Metallothionein is crucial for safe intracellular copper storage and cell survival at normal and supra-physiological exposure levels

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MTs (metallothioneins) increase the resistance of cells to exposure to high Cu (copper) levels. Characterization of the MT–Cu complex suggests that MT has an important role in the cellular storage and/or delivery of Cu ions to cuproenzymes. In this work we investigate how these properties contribute to Cu homoeostasis by evaluating the uptake, accumulation and efflux of Cu in wild-type and MT I/II null rat fibroblast cell lines. We also assessed changes in the expression of Cu metabolism-related genes in response to Cu exposure. At sub-physiological Cu levels (0.4 μM), the metal content was not dependent on MT; however, when extracellular Cu was increased to physiological levels (10 μM), MTs were required for the cell's ability to accumulate the metal. The subcellular localization of the accumulated metal in the cytoplasm was MT-dependent. Following supra-physiological Cu exposure ($>$ 50 μM), MT null cells had a decreased capacity for Cu storage and an elevated sensitivity to a minor increment in intracellular metal levels, suggesting that intracellular Cu toxicity is due not to the metal content but to the interactions of the metal with cellular components. Moreover, MT null cells failed to show increased levels of mRNAs encoding MT I, SOD1 (superoxide dismutase 1) and Ccs1 (Cu chaperone for SOD) in response to Cu exposure. These results support a role for MT in the storage of Cu in a safe compartment and in sequestering an intracellular excess of Cu in response to supra-physiological Cu exposure. Gene expression analysis suggests the necessity of having MT as part of the signalling pathway that induces gene expression in response to Cu.

Key words: copper, efflux, iron, metallothionein, uptake, zinc.

INTRODUCTION

Cu (copper) is an essential micronutrient for life. It is required by a wide range of species, from bacteria to yeast, plants and mammals, including humans [1]. The essential requirement for Cu is based on its capacity to act as an intermediary in the transfer of electrons. The metal is found in the prosthetic groups of enzymes that participate in processes such as cellular respiration, iron transport and metabolism, and neurotransmitter synthesis. As a consequence of its redox properties, excess Cu is potentially toxic to membranes, DNA and proteins via Fenton reactions [2]. Dramatic examples of the essential requirement for and toxicity of the metal are given by two inherited human disorders, Menkes' disease and Wilson's disease [3]. To prevent the consequences of excess or deficit, living organisms have evolved cellular and molecular mechanisms that regulate the uptake, efflux, storage and utilization of the metal. The available evidence indicates that Cu handling is tightly regulated; thus, under normal conditions, it is virtually impossible to find free Cu ions inside the cell [4].

MTs (metallothioneins) are the major intracellular proteins that bind Cu and Zn (zinc) under physiological conditions [5]. They are small proteins (7 kDa) that are rich in cysteine residues (20–30 %) and lack aromatic amino acids and histidine [1]. MT genes coding for MT isoforms show a high degree of similarity, and the proteins have identical metal binding geometry, throughout the spectrum of evolution from a single-cell eukaryotic organism to humans [6]. In mammals, there are two major forms, MT I and MT II, which are expressed in most of the tissues and stages of development. It has been shown that MT binds several atoms of metal with a high thermodynamic stability, but also with high kinetic lability, suggesting that at least some of these atoms are transferable to a metalloprotein, thus regulating the activity of metalloenzymes [7–10].

Various lines of evidence have shown that the intracellular MT content correlates directly with resistance to Cu exposure in yeast, cell cultures and mice [11–15]. MT I/II knockout mice serve to demonstrate that MT expression is not essential for the development, growth or reproductive capacity of these animals, although they were more sensitive to cadmium exposure [15,16]. Nevertheless, these mutant mice and the cell lines derived from them have been used extensively in recent years to investigate the role of MT. Most results show that animals or cells lacking MT I/II are more sensitive to a wide range of stressors, such as oxidative stress, excess Cu and Zn, and infectious and inflammatory agents [17–21]. Moreover, when the function of Cu efflux transporters (ATP7A; Cu-transporting P-type ATPase) is altered, the presence of MT becomes essential for the survival of the cells [15], suggesting that these two proteins may be responsible for the regulation of intracellular Cu levels during adaptation to extracellular Cu excess.

Here we have investigated the mechanisms by which MT increases cellular resistance to Cu exposure by analysing different aspects of Cu metabolism, such as traffic, storage and gene expression. To this end, we exposed both wild-type and mutant fibroblasts that lack the expression of MT I/II to sub-, iso- and supra-physiological Cu concentrations [15]. Our results show that, under physiological and supra-physiological metal exposure, MT null cells have less intracellular Cu, particularly in the cytosol, and

Abbreviations used: AAS, atomic adsorption spectrophotometry; ATP7A, Cu-transporting P-type ATPase; Ccs1, Cu chaperone for superoxide dismutase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; MT, metallothionein; MTF, metal regulatory element binding transcription factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT-PCR, reverse transcriptase–PCR; SOD, superoxide dismutase.

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are more sensitive to it. These results suggest that, at physiological levels of Cu, this metal forms a safe molecular association with MT that permits its storage in the cytoplasmic compartment. When the extracellular metal concentration rises, the induction of MT expression provides more sites for the safe binding of Cu. Interestingly, we show that MT may also regulate the expression of genes involved in Cu metabolism. In the absence of this protein, cells fail to increase the expression of the mRNAs for MT itself, SOD1 (superoxide dismutase 1) and Ccs1 (Cu chaperone for SOD1) in response to Cu exposure.

