intracellular zinc in the parental LTK− cells were measured to be 0.44 ± 0.09 fmol zinc/cell in n = 4 experiments (Fig. 1A). There was little increase in intracellular zinc levels in LTK− cells until [Zn] media exceeded 20 μM. A 3.5-fold increase of

![Fig. 1. Measurements of intracellular zinc from LZA-LTK− and LTK− cells grown in various [Zn] media. A, after 3 days of growth, LZA-LTK− (open circles) and LTK− cells (filled circles) were harvested, counted, and measured for intracellular metal content by ICP-AES. B, the zinc remaining in the spent media from the LZA-LTK− (open circles) and LTK− cells (filled circles) was determined by ICP-AES. The straight dashed line, with a slope of 1, represents the amount of zinc that would be present in the media if growth of the cells did not perturb the zinc concentrations.](image-url)
intracellular zinc concentration was observed in the presence of the maximum amount of zinc added into the medium (50 μM [Zn]media). Consistent with previous observations (19), LZA-LTK² cells grown in unsupplemented zinc-deficient medium maintained basal intracellular zinc levels (0.54 ± 0.13 fmol zinc/cell n = 4 experiments) similar to those measured in the LTK² cells (Fig. 1A). As [Zn]media was increased, however, the intracellular zinc levels within the LZA-LTK² cells increased substantially, reaching a maximal level of 8.68 ± 0.86 fmol zinc/cell when [Zn]media was 3.5 μM (a 16-fold increase). Additional zinc supplemented into the media, up to 50 μM, resulted in no further increase in intracellular zinc levels.

The concentration of zinc remaining in the spent medium following growth was also measured by ICP-AES (Fig. 1B). The dashed line represents the concentration of zinc expected in spent media if the growth of the cells did not significantly perturb [Zn]media; this was the case with the LTK² cell line. Intriguingly, no measurable zinc was observed in the spent media from LZA-LTK² cells grown with [Zn]media, 3.5 μM. Furthermore, there was a striking relationship between the amount of intracellular zinc in LZA-LTK² cells and the concentration of extracellular zinc remaining in spent media (Fig. 1A and B). Although measurable amounts of metal were added, zinc was undetectable in spent media until the intracellular zinc levels in the LZA-LTK² cells reached the “plateau” level of approximately 8.6 fmol zinc/cell. One interpretation of these data is that zinc-scavenging mechanisms are present in the LZA-LTK² cells that enhance uptake and intracellular sequestration of zinc from the extracellular medium and that this mechanism fills the zinc storage molecules with all available environmental zinc until these sites are saturated.

High Constitutive Levels of MT mRNA Are Present in LZA-LTK² Cells—Intracellular metal concentrations of zinc or cadmium are often directly proportional to the amount of MT protein present within cells (36–38). In the LTK² cells, the rate of increase in intracellular zinc levels as a function of [Zn]media is similar to previously published patterns for metal-induced MT gene transcription (39). However, the LZA-LTK² cells exhibit a strikingly different intracellular zinc profile in response to increased extracellular zinc. MT mRNA levels were therefore examined in both cell lines to determine what role, if any, MT plays in assisting the cell to scavenge zinc from the media. As expected from previous studies, MT mRNA levels rise in LTK² cells in response to elevated [Zn]media (>20 μM) in a dose-dependent manner that is presumably mediated through transcriptional activation of the MREs (Fig. 2A). A 5-fold induction of MT mRNA was detected between LTK² cells grown in the lowest and highest [Zn]media (Fig. 2B). In contrast, high, constitutive levels of MT mRNA were present in LZA-LTK² cells regardless of the external [Zn]media (Fig. 2A). There were no significant [Zn]media-dependent changes in the high levels of MT mRNA in LZA-LTK² cells (Fig. 2B).

