A mutant mouse (*tx*) with increased hepatic metallothionein stability and accumulation

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Metallothioneins (MTs) are low-molecular-mass cysteine-rich proteins implicated in metal homeostasis and resistance to toxicity induced by heavy metals and alkylating agents. We report high hepatic MT protein accumulation (greater than 100-fold compared with wild-type mice) in *toxic milk* (*tx*) mice, along with markedly higher cytosol copper and zinc levels. Increased MT-gene transcription alone could not account for the high constitutive MT protein levels, since MT mRNA levels were not increased in *tx* mouse livers. However, hepatic MT was sig-

nificantly more stable in adult *tx* mice: MT half-life ($t_{1/2}$) was 79 or 77% greater than in wild-type mice before and after Cd induction respectively. Cd or Zn treatment increased MT mRNA, but not MT protein, accumulation in *tx* mouse livers: Cd displaced MT-bound Zn and Cu in preexisting MT. Thus *tx* mice appear to accumulate hepatic MT as a result of decreased protein degradation. These animals may provide a useful model to study the physiological role of MT, and human diseases (such as Wilson's disease) with abnormal copper metabolism.

INTRODUCTION

Metallothioneins (MTs) are low-molecular-mass metal-binding proteins involved in resistance to toxic heavy metals [1,2]. They are bound primarily to Zn and Cu in humans and rodents untreated with heavy metals [3], and are encoded by a family of genes in primates (a single functional MT-2 gene, a brain-specific MT-3 gene, and at least five functional MT-1 genes) and rodents (by single copies of MT-1, MT-2 and MT-3 genes). MT-gene expression is induced by heavy metals and a variety of other agents, including hormones and γ and u.v. irradiation [4–7]. MT genes are developmentally regulated in rodents [8–10] and humans [11], and are overexpressed in some solid human tumours [12]. Because of the wide range of conditions affecting MT expression, a homeostatic role in Zn and Cu regulation has been postulated for the protein [2].

In order to understand this putative homeostatic role and relate it to human diseases known to involve abnormal metal metabolism, it is desirable to study organisms with altered metallothionein expression. At present, only cultured cells with naturally occurring high (B16F1 mouse melanoma cells [13]; IGR 39 human melanoma cells [7]) or low (S49.1 mouse lymphoma and W7 mouse thymoma cells [14]) MT expression, or high MT expression due to transfection of transcriptionally active foreign MT genes [15–18] exist. Animals with mutations resulting in altered MT tissue levels have not been available.

Toxic milk (*tx*) is an autosomal recessive mutation which alters Cu homeostasis in mice [19,20], and results in markedly reduced hepatic Cu concentration, hypopigmentation, reduced growth, abnormal locomotor behaviour, tremors and death at 2 weeks of age in newborn animals. Mice allowed to suckle from normal lactating dams survive and accumulate as much as 30 times as much hepatic Cu in adulthood as normal mice, and have enlarged livers with large, irregular nodules separated from the remainder of the parenchyma by depressed fibrotic septa [20]. Although the mechanism of hepatic damage in *tx* mice is still unclear, the

increased accumulation of Cu may cause toxicosis and hepatic damage. Little is known of changes in hepatic Cu-binding proteins in *tx* mice. Increased superoxide dismutase (SOD) activity has been reported in livers of *tx* mice, while serum caeruloplasmin is low [21]. In the present study we found that the major portion of hepatic Cu in *tx* mice was bound to MT. We describe the expression of MT protein and mRNA under various experimental conditions. We also report increased stability (significantly longer half-life) of metallothionein protein in *tx* mice, both before and after induction with Cd salts.

EXPERIMENTAL

Animals

tx and adult wild-type mice were obtained from Dr. H. Rauch (University of Massachusetts, Amherst, MA, U.S.A.) and bred according to the published protocol [19]. *tx* mice were fostered at birth to wild-type lactating dams, and mice were fostered by their natural mothers. Male or female mice (3–5 months old) were injected intraperitoneally (i.p.) with 20 mg/kg Zn (as $ZnCl_2$) or 1 or 5 mg/kg Cd (as $CdCl_2$). Metal salts were dissolved in 0.9% NaCl; control mice were injected with 0.9% NaCl alone. After 6 or 24 h, mice were killed by cervical dislocation, and livers and kidneys were removed and kept on ice. Because metal-induced MT mRNA accumulation is maximal at 4–6 h after metal treatment, while MT protein accumulation is highest at 24 h [22], MT mRNA was measured in 6 h samples and MT protein accumulation was measured in 24 h samples, as described below.

MT mRNA measurement

Total cellular RNA was isolated by standard methods [10,23] and analysed by separation on denaturing formaldehyde/agarose gels followed by transfer to Hybond nylon filters, or immobilization on nylon filters by RNA dot-blotting [13].

Abbreviations used: MT, metallothionein; SOD, superoxide dismutase; *tx*, *toxic milk* mouse mutant.