MATERIALS AND METHODS

Cell culture and Cu treatment

The fibroblast cell lines used in the present study correspond to a wild type (MT+/−) and a MT I/II mutant (MT−/−). Kelly and Palmiter [15] created various cell lines from embryos generated by crossing a female MT−/+, Mo-brJ+/− mouse with a male MT−/− mouse. The Mo-brJ (Mottled Brindled) gene corresponds to the murine model of Menkes' disease. The MT−/− cell line used here is wild type for the Menkes' transporter, as determined by immunoblotting using an antibody against ATP7A (Cu-transporting P-type ATPase) (results not shown). The cell lines were characterized with regard to their shape, duplication time and capacity to adhere to the matrix, and no differences were found between them. The expression of MT in the fibroblasts was compared with that in a human hepatic cell line, HepG2, as a reference. All cell lines were grown in complete Dulbecco's modified Eagle's medium containing 10 % (v/v) fetal bovine serum and penicillin/streptomycin (Gibco BRL). Element concentrations in the culture medium were 0.44 μM Cu, 2.69 μM Fe and 3.80 μM Zn [22]. For all treatments, Cu was supplemented in the culture medium as a Cu–His complex (1:10 molar ratio) [23], and Zn was added as $ZnCl_2$, at the concentrations and the times specified in each Figure legend.

MT extraction and Western blot

A protein extract enriched in MT was prepared as described in [24]. Briefly, cells were homogenized by mechanical breakage in lysis buffer (150 mM NaCl, 3 mM $MgCl_2$, 0.5 % Nonidet P40 and 10 mM Tris/HCl, pH 7.0). After centrifugation (15 min at 4 °C and 12 000 ***g***), the supernatant was boiled (5 min) and centrifuged again. The resulting supernatant was freeze-dried and resuspended in deionized distilled water. The protein content was determined using Protein Assay reagent (Bio-Rad). The samples were carboxymethylated by adding dithiothreitol and freshly made 1 M iodoacetamide, and the reaction was stopped by the addition of sample loading buffer at the end of the incubation time. A portion of 10 μg of each sample was used for SDS/PAGE analysis with special modifications as described in [25]. MT was detected using a monoclonal antibody against MT I and II (Dako) and a second antibody conjugated to horseradish peroxidase; the antibody–antigen complex was detected using enhanced chemiluminescence (ECL®; Amersham).

Viability

Cells were grown in 24-well cell culture plates (Nunc) and exposed to 50, 100 or 250 μM Cu–His in the culture medium for 24, 48 and 72 h in triplicate. The relative survival of the cells treated with Cu was evaluated using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma] reduction assays as described in [26]. The percentage viability was determined by comparing the measurements of the average absorbance for a given treatment group with that obtained with a reference sample of the same cell line treated for an identical period of time with control medium (100 % viability).

Quantification of Cu and Zn

For quantification of the total metal content, cells were processed as described [27]. Briefly, cells [$(2–4) \times 10^5$] at 80 % confluence were disrupted in concentrated suprapure nitric acid (Merck) in a vortex and then diluted with distilled deionized water. Samples were digested at 60 °C overnight. The Cu concentration was determined by means of a graphite furnace atomic adsorption spectrophotometer (Perkin Elmer; SIMMA 6100). The content of Zn was determined by flame AAS (atomic adsorption spectrophotometry). Calibration was against a standard curve prepared using dilutions of a Cu or Zn standard (J. T. Baker), and the sample values were normalized to the total protein content.

Cu uptake and efflux

Radioactive Cu (^{64}Cu) was purchased from the Comision Chilena de Energia Nuclear (Santiago, Chile). The protocols for uptake experiments were performed as reported previously [28] with modifications [23]. For the uptake assay, cells at 80 % confluence on 24-well cell culture plates were incubated with Dulbecco's modified Eagle's medium containing ^{64}Cu–His (0.25–10 μM) for 15 min. Uptake was stopped by washing the cells with cold PBS containing an excess of Cu–His (500 μM). Cells were disrupted with 0.5 M NaOH/0.1 % Triton X-100, and an aliquot was kept for protein determination. The cellular content of ^{64}Cu was determined by measuring the radioactivity with a γ-counter. The concentration of newly incorporated Cu was interpolated from a standard curve of 1–100 pmol of ^{64}Cu. To assess the effects of a supra-physiological Cu concentration, fibroblasts were pre-exposed to 250 μM Cu–His for 24 h before uptake or efflux assays in some experiments. For the efflux experiments, fibroblasts were exposed to 1 μM ^{64}Cu–His for 1 h and washed immediately with cold PBS containing an excess of Cu–His. Cells were incubated in fresh medium and the cellular ^{64}Cu content at different times of chase (0–90 min) was determined as in the uptake assay.

Intracellular distribution of Cu

Fibroblasts were exposed to 5 μM ^{64}Cu–His for 3 or 24 h. After the treatment, the cells were washed in cold PBS and recovered by trypsinization. Pellets of 1×10^6 cells were resuspended in 1 ml of cold Hepes/sucrose buffer (10 mM Hepes, pH 7.3, and 250 mM sucrose) and disrupted by five strokes of a glass/glass homogenizer. To measure Cu accumulation in the cytosol and pellet fractions, the cellular lysates were centrifuged at 100 000 ***g*** for 60 min at 4 °C (Beckmann GS-15R) to yield the supernatant (cytosol) and a pellet fraction. The latter fraction was resuspended in 50 μl of 0.1 % Triton X-100 in Hepes/sucrose buffer. An aliquot of each fraction was used to determine the protein concentration using a BSA standard.

cDNA array and reverse Northern blot analysis to assess the expression of Cu-related genes

Using proprietary software named Redigen (M. F. Cisternas and M. Gonzalez, unpublished work; details available from M.G. on application), we defined a 200 bp region of the genes encoding Ccs, SOD1, ATP7A and MT that showed the lowest similarity

within this group of genes. Sequences were amplified from a wild-type mouse fibroblast cell line or a mouse liver cDNA sample using specific primers that spanned the low-identity regions.