MT Protein Levels Do Not Correspond to MT mRNA Levels in LZA-LTK² Cells—To determine if the constitutively elevated MT mRNA levels in the LZA-LTK² cells result in correspondingly high steady-state MT protein levels, metabolic labeling
studies were conducted. The high cysteine content of MT and relatively low molecular weight (≈10 kDa) make the protein readily detectable in \[^{35}\text{S}\]cysteine labeling experiments. LTK\(^2\) and LZA-LTK\(^2\) cells were grown in \[^{65}\text{Zn}\] media similar to those used in the RNA and intracellular zinc measurements, and \[^{35}\text{S}\]cysteine was added 24 h prior to harvesting to achieve steady-state labeling. Extracts were carboxymethylated to prevent oxidation of MT sulfhydryl groups (28), and equivalent amounts of protein were loaded and resolved on Tricine-based SDS-PAGE gels (29). In the parental LTK\(^2\) cell line (Fig. 3A), increases in \[^{65}\text{Zn}\] media lead to increases in MT as well as increases in intracellular zinc and MT RNA levels (Figs. 1A and 2B, respectively). Each of these parameters remained low until \[^{65}\text{Zn}\] media was 20 \(\mu\text{M}\) or greater. The increase in MT protein levels occurred at lower \[^{65}\text{Zn}\] media in the LZA-LTK\(^2\) cell line and paralleled the rise in intracellular zinc in LZA-LTK\(^2\) cells at each \[^{65}\text{Zn}\] media (Fig. 1B). Grown at a wide range of \[^{65}\text{Zn}\] media, the amount of MT protein in cells grown in the lower zinc concentrations was relatively low. It is possible that differential translation rates of the MT mRNA might contribute to the differences in MT protein abundance in the LZA-LTK\(^2\) cell line at the different \[^{65}\text{Zn}\] media conditions. However, it is more likely that differences in the rates of degradation of the apoprotein and the metal-bound protein account for the observed steady-state protein levels. Specifically, Klaassen et al. (40) have shown in proteolysis experiments performed in lysosomal extracts that apo-MT, MT complexed with Cd(II), or MT complexed with Zn(II) had rates of degradation of 50, 200, 35, and 20 pmol/mg of lysosomal protein/min, respectively. Thus, in the absence of enough saturating metal to stabilize the protein, it is expected that apo-MT will be rapidly proteolyzed in the LZA-LTK\(^2\) cells.

To test whether restriction of zinc availability leads to rapid proteolytic turnover of MT protein, LZA-LTK\(^2\) cells grown in 20 \(\mu\text{M}\) \[^{65}\text{Zn}\] media and labeled for 24 h with \[^{35}\text{S}\]cysteine were exposed to the intracellular metal-ion chelator TPEN or the extracellular agents EDTA and EGTA for various lengths of time. Dose-dependent and time-dependent degradation of MT protein occurred in cells treated with the intracellular chelator TPEN (Fig. 4). The \(t_{1/2}\) of MT in cells treated with TPEN was approximately 90 min, while the \(t_{1/2}\) for MT in the untreated cells was greater than the 4-h experiment. Other labeled proteins were not affected by TPEN, suggesting that MT degradation is due to metal chelation and not general degradation of all proteins. EDTA and EGTA, unable to traverse the plasma membrane because of negatively charged moieties, had no effect on the labeled MT although both have high affinities for zinc.

The Majority of Zinc Accumulated by LZA-LTK\(^2\) Cells Is Associated with MT—To determine if MT protein was directly involved in the increased zinc, the distribution of intracellular zinc accumulation in the LZA-LTK\(^2\) cell line at various \[^{65}\text{Zn}\] media concentrations was examined. Cells grown in various \[^{65}\text{Zn}\] media were subjected to subcellular fractionation following

![Fig. 3. MT protein levels from LTK\(^{-}\) and LZA-LTK\(^{-}\) cells grown at various \[^{65}\text{Zn}\] media. Following steady-state labeling with 10 \(\mu\text{Ci}\) of \[^{35}\text{S}\]cysteine for 24 h, LTK\(^{-}\) (A, filled circles) and LZA-LTK\(^{-}\) cells (B, open circles) were harvested, and extracts were prepared. Following carboxymethylation, equivalent amounts of protein per lane were separated on Tricine-based polyacrylamide gels. The gels were treated with Enhance prior to autoradiography. The data from quantification of the gels by scanning densitometry are shown below each autoradiogram.](image-url)
Fig. 4. Effect of chelators of extracellular and intracellular metals on MT levels in LZA-LTK cells grown in 20 μM [Zn]media. Following steady-state labeling with 10 μCi of [35S]cysteine, cells were treated with chelators for the indicated times, the cells were harvested, and extracts were prepared. Following carboxymethylation, equivalent amounts of protein per lane were separated on a Tricine-based polyacrylamide gel. The gel was treated with Enhance prior to autoradiography.