Primers were synthesized by Invitrogen. The sequences were determined using the software Primer Premier 3: Ccs1, 5′-TCCCTTATCCAAGATCACAG-3′ and 5′-CGAGGACCAAATAACCTGA-3′; SOD1, 5′-GACTGCTGGAAAGGACG-3′, and 5′-AGTTTAATGGTTTGAGGGTA-3′; ATP7A, 5′-CAGGCTGTGGTATTAGCT-3′ and 5′-AGTCCGACCTCTTCTTTC-3′; MT I, 5′-CCGTGGGCTGCTCCAAAT-3′ and 5′-TAGGAAGACGCTGGGTTG-3′. In addition, the following primers were used to amplify amyloid β precursor: 5′-ATGCTGCCCGGTTTGGCAC-3′ and 5′-CTGTCCAACTTCAGAGGCT-3′. Finally, two housekeeping genes were amplified to be used as internal controls: G3PDH (glyceraldehyde-3-phosphate dehydrogenase) (5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCCTGTTGCTGTA-3′) and tubulin (5-CATTGCCACCATCAAGACCA-3′ and 5′-TCAACGCCAACCTCCTCATA-3′).

The amplification products were cloned into the pGEM-T vector (Promega) and sequenced. Inserts were amplified by PCR using the T7/SP6 forward/reverse primers. Products were precipitated and arrayed in 96-well plates at two concentrations (0.5 and 1 μg/μl). DNA (350 ng) was spotted on to a positively charged Nylon membrane (Millipore) in duplicate filters using a 96-pin multi-blot replicator (V&P Scientific). Each clone was spotted in quadruplicate, keeping five neighbouring spots free of cDNA to determine local background. The filters were washed twice with denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 7 min and with neutralizing solution (0.5 M Tris/HCl, pH 7.4, 1.5 M NaCl) for 3 min. DNA was permanently attached to the membrane by UV cross-linking. For reverse Northern blot analysis, membranes were pre-hybridized for 2 h at 42 °C in 10 ml of 2 × Prehybridization/hybridization Solution (Life Technologies) plus 0.2 × sodium phosphate buffer, pH 6.5, 40 % formamide and 1 % SDS, and hybridized overnight at 42 °C with agitation in the same buffer. The samples were hybridized in individual membranes, and after hybridization the filters were washed six times as follows: once with 2 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) for 10 min at room temperature, once with 2 × SSC/0.1 % SDS for 10 min at room temperature, twice with 1 × SSC/0.1 % SDS for 15 min at 65 °C, and twice with 0.1 × SSC for 15 min at room temperature. A phosphor scanner (Molecular Images FX; Bio-Rad) was used to recover the signal associated with each spot, and the intensity of the spots was determined with Kodak Digital Science 1D software. We performed duplicate hybridization experiments. The average intensity of the four spots for each gene minus the average of local background intensity was normalized using the values obtained for G3PDH. Only spots with a hybridization intensity two times above the local background measurements were included in the gene expression analysis.

RNA extraction and probe preparation

Total RNA was extracted from wild-type and MT−/− mutant cell lines maintained under basal cell culture conditions or exposed to 100 μM Cu–His using RNA_{WIZ} reagent (Ambion). A 2 μg aliquot of total RNA was used to synthesize double-stranded cDNA with MMLV (Moloney murine leukaemia virus) reverse transcriptase (Promega) and Second-Strand Enzyme Cocktail contained in the PCR-Select cDNA Subtraction kit (Clontech), following the manufacturer's recommendations. Double-stranded cDNA samples from Cu-treated and control cells were labelled with ^{33}P using the random primer labelling method (Invitrogen). Probes were cleaned using the Wizard DNA Clean-up System (Promega), and approx. 2×10^6 c.p.m. of the probe was mixed with 1 μg of salmon sperm single-stranded DNA, heat denaturated and added to the pre-hybridized membrane.

Semi-quantitative PCR

Aliquots of 1 μl of cDNA (0.2 μg/μl) were amplified with 1 unit of *Taq* DNA polymerase (Invitrogen) in the buffer provided by the manufacturer, and in the presence of the specific primers for MT (10–50 μM) and G3PDH (used as an internal control). Reactions were carried out in a PTC-100 thermocycler (MJ Research). The standard programme comprised 30 s at 94 °C, 30 s at 56 °C and 2 min at 68 °C for a number of cycles determined previously so that amplification was in the exponential range (26 cycles for *mt I* and 24 cycles for *sod1* and *ccs*). The PCR products were analysed in a 1.2 % (w/v) agarose gel, and further analysis of the MT product was carried out in an 8 % (w/v) denaturating acrylamide gel. Images of the ethidium bromide-stained agarose gels were acquired with a Kodak DC-290 camera, and quantification of the bands was performed using the program Kodak 1D, version 3.5. Band intensity was expressed as arbitrary units. The ratio between the sample cDNA to be determined and G3PDH cDNA was calculated to control for initial variations in sample concentration and for reaction efficiency. Means and S.D.s were calculated for all experiments after normalization to G3PDH.

Statistical analysis

In all experiments, variables were tested in triplicate samples, and most of the analyses of each sample were repeated at least twice. One-way ANOVA [29] was used to test differences between means, and a post-hoc *t* test was used for comparisons using STATA 6.0 (Stata Statistical Software).

RESULTS

MT mutant cells express a modified mRNA that is not translated to protein

First we characterized the two cell lines utilized. We determined that the cells had a similar shape, duplication time, attachment to the matrix and expression of ATP7A (see the Materials and methods section). Wild-type fibroblasts growing in culture medium supplemented with 100 μM Cu–His for 24 h responded to Cu exposure by increasing the abundance of MT protein, demonstrated by using a monoclonal antibody against MT I/II (Figure 1A). The same response was observed in HepG2, a hepatic cell line that represents a tissue that is able to store Cu. Densitometric quantification revealed 3- and 7-fold rises in MT relative abundance in wild-type fibroblasts and HepG2 cells respectively. In contrast, the MT mutant cells (Figure 1A) failed to reveal any MT immune-stained band in cells grown in media either with or without Cu supplementation. Since the mutation in the MT mutant cell line was achieved by introducing translation stop sequences in the MT I and II exons [15], we analysed the sizes of the transcripts. As expected, the MT I transcript was slightly larger in the mutant cells (Figure 1B), confirming the origin of this cell line.

MT mutant cells are more sensitive than wild-type fibroblasts to an increased intracellular content of Cu

The viability of MT mutant and normal fibroblasts supplemented with 50, 100 or 250 μM Cu–His for 24, 48 and 72 h decreased in a time-dependent manner. MT mutant cells were more sensitive than wild-type fibroblasts to Cu–His at all doses and times

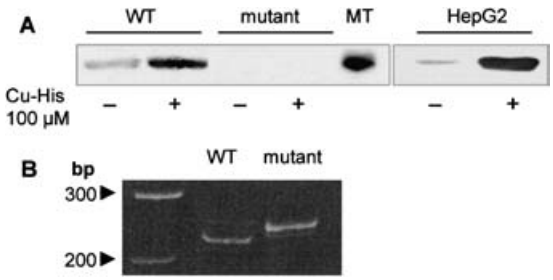

Figure 1 The mutant cell line expresses a modified MT I mRNA and fails to express the protein

(**A**) Cell lines were treated with (+) or without (−) 100 μM Cu–His for 24 h. Aliquots of a MT-rich fraction [10 μg from wild-type (WT) and MT mutant fibroblasts, and 3.5 μg from HepG2 cells] were resolved by SDS/PAGE and subjected to immunoblot analysis with an antibody against MT I/II. A MT standard from rabbit liver was included in the assay. (**B**) cDNA was synthesized from samples of fibroblast total RNA. The MT I transcript was amplified by RT-PCR with the primer pair indicated in the Materials and methods section. PCR products were resolved in an 8 % (w/v) denaturing polyacrylamide gel.

(Figure 2A). Significant differences were observed after 48 h of treatment, at which time cell viability had fallen in MT mutant fibroblasts treated with 100 or 250 μM Cu, whereas the wild-type cells were affected only after 72 h of treatment. MT mutant cells had lower viability when exposed to supra-physiological concentration of the metal, yet there was close to 80 % survival even at the highest Cu exposure. To explain the apparent resistance of MT mutant cells to exposure to high Cu levels, we determined the changes in the intracellular Cu content in response to progressively increasing metal exposure using AAS. No significant differences were observed in the Cu content of mutant and normal cell lines growing in standard culture conditions (0.75 ± 0.07 and 0.77 ± 0.26 nmol/mg of protein respectively). When the fibroblasts were exposed to a supra-physiological concentration of Cu, the intracellular metal content increased in both cell lines, but that in wild-type fibroblasts rose significantly more than in MT mutant fibroblasts (Figure 2B).

To analyse the correlation between viability and intracellular Cu content, we re-assessed the viability data with respect to the intracellular Cu content of the fibroblasts exposed to different Cu concentrations for 72 h, as presented in Figure 2. The results suggested that there is an inverse correlation between intracellular Cu content and the percentage of viable cells; the simple linear correlation coefficient for wild-type cells was 0.89 and that for MT mutant cells was 0.88 (Figure 3). Furthermore, the slopes were significantly different, indicating a differential sensitivity to increasing intracellular concentration that is explained by the presence of MT. MT mutant cells were 4 times more sensitive to the increased intracellular Cu content than wild-type cells (5.1 % and 22.1 % loss of viability/nmol of Cu per mg of protein), supporting a protective role for MT in response to Cu excess.

Since MT also binds Zn, and since there is considerable amount of evidence of metabolic interaction between Cu and Zn, we analysed whether the Zn content was affected in MT mutant cells exposed to Cu. The Zn content was similar in these two fibroblast cell lines grown under standard culture conditions (15.07 ± 2.9 and 13.21 ± 0.85 nmol/mg of protein for wild-type and MT mutant cells respectively), and this was unaffected by 72 h of exposure to 100 μM Cu–His (12.35 ± 2.09 and 13.89 ± 2.13 nmol/mg of protein for wild-type and MT mutant cells respectively). Thus the data indicate that the major element affected by a rise in the extracellular Cu concentration is Cu itself in both the MT mutant and wild-type cell lines.