disruption of the plasma membrane by extrusion through polycarbonate membranes with pore sizes slightly smaller than the diameters of the cells. Latency, a measurement of the structural integrity of cellular organelles, was examined by β-hexosaminidase enzyme activity measurements on aliquots from the postnuclear supernatants (30). Only samples with greater than 90% latency were used in subsequent procedures to ensure that the distribution of zinc among the fractions was not altered by contamination from disrupted organelles. Additionally, a wide variety of buffers were utilized to ensure that zinc was not being chelated from the organelle fractions (data not shown). Subcellular fractionation by differential velocity centrifugation was used to partition the homogenate into four fractions (31). ICP-AES analyses (Fig. 5A and Table I) demonstrated that the majority of the zinc taken up by the cells at increasing [Zn]media is found in the cytosol. Additionally, there were small, but measurable, increases in the other membranous compartments (Table I).

The cytosolic fraction was further resolved by gel filtration chromatography. The majority of zinc in the cytosolic fraction was associated with a single peak that increased in parallel with [Zn]media (Fig. 5B) and eluted with an apparent molecular mass of 10,000 daltons. This peak coincides with the elution profile of purified cadmium-saturated MT (data not shown). The problems associated with the detection of MT by Western blot analysis have been well documented, and specific modifications have been suggested to improve the performance of these assays (41). Even employing these changes, we were still unable to detect MT by Western analysis in fractions obtained by gel filtration or from commercially available purified MT (data not shown). However, competitive displacement of MT-bound zinc by Cd(II), which relies upon a rapid exchange of Cd(II) into the protein, has frequently been used to quantify MT (42). Aliquots of the cytosols were incubated with excess cadmium in the presence of thiols followed by gel filtration chromatography. Zinc was completely removed from each of the 10,000 molecular weight fractions and replaced with cadmium (Fig. 5C). The zinc displaced from MT (data not shown), along with excess cadmium, could be detected in the flow-through fractions (peaks 28–33). These data indicate that the principle zinc-binding species in the 10,000 fraction is metallothionein.

MT mRNA Levels Do Not Rapidly Increase in LTK Cells—MT mRNA levels remain relatively constant in the initial hours of exposure to zinc deficiency, then the high constitutive levels of MT mRNA probably represent a long term adaptation of the LZA-LTK cell line and suggest that other components of zinc homeostasis machinery are altered to respond to the zinc-deficient conditions. Northern blot analysis was performed on mRNA from LTK cells isolated after transfer of the cells into zinc-deficient medium for several days. Individual plates were harvested on each of the first 3 days. On the third day, an additional plate of the LTK cells was passaged into several more plates containing zinc-deficient medium for time points extending to 124 h. No significant increase in MT mRNA occurred (Fig. 6), indicating that increased MT expression is not a simple adaptive response of LTK cells to low zinc conditions and is likely to have arisen from a genetic event.

MT MRE Elements Are Not Constitutively Activated in LZA-LTK Cells—Another mechanism that can lead to increased levels of specific MT mRNA is the regulation of the strong, constitutively active Rous sarcoma virus long terminal repeat (44). In the parental LTK cell line, zinc-dependent activation of the MRE-βGeo construct (MRE-βGeo) (7) was transiently transfected into the LTK and LZA-LTK cell lines, and β-galactosidase activity was examined as a function of increasing [Zn]media. Differences in transfection efficiencies between the two cell lines were controlled by cotransfection with a RSV-CAT construct, which is under the regulation of the strong, constitutively active Rous sarcoma virus long terminal repeat (44). In the parental LTK cell line, zinc-dependent activation of the MRE-βGeo construct (Fig. 7A) paralleled the Northern blot results in Fig. 2, indicating that the increase of metallothionein message in this case is probably mediated through the metal-responsive elements. In the LZA-LTK cell line, however, there was no indication of β-galactosidase activity for any of the [Zn]media conditions (Fig. 7A). While CAT activity assays demonstrated that the LZA-LTK cells had lowered transfection efficiencies relative to the LTK cells (data not shown), it was not sufficiently decreased to account for the lack of β-galactosidase activity. At 50 μM [Zn]media, LTK cells possessed 100-fold more β-galactosidase activity than the LZA-LTK cells grown at similar [Zn]media. The lack of responsive transfected MRE elements led us to examine other mechanisms for elevation of MT mRNA.