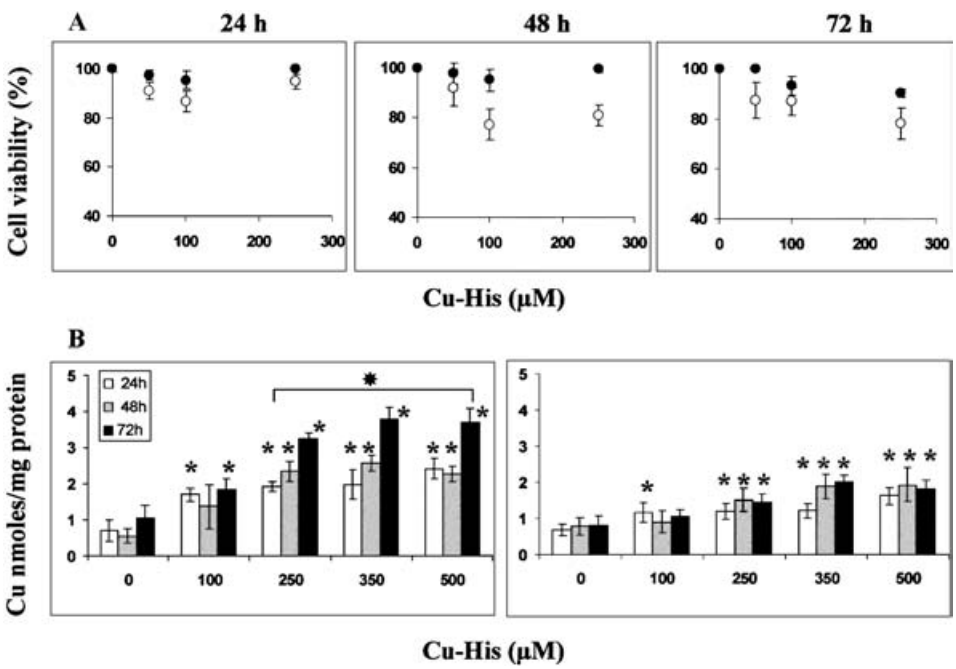

Figure 2 Cells expressing MT store more Cu and are more resistant to Cu exposure

(**A**) Cell viability at 24, 48 and 72 h in response to graded concentrations of Cu–His assessed by reduction of MTT, and was expressed as percentage viability relative to unexposed cells. ●, Wild-type; ○, MT mutant. (**B**) Total Cu content of wild-type cells (left panel) and MT mutant cells (right panel) in response to graded concentrations of Cu–His at 24, 48 and 72 h. Cu content was determined by AAS. Mean values from triplicate experiments are shown with corresponding S.D.s. ANOVA demonstrated a significant effect induced by Cu exposure compared with unexposed cells ($^*P < 0.05$); and an effect of exposure time, as indicated by the horizontal line ($P < 0.05$).

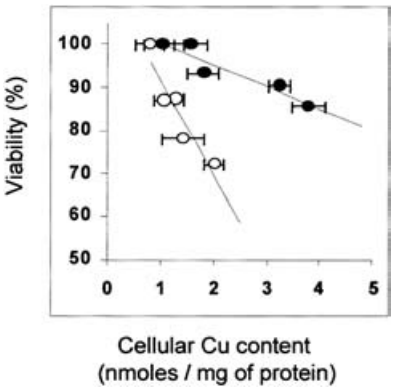

Figure 3 Correlation between intracellular Cu content and cell viability

The percentage viability of wild-type (●) and MT mutant (○) fibroblasts in relation to the intracellular Cu content after 72 h of exposure to graded concentrations of the metal (data from Figure 2A, 72 h) is shown. A significant effect of intracellular Cu content on viability was found, with correlation coefficient values for wild-type and MT mutant cells of 0.89 and 0.88 respectively (Pearson's test, $P < 0.001$). ANOVA showed a significant difference between the two response curves ($P < 0.05$).

Uptake of ^{64}Cu is decreased in MT mutant cells pre-exposed to supra-physiological Cu concentrations

We compared the uptake and efflux of ^{64}Cu in the two cell lines to assess whether the decreased Cu storage of MT mutant cells was the result of differences in either or both of these parameters. The time dependence of ^{64}Cu uptake in wild-type cells was linear for at least 30 min after exposure to 1 or 5 μM Cu (results not shown). Following 15 min of ^{64}Cu–His exposure in the concentration range 0.25–10 μM, the initial rates of ^{64}Cu incorporation exhibited similar hyperbolic curves (Figure 4A). A double-reciprocal analysis indicated similar values of apparent kinetic parameters: 4.25 ± 0.78 and 6.22 ± 0.38 pmol of Cu/min per mg of protein for V_{max} and 1.46 ± 0.33 and 2.32 ± 0.70 μM for K_m in wild-type and mutant cells respectively. This suggests that the absence of MT does not affect the uptake kinetics. Cu efflux was determined as ^{64}Cu retention at different chase times (0–30 min) after a pulse of 1 μM ^{64}Cu–His for 1 h. As shown in Figure 4(B), intracellular ^{64}Cu retention decreased in a biphasic manner, with a rapid phase during the first 10 min followed by a low-rate efflux that continued over the next 20 min (and for at least 90 min; results not shown). We suggest that the first phase corresponds to the fraction of newly incorporated ^{64}Cu that is delivered directly to efflux/transport system, and that the second phase may correspond to the ^{64}Cu that is incorporated into the pool of apo-cuproproteins or that is associated with the storage compartment (MT and/or GSH).

In order to investigate if there are any differences in Cu uptake and/or efflux parameters between these cell lines when they are exposed to a supra-physiological concentration of Cu, we performed the same experiments in cells pre-exposed to 250 μM Cu–His for 24 h. The uptake of Cu is expressed as the percentage of ^{64}Cu incorporated after 15 min of ^{64}Cu exposure in pre-exposed cells with respect to uptake by the same cell line not pre-exposed. Both cell lines decreased their Cu uptake in response to the pre-exposure, as shown in Figure 4(C). However, the decrease in uptake was more marked in the MT mutant cells. This result explains, at least in part, the observation of a decreased capacity for Cu accumulation in the mutant cells. Under these study conditions, no significant differences were observed in ^{64}Cu efflux between wild-type and MT mutant cells pre-exposed to excess Cu (results not shown).

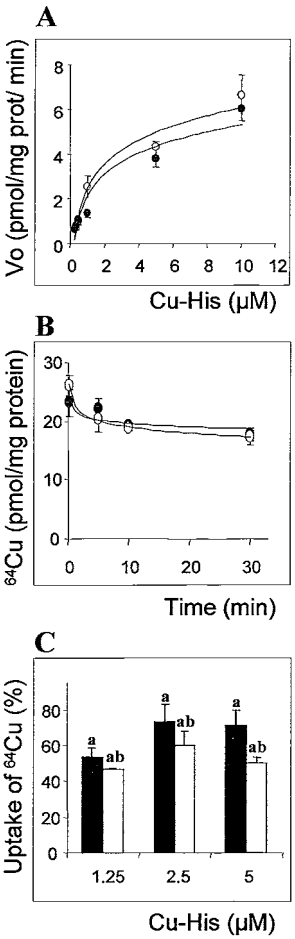

Figure 4 Wild-type and MT mutant fibroblasts differ in their ^{64}Cu uptake rate after pre-exposure to a high concentration of Cu

(**A**) Initial velocity (Vo) was determined as the radioactivity content in wild-type (●) and MT mutant (○) fibroblasts after 15 min of exposure to ^{64}Cu–His (0–10 μM). (**B**) The efflux from fibroblasts was measured as the radioactivity retained at different times of chase after exposure to 1 μM ^{64}Cu–His for 1 h. (**C**) ^{64}Cu uptake by wild-type (■) and MT mutant (□) fibroblasts pre-exposed to 250 μM Cu–His for 24 h was assessed as described in (**A**) and expressed as a percentage relative to uptake by the respective cell line not pre-exposed to Cu (100 %). Significant differences were analysed by ANOVA alone or ANOVA followed by a Bonferroni test analysis ($P < 0.05$). Bars labelled 'a' indicate a significant difference in uptake between pre-exposed and non-pre-exposed cells, and those labelled 'b' indicate a significant difference between pre-exposed wild-type and MT mutant cells. All values are means for triplicate determinations and the corresponding S.D.s.

Cu is distributed differently in wild-type and MT mutant cells

Wild-type and MT mutant cells were exposed to 5 μM ^{64}Cu–His for 3 or 24 h, and the distribution of the intracellular radioactivity was analysed after high-speed centrifugation. The amounts of ^{64}Cu in the cytosol and pellet fractions were measured for each cell line and expressed per unit of protein mass. After 24 h of exposure, the total ^{64}Cu accumulated was 2-fold greater in wild-type cells compared with MT mutant cells (126.8 ± 13.6 and 54.6 ± 1.9 pmol/mg of protein respectively). In addition, the subcellular distribution of the retained ^{64}Cu differed between wild-type and MT mutant cells. Newly incorporated Cu (^{64}Cu) was found principally in the cytosolic fraction in wild-type fibroblasts, whereas the Cu was more evenly distributed between pellet and cytosol in the MT mutant cells (Figure 5).

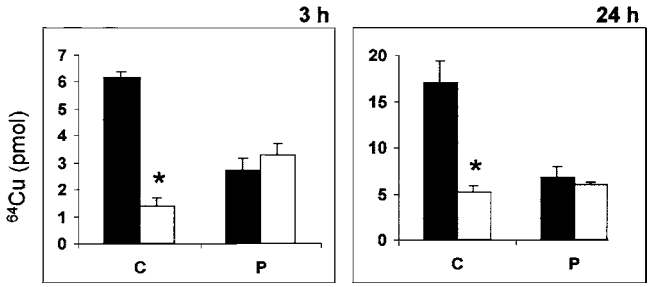

Figure 5 MT mutant cells store less Cu in the cytosolic fraction

Wild-type (■) and MT mutant (□) cell lines were exposed to 5 μM ^{64}Cu–His for 3 or 24 h. The distribution of the intracellular radioactive Cu was analysed in the cytosolic (C) and the pellet (P) fractions after high-speed centrifugation of the homogenates. The values correspond to means for triplicate determinations and the corresponding S.D.s. A significant difference in Cu content in the cytosolic fraction was noted between wild-type and MT mutant cells (Student's *t* test; $^*P < 0.01$).

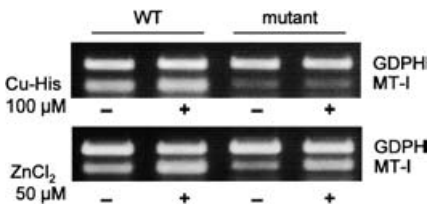

Figure 6 Induction of MT I mRNA by exposure to Cu and Zn

cDNA was generated from total RNA extracted from wild-type (WT) and MT mutant cell lines exposed to 0 or 100 μM Cu–His and 0 or 50 μM $ZnCl_2$ for 24 h. Samples of 0.2 μg of these cDNAs were used to amplify simultaneously the MT I and G3PDH (GPDH) transcripts by semi-quantitative PCR. The PCR products were separated in a 1.2 % (w/v) agarose gel and stained with ethidium bromide.

Wild-type and MT mutant cells show differences in expression of the genes encoding MT I, SOD1 and Ccs1 in response to Cu exposure

During characterization of the MT mutant cells, we observed differences in the relative abundance of the MT transcript in response to Cu exposure. We conducted a semi-quantitative RT-PCR (reverse transcriptase–PCR) assay to amplify MT I and G3PDH mRNAs simultaneously (Figure 6). Densitometric analysis of the amplification products revealed that, in the presence of a Cu excess (100 μM for 24 h), the relative abundance of the MT transcript rose 1.8–2.0-fold in wild-type cells, whereas no induction was detected in the MT mutant cells. This loss of response of MT expression was specific to Cu, since treatment with 50 μM Zn elicited a similar response in wild-type and MT mutant cells, i.e. both cell lines were able to increase the relative abundance of the MT transcript to a comparable extent (Figure 6). These results suggest that MT plays a role in the Cu-dependent induction of its own transcription.