MT Gene Copy Number Is Amplified in LZA-LTK Cells—Another mechanism that can lead to increased levels of specific
mRNAs, selective gene amplification, was also examined. Cell lines, including LTK\(^{2}\) cells, have been shown to amplify the copy number of the MT locus in response to continuous exposure to high concentrations of cadmium or copper (27, 45–48). Increased amounts of MT protein produced from the amplified genes provide a selective advantage to the cell by binding and sequestering toxic metals. To determine if the copy number of the MT-I gene had been increased in the LZA-LTK\(^{2}\) cell line relative to the parental LTK\(^{2}\) cells, Southern blot analysis was performed. Fig. 8 shows an autoradiograph of EcoRI-digested total DNA isolated from LZA-LTK\(^{2}\) and LTK\(^{2}\) cell lines and probed with radiolabeled MT-I cDNA. The radiolabeled MT-I cDNA hybridized to a band of approximately 4 kilobase pairs, a size consistent with other studies of the murine MT-I gene locus isolated by EcoRI digestion (27). Quantitative PhosphoImager (Molecular Dynamics, Inc.) analysis showed that the LZA-LTK\(^{2}\) cell line had 7–10 times the number of the MT-I gene copies relative to the parental LTK\(^{2}\) counterpart, clearly demonstrating MT-I gene amplification in the former. Analysis of the copy number of the GAPDH gene indicated an equivalent number of that gene in both cell lines (data not shown).

The clear cut amplification of the MT locus has important
Implications for Metal-responsive Regulation. If genomic amplification of the MT locus in the LZA-LTK<sup>2</sup> cell line leads to multiple endogenous MREs, these could sequester a limiting MRE-binding factor and thus prevent zinc-responsive expression from the transfected reporter construct (Fig. 7A). Alternatively, the overexpression of the metallothionein protein in the LZA-LTK<sup>2</sup> cell line may abrogate activation of the metal-responsive element regulatory system. Since the high constitutive levels of MT would immediately bind labile metal as it entered the cell, the availability of the cellular Zn(II) would be limited and perhaps prevented from reaching levels necessary to activate MRE-binding factors such as MTF-1. Experiments were conducted in which increasing amounts of the MRE-βGeo plasmid were transfected into the parental LTK<sup>2</sup> and LZA-LTK<sup>2</sup> cells grown in 50 μM [Zn]<sub>medium</sub>. Increased β-galactosidase activity was observed as the amount of plasmid transfected into the LTK<sup>2</sup> cell line was increased (Fig. 7B), indicating that increased levels of MRE from the transfected constructs can recruit the requisite zinc-responsive activating factors. Conversely, there was a lack of increased β-galactosidase activity in LZA-LTK<sup>2</sup> cells grown in 50 μM [Zn]<sub>medium</sub> as the amount of transfected construct was increased. Although it is possible that the endogenous, amplified MREs within the LZA-LTK<sup>2</sup> cell line monopolize MRE-binding activation factors such as MTF-1 and thus deprive the transfected constructs, we consider it more likely that the overexpressed MT protein suppresses labile intracellular zinc to levels below the threshold needed to activate MTF-1. Supporting evidence for this conclusion comes from previous studies, which demonstrated that cell lines in which the MT locus had been amplified 10–12-fold still retained the ability to induce metallothionein by heavy metals at the transcriptional level (49, 50). Furthermore, transgenic mice that carry 56 additional copies of the mouse MT-I locus also retain the ability to induce MT protein levels in response to high levels of zinc and cadmium (51, 52). The simplest interpretation is that the high constitutive levels of MT mRNA in the LZA-LTK<sup>2</sup> cells are not regulated at the level of MRE/MTF-1-dependent transcription.

**LTK<sup>−</sup> Status of the LZA-LTK<sup>−</sup> Cells Does Not Contribute to Their Survivability in Zinc-deficient Conditions—Experiments**

![Fig. 6. MT mRNA levels of LTK<sup>−</sup> cells after their transfer into zinc-deficient media.](image)

Each lane contains 10 μg of total RNA harvested at the indicated times, which was separated on formaldehyde-agarose gels and then transferred to nitrocellulose. The blots were probed with radiolabeled cDNA of mouse MT-I or GAPDH, the latter used as a control for the amount of total RNA loaded per lane.

![Fig. 7. Induction of the MRE-βGeo reporter construct in LZA-LTK<sup>−</sup> and LTK<sup>−</sup> cells in response to elevated [Zn]<sub>medium</sub>.](image)

A. β-galactosidase activity (normalized per μg of DNA from harvested cells) from LTK<sup>−</sup> (filled circles) and LZA-LTK<sup>−</sup> (open circles) cells, which were grown for 24 h in increasing [Zn]<sub>medium</sub> and then co-transfected with 10 μg each of MRE-βGeo and RSV-CAT plasmids. B. β-galactosidase activity of LTK<sup>−</sup> (filled circles) and LZA-LTK<sup>−</sup> (open circles) cells grown for 24 h at 50 μM [Zn]<sub>medium</sub> and then co-transfected with 10 μg of RSV-CAT and increasing amounts of MRE-βGeo.

These results indicate the thymidine kinase status of the LTK<sup>−</sup> cells does not affect their ability to proliferate in zinc-deficient media. These findings also demon-
Involvement of Metallothionein in a Zinc-scavenging System

First detected in the parental LTK\(^{-}\) cells. A 2.5-fold increase in intracellular zinc was not observed in the parental LTK\(^{-}\) cell line until \([\text{Zn}]_{\text{media}}\) reached 40 \(\mu\text{M}\), or 100 times the level of \([\text{Zn}]_{\text{media}}\) required for a similar increase of intracellular zinc in LZA-LTK\(^{-}\) cells. Third, the amounts of MT protein and intracellular zinc correlate well with the observed depletion of extracellular zinc in the spent growth medium in the LZA-LTK\(^{-}\) cell line. MT protein levels and intracellular zinc levels rose in parallel with increasing \([\text{Zn}]_{\text{media}}\). No measurable zinc remained in the spent growth medium until intracellular MT levels reached a plateau at 3.5 \(\mu\text{M}\) \([\text{Zn}]_{\text{media}}\). Thus, in these zinc-limiting conditions, MT appears to act as a labile sponge or a buffering component that facilitates cellular retention of the available portion of zinc that exceeds the basal cellular requirements of \(-0.4\) fmol zinc/cell. Once this pool of MT protein is saturated with the metal, then external zinc is readily measured in the spent growth medium.

Recent genetic studies have provided complementary evidence that MT is able to exert a cytoprotective role in organisms exposed to zinc deficiency. Transgenic mouse embryos that express multiple copies of the MT gene demonstrated a greater resistance to developmental defects caused by zinc deficiency than did wild-type embryos (55). Furthermore, mouse strains in which the MT-I and MT-II alleles were disrupted demonstrated a much greater sensitivity to the phenotypic effects of zinc deficiency, such as malformed kidney structures, than their wild-type counterparts (56). These authors suggested that MT-bound zinc may serve as a reservoir that can be accessed and utilized at times of zinc deficiency. However, we propose that MT also plays a role in scavenging zinc from extracellular sources when intracellular supplies of this metal are low. Indeed, MT may bind zinc entering the cell and subsequently chaperone the metal to specific storage sites or proteins in a manner that is analogous to the protein metallochaperone, Atx1, which delivers copper to specific intracellular vesicle proteins (57). It is clear that zinc-laden MT can readily donate its metal cargo to restore the function of inactive apoenzymes in vitro, including alcohol dehydrogenase, aldolase, thermolysin, alkaline phosphatase, carbonic anhydrase, TFIIA, and Sp1 (22, 58–60). To date, however, there is no evidence for direct MT interaction with specific partners. This leaves open the question of a metallochaperone function for MT.

**DISCUSSION**

Upon encountering conditions of extreme zinc deficiency, mouse fibroblasts undergo a series of changes that ultimately allow them to survive deprivation of this essential nutrient. One dramatic difference observed between the cells that have been subjected to prolonged zinc deficiency (LZA-LTK\(^{-}\) and the parental LTK\(^{-}\) cells is a large increase in basal MT mRNA levels. The opposite was anticipated, because MT gene transcription is normally induced by high, not low, concentrations of heavy metals and because the only known role attributed to MT is in metal ion detoxification. Furthermore, high levels of the cysteine-rich MT protein would be expected to compete with cellular zinc-binding molecules for the limited available supply of the metal. Clearly, MT is not simply sequestering or irreversibly tying up zinc.