In order to examine whether the transcript abundances of other genes related to Cu metabolism were altered in the mutant cells, we generated a cDNA array filter containing the DNA sequences of five Cu-related and two housekeeping genes. The membranes were hybridized with ^{33}P-labelled cDNA probes from wild-type or mutant cells exposed or not to 100 μM Cu for 24 h. In order to calculate the fold induction of Cu-related genes, the ratios between the normalized signals of membranes hybridized with cDNAs from Cu-exposed and -unexposed cells were determined for each spot, as described in the Materials and methods sections. The results of this assay indicated that the expression of three genes, *ccs1*, *mt I* and *sod1*, was up-regulated in wild-type cells after exposure to Cu, whereas in mutant cells the relative expression levels of *mt I* and *ccs1* decreased slightly and that of *sod1* did not change (Table 1). The differential expression of *mt I*, *ccs1* and *sod1* identified by the reverse Northern blot was confirmed by means of a semi-quantitative RT-PCR analysis (results not shown). These results indicate that cells respond differentially to Cu exposure depending on the presence of MT, suggesting that this protein is responsible in part for the regulation of expression of Cu-related genes associated with cellular Cu homoeostasis.

Table 1 Gene expression analysis of genes related to Cu metabolism

Induction values correspond to the ratio of normalized hybridization intensity from Cu-treated/untreated cells.

Accession ID (GenBank)	Protein	Induction (fold)	
		Wild type	Mutant
AB007134	ATP7A	1.1	0.9
AF173379	Ccs1	2.4	0.4
M35725/	SOD1 (Cu,Zn-SOD)	2.0	0.4
NM_000484	Amyloid β precursor	0.9	0.3
J00605	MT I	2.8	0.5
Nm_011655	Tubulin	1.0	1.1
M32599	G3PDH	1.0	1.0

DISCUSSION

The role of MT has been extensively studied, but it remains elusive. Part of the reason is that MT is implicated in multiple physiological processes, such as metal storage (Cu, Zn), metal detoxification (Cd, Cu, Zn), oxidative scavenging and the inflammatory response [5,30]. It has been well demonstrated that MT protects cells against supra-physiological levels of extracellular Cu [12–14]. In the present study our goal was to analyse the role of MT in Cu metabolism, monitoring in the same system a wide range of parameters such as viability, Cu content, Cu uptake and efflux, subcellular localization, and the expression of MT as well as Cu metabolism-related genes. We took advantage of the simultaneous generation of two cell lines from littermate mouse fetuses [15]: one expresses MT (wild type), while the other expresses modified mRNA isoforms of MT I and II that are not able to be translated into proteins (Figure 1). A considerable number of studies on Cu metabolism and MT have been carried out using cellular models resembling tissues specialized in Cu detoxification and absorption, such as hepatic and intestinal tissues. The fibroblasts used in the present study represent a cell that is not specialized in organic Cu management, and thus we were able to evaluate the role of MT in cells that are normally not exposed to substantial fluctuations in Cu availability. In agreement with previous studies [22,31,32], our results showed that exposure of fibroblasts to Cu elicits (to a minor extent compared with hepatic and intestinal cells) metal storage and the induction of MT.

Analysis of Cu content by AAS showed that, under standard culture conditions (0.4 μM extracellular Cu), the amount of Cu in the two fibroblast cell lines was similar. At this sub-physiological exposure of Cu, the cells lines studied showed the same viability and duplication rate. This has also been shown in embryo fibroblast cultures from MT mutant mice, where the absence of MT did not affect their growth rate with respect to normal fibroblasts [33]. Considering that other molecules, such as GSH and Cu chaperones, are able to bind and transfer the metal to

ensure the storage and delivery of Cu to cuproenzymes [34,35], the results suggest that MT is not essential for the delivery of Cu to the cuproenzymes at sub-physiological Cu concentrations.

However, at a physiological concentration of Cu, MT mutant cells accumulated two times less metal than wild-type cells (24 h exposure to 5 μM ^{64}Cu–His), suggesting that the presence of MT is key for increasing Cu storage capacity within the physiological range. Interestingly, we observed that in wild-type cells the metal increment was located mainly in the cytosolic fraction. Since most MT is also cytosolic, these results suggest that the increment in the intracellular Cu content is associated with MT in wild-type cells. This preferential distribution of Cu in the presence of MT has been shown previously in hepatocytes exposed to physiological concentrations of Cu [13,36], in hepatic tissue induced to increase the expression of MT [37] and in hepatic cell lines (Huh7 and HepG2) pre-exposed to Cu [13,22]. Moreover, it has been shown that the increase in the cellular Cu content is correlated with an increase in the amount of Cu bound to MT [37,38]. In particular, in ATP7A mutant fibroblasts (which have a defect in Cu efflux), excess Cu is mostly associated with MT [38,39], probably through the binding of Cu to newly synthesized MT or to a pre-existing Zn–MT form by displacing Zn [1,34]. This evidence and our present results support the idea that, within the physiological range of Cu exposure, MT has a role in the cytosolic storage of Cu, serving as a reservoir to be used for cuproenzyme synthesis in the event of Cu deficit, a function that is not provided by other proteins involved in Cu metabolism.