Three key biochemical observations establish that MT protein participates in a zinc-scavenging system in the LZA-LTK\(^{-}\) cell line. First, LZA-LTK\(^{-}\) cells grown at the highest \([\text{Zn}]_{\text{media}}\) reached 20 \(\mu\text{M}\) of zinc/cell versus 1.4 fmol of zinc/cell in the parental LTK\(^{-}\) counterparts (8.6 fmol of zinc/cell). Fractionation experiments led us to conclude that >90% of the zinc is associated with MT protein. Second, intracellular zinc levels increase in the LZA-LTK\(^{-}\) cells in response to the addition of very small amounts of zinc into the zinc-deficient medium. For example, a 2.5-fold increase in intracellular zinc was measured in the LZA-LTK\(^{-}\) cell line upon the addition of 400 nm zinc into the growth medium. Conversely, intracellular zinc does not change in the parental LTK\(^{-}\) cell line until \([\text{Zn}]_{\text{media}}\) exceeds 20 \(\mu\text{M}\). This threshold value of 20 \(\mu\text{M}\) \([\text{Zn}]_{\text{media}}\) corresponds to the concentration of zinc at which increases in MT mRNA and protein levels are

![FIG. 8. Copy number of the MT-I gene in genomic DNA isolated from LZA-LTK\(^{-}\) and LTK\(^{-}\) cells. The DNA was digested with EcoRI and separated on a 0.75% agarose gel in the quantities listed. Following transfer to nitrocellulose, the blot was hybridized with \(32^P\)-radiolabeled MT-I or GAPDH cDNA. Quantitative analysis of the blot was conducted on a PhosphorImager. Kb, kilobase pairs.]](image)

**FIG. 9. Analysis of intracellular zinc levels of LTK\(^{-}\) and L cells after their transfer into zinc-deficient media.** LTK\(^{-}\) (filled circles) and L (open triangles) cell cultures were established from frozen ATCC stocks in unmanipulated 10% calf serum/Dulbecco’s modified Eagle’s medium prior to exposure to the zinc-deficient media. During the course of the experiment, the cultures were passaged every fourth day. Periodically, the cells were harvested, counted, and measured for intracellular metal content by ICP-AES. S.D. was calculated from \(n = 3\) plates at each time point.
Amplification of the MT gene locus in the LZA-LTK\(^{-}\) cell line explains, at least in part, the high levels of MT mRNA. Cell lines or organisms that have increased copy number of specific genes can be selected by environmental stress (61). Numerous studies have demonstrated that amplification of the MT gene locus in mammalian and yeast cells can be selected by continuous exposure to high concentrations of cadmium, copper, or metal-based drugs such as cisplatin (27, 45, 47, 62). In these cases, cells containing the amplified genes produce elevated levels of MT protein compared with those that contain only a single copy of the gene. Under conditions of exposure to high levels of heavy metals, abundant MT provides a cytoprotective role by sequestering the potentially harmful ions from the rest of the cell. We propose that elevated levels of MT can also provide a zinc-scavenging mechanism and thus select for amplification of the MT gene in cells exposed to zinc-deficient conditions. Yet, mechanisms in addition to gene amplification cannot be ruled out to explain the high levels of MT mRNA in response to the zinc-deficient conditions. For instance, it has been recently demonstrated that interleukin-1\(\alpha\)-induced MT-I gene expression is markedly enhanced in rats fed zinc-restrictive diets (15). Additional processes may also participate in elevation of MT mRNA levels in the zinc-deprived cell lines.

A Model for the Zinc-scavenging Role of MT in Conditions of Extreme Zinc Deprivation—Regardless of the mechanism by which the LZA-LTK\(^{-}\) cells accumulate high levels of MT mRNA, an increase in the steady-state concentration of apo-MT is expected. The apoprotein is predicted to be rapidly proteolyzed \textit{in vivo} when [Zn]\textsubscript{median} levels are insufficient to saturate the MT protein pool. Yet even at low [Zn]\textsubscript{median}, small “steady-state” levels of apo-MT may persist and bind zinc as it is transported from the environment, trapping the ion within the cell. Since MT is being continuously produced from the large, constitutive MT mRNA pool in the LZA-LTK\(^{-}\) cells, enough molecules of apo-MT are present at any given time in the cell to retain additional zinc as it becomes available (Fig. 10). Although the assiduous production and degradation of MT may seem like a futile and energetically unfavorable cycle, it apparently provides a mechanism by which the LZA-LTK\(^{-}\) cells can efficiently scavenge zinc from the environment, thus permitting survival under conditions in which the availability of the ion is severely restricted.

This amplification of the MT gene locus is the result of a long-term process that requires many cell doublings. Increased MT levels may not be utilized by a cell to deal with short-term metal ion deficiencies. Indeed, no appreciable increase in MT mRNA levels was noted in the parental LTK\(^{-}\) cells after transfer into zinc-deficient media, even over a period of several days. Since BALB/c 3T3 cells were unable to tolerate even one passage in the zinc-deficient medium without entering quiescence (19), the LTK\(^{-}\) cell line may have characteristics in addition to the ability to amplify the MT gene locus that allow survival and that are not shared by all immortalized mouse cell lines. One difference between the BALB/c 3T3 and LTK\(^{-}\) cell lines is that the latter lacks functional thymidine kinase, an enzyme that has previously been reported to be extremely sensitive to zinc-deficient conditions (53, 54). However, both L and L-M(TK\(^{-}\)) cell lines had similar intracellular zinc profiles when exposed to zinc-deficient conditions, suggesting that the ability to utilize functional thymidine kinase does not affect the ability of these two cell lines to proliferate in zinc-deficient media. Consistent with the requirement of events in addition to MT amplification, the constitutive overexpression of either MT-I or MT-III was insufficient for long-term survival of baby hamster kidney cells, a line that also lacks thymidine kinase, under zinc-restrictive conditions (63). These results lead us to conclude that high levels of MT expression alone are not responsible for the robustness of the adapted L cells in response to zinc limitation.

Thus, in addition to increasing the steady-state concentration of apo-MT, it is likely that the LZA-LTK\(^{-}\) cell line upregulates other components of the zinc homeostasis machinery (Fig. 10) in response to the zinc-deficient conditions. For example, a family of homologous mammalian proteins, designated as ZnT-1, ZnT-2, ZnT-3, and ZnT-4, thought to encode zinc transport proteins, has recently been identified (24, 64–66). ZnT-1, the first member isolated, appears to be responsible for pumping excess zinc out (efflux) of the cell. ZnT-2 encodes a protein that sequesters zinc into intracellular storage compartments. However, none of these proteins appears to play an important role in shuttling zinc into the cell. Eide and co-workers (67, 68) have recently identified two genes in \textit{Saccharomyces cerevisiae}, designated as \textit{ZRT1} and \textit{ZRT2} (zinc-regulated transporters), which appear to encode zinc transporters of a high affinity and

![Fig. 10. Model of zinc ion homeostasis in the LZA-LTK\(^{-}\) cell line. Zinc ions are represented by the shaded circles, and apo-MT was drawn to resemble a cylinder on a revolver. The packed bundles represent transporter proteins that have been identified in mammalian or yeast cells (24, 64, 67, 68). Black boxes represent putative interactions of zinc-loaded MT that may donate its cargo to nascent, zinc-requiring polypeptides or to cellular compartments responsible for the storage of loosely complexed zinc. See text for the discussion of the interactions between the components.](image-url)
low affinity uptake system, respectively, the former of which is up-regulated under conditions of zinc deficiency.

Zinc is an essential structural component in a wide variety of proteins, and therefore the maintenance of appropriate intracellular zinc levels is critical for cell survival. Although the exact function of the metallothionein proteins remains elusive (69), one proposed role for the protein is to maintain cellular homeostasis by metal ion detoxification, because transcription of MT can be induced by high levels of heavy metals. We have shown that the amplification of the MT gene locus can occur in response to low concentrations of metals and implies that MT can act as a survival factor by assisting cells in sequestering limited supplies of available zinc from the environment. The derivation and characterization of this cell line should also be useful in identifying other components of the zinc homeostasis system.

Acknowledgments—We thank Richard Palmiter for the generous gift of the mouse MT-I cDNA and the MRE-ßGeo plasmid; Richard Morimoto for the GAPDH cDNA; and members of the O’Halloran group, in particular Rama Dwevedi, Sarwar Nasir, and Christoph Fahrni for helpful discussions. Additionally, we thank Kelly Mayo, Richard Gaber, Val Culotta, and Karla Kirkegaard for constructive discussions and critical reading of this manuscript.

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Involvement of Metallothionein in a Zinc-scavenging System

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