At physiological concentrations of extracellular Cu, the rate of uptake of ^{64}Cu was similar in the two cell lines, suggesting that this process is not regulated by the presence of MT. However, when the cells were pre-exposed to supra-physiological Cu concentrations, both cell lines showed decreased uptake, suggesting activation of a mechanism working against the accumulation of Cu. This response has been shown in yeast, and recently also in human cell lines by means of the internalization and degradation of hCTR1, the high-affinity human Cu transporter [40], which has a murine homologue, mCTR1. Our observation that wild-type cells decreased their ^{64}Cu uptake to a lesser extent than the mutant cells may explain why the mutants failed to accumulate Cu to the same level as wild-type cells during Cu treatment. The efflux of newly incorporated Cu (^{64}Cu) was similar in the two cell lines, presenting rapid efflux in the first 10 min and slower efflux lasting for at least 90 min. The existence of these two pools (rapid and slow interchangeable) has been reported previously [23,41]. In view of these intracellular Cu pools, and since under our experimental conditions there was radioactive and unlabelled Cu being secreted from the cells at any specific moment, we propose that in wild-type fibroblasts the main destination of the slow interchangeable pool of Cu is MT and cuproenzymes, while in the mutant cells only cuproenzymes will bind the available Cu. Excess Cu will be released eventually from these proteins into the cytosol, and in the case of the wild-type cells a fraction of this Cu may re-bind to MT, preventing it from entering the efflux pathway and thus increasing the intracellular content of Cu.

Following supra-physiological metal exposure, both cell lines increased their cellular content, although wild-type cells had a higher metal content (Figure 2B). We and others have reported previously that distinct cells lines from different mammalian tissues increased their intracellular metal content in response to a progressive rise in Cu exposure [12–15,22,27]. Our present results corroborate those findings and suggest that the magnitude of the Cu increment is dependent, at least in part, on the presence of MT, which is able to enhance its own expression under conditions of excess extracellular Cu. We observed only a small increase in sensitivity to Cu toxicity in the MT mutant cells, and the major differences were observed with longer exposure times and only when extracellular concentration of Cu used was very high [42]. These results indicate that the major contribution of MT to Cu metabolism is to increase the capacity to store Cu, and that protection from extracellular Cu excess appears to represent a secondary contribution of this protein to cellular Cu homoeostasis. In addition, our results suggest that it is not the amount of intracellular Cu *per se* that is toxic or safe, but the capacity of the Cu ions to establish appropriate or abnormal molecular associations. In this context, the binding of Cu to MT may provide a safe environment for the localization of Cu (as stable Cu) for metal storage or in transit to cuproenzymes.

A critical consequence of the relationship between the increment of intracellular Cu and MT was observed at the gene expression level. During our analysis of the role of MT in Cu metabolism, we noted that an excess of extracellular Cu failed to induce MT I mRNA in the mutant cell line. Intrigued by this finding, we stimulated the cells with Zn, and MT I transcript levels rose in response to Zn exposure, as in the control cell line. The induction of MT expression in response to Zn in these mutant cells has been shown by others [43,44], but there is no previous report of a lack of induction by Cu. Our results suggest that the stimulation of MT expression by Cu is mediated by the MT protein through its capacity to affect the intracellular amount of Cu and by modulating the availability of Zn for Zn-dependent transcription factors. In mammals it has been proposed that MTF-1 (metal regulatory element binding transcription factor-1) is the transcription factor that senses the Zn status, since one ion of its Zn-fingers is interchangeable at physiological concentrations of the metal [45]. MTF-1 induces the transcription of MT,ZnT-1 (a Zn transporter), the heavy-chain subunit of γ-glutamylcysteine synthetase (the rate-limiting enzyme for GSH biosynthesis) [45] and other genes related to oxidative stress, hypoxia and amino acid starvation [46]. Thus an increment in the Zn concentration can stimulate the activity of MTF-1, and MT expression. We hypothesized that Cu induces MT expression by displacing Zn from Zn-binding proteins, including MT, in wild-type cells. This idea is supported by the fact that MT has a greater affinity for Cu than for Zn [1], and by our observation that an excess of Zn may restore the induction of MT transcripts in the absence of MT. Moreover, Cano-Gauci and Sarkar [7] reported reversible Zn exchange between MT and the oestrogen receptor in a gel mobility shift assay using nuclear extracts.

Finally, we assessed whether the expression of other genes involved in Cu metabolism was affected by the absence of MT. In a previous study, a differential display assay performed using MT I/II mutant and wild-type 7-day-old mice revealed the differential expression of five cDNAs in the MT mutant mice, including MT I wild-type and mutant cDNAs [47]. Here, by using a cDNA array filter and a semi-quantitative PCR, we observed a lack of induction of the expression of SOD1 and its Cu chaperone, Ccs1, in response to Cu in MT mutant relative to wild-type cells. Ccs1 activates SOD1 by directly inserting the Cu cofactor [35], and thus both SOD1 and Ccs1 play a significant role in protecting cells against oxidative damage [48]. These results suggest a role for MT as a modulator of gene expression; however, further investigation is required to elucidate the mechanism involved in the MT-mediated changes in gene transcription by metals.

The results discussed here indicate that, within the physiological range, the role of MT is to provide a safe storage compartment for Cu. As a consequence of this function, at supra-physiological levels of Cu exposure MT has a role in detoxification. Additionally, MT is a modulator of gene expression, since in its absence exposure to high Cu concentrations failed to induce the expression of MT and other Cu-related genes such as those

encoding SOD1 and Ccs1, suggesting a second mechanism by which MT may contribute to cellular resistance to Cu exposure.

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