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Immobilized RNA was hybridized to a ^{32}P -labelled genomic MT-1/MT-2 DNA probe (9.8 kb, including the entire coding regions of both mouse MT-1 and MT-2, bounded by *Kpn*I and *Hind*III restriction sites [24]). Rehybridization of the same filters to a mouse β -actin cDNA probe [25] provided comparative quantification within the same sample of RNA. Regression analysis of a plot of probe hybridized versus mg of RNA dotted yielded values of total MT and β -actin mRNA accumulated ($\pm$ S.E.M.), and total mouse MT mRNA accumulation was expressed relative to β -actin mRNA accumulation [13].

Tissue MT, Zn, Cu, Cd and GSH measurement

Tissue samples from mice killed 24 h after saline or metal-salt treatment were homogenized in 2 vol. of 0.25 M sucrose (BDH Canada) and centrifuged at 10000 *g* to precipitate particulate material. MT was measured in the supernatant fractions by the Ag-haem method [26]. Zn and Cu were measured in separate whole-tissue aliquots digested in concentrated HNO_3 by flame spectrophotometry using a Varian AA-30 flame spectrophotometer [27].

Cytosolic fractionation of MT by column chromatography

Hepatic cytosols were obtained from *tx* mice treated with iso-osmotic saline or 5 mg/kg Cd 24 h prior to organ removal. Supernatants of liver and kidney were prepared by homogenizing tissue samples in 0.5 M glycine, pH 8.5, and centrifuging at 20000 *g* for 20 min. Proteins from resulting supernatants were separated on a Sephadex G-75 column (Pharmacia; 0.9 cm $\times$ 60 cm) and analysed for Cu, Zn and Cd by the methods described above. Metal concentrations were expressed per g of wet tissue. The percentage changes in Cu and Zn content in high-molecular-mass proteins (column fractions 10–19) and MT-containing protein fractions (column fractions 19–35) were calculated according to changes in areas under graphs of metal content (p.p.m.) per column fraction.

Measurement of the fractional rate of degradation of MT

(a) Radiolabelling of hepatic protein *in vivo*

tx or wild-type male mice (4–6 months old) were injected subcutaneously with 1 mg/kg CdCl_2 in iso-osmotic saline at zero time. Control animals were treated with iso-osmotic saline alone. At 6 h after Cd injection (at the point of maximum accretion of murine hepatic MT protein [22,28,29], each animal was injected with 15 μCi of L- ^{35}S cysteine (ICN Biomedicals) (sp. radioactivity 1080 Ci/mmol) in PBS, pH 6.8. All animals were injected with unlabelled L-cysteine (1 mmol/kg) in PBS at 24, 48, 72, 96, and 120 h following Cd treatment, depending on the time of sacrifice. Animals were killed by cervical dislocation and exsanguination at the times indicated in the Figure legends.

(b) Measurement of ^{35}S label incorporated in MT

Mouse liver tissues were homogenized in 0.5 M glycine buffer, pH 8.6, and centrifuged at 23700 *g* for 20 min at 4 $^\circ\text{C}$. Supernatants were heated to 70 $^\circ\text{C}$ for 10 min and re-centrifuged at 23700 *g* for 20 min followed by separation through Sephadex G-25 (pre-prepared Pharmacia PD-10 columns) to remove unincorporated ^{35}S cysteine. Particulate fractions precipitated by the two centrifugation steps showed no MT signal, either by

radioimmunoassay or Ag-haem analysis (results not shown). Hepatic proteins were separated by electrophoresis through SDS/12%-PAGE gels and the MT band identified by comparison with rabbit MT-1 and MT-2 (Sigma) and radioimmunoassay of pooled high- and low-molecular-mass fractions [30]. Polyacrylamide slivers containing MT protein bands were excised from polyacrylamide gels and eluted by electrophoresis within dialysis tubing that allowed pass-through of only molecules of 5000 Da or less. ^{35}S radiolabel was measured in solubilized MT bands by scintillation counting, and MT was measured by competitive solid-phase radioimmunoassay [30]. [^{35}S]Cysteine d.p.m. (total activity/mg of protein) was calculated by dividing the amount of MT-associated label by the total amount of soluble protein separated on the gel. ^{35}S radiolabel in non-MT proteins was determined by excising a polyacrylamide sliver containing proteins of greater than 30 kDa from each lane, immersing the sliver in water-soluble scintillation fluid (PCS, Amersham Corp.) and scintillation counting. Total [^{35}S]cysteine d.p.m. in the non-MT fraction/mg of total protein was determined as for MT-associated radiolabel.

(c) Calculation of MT and non-MT-protein decay rate and half-life

The rate of loss of MT and non-MT proteins in the soluble fraction of liver proteins from *tx* and wild-type mice was calculated as the slope of the regression of the natural logarithm of [^{35}S]cysteine d.p.m. (total activity) versus time. MT half-life ($t_{1/2}$) was calculated from decay rate values by the relationship [31,32]:

$$t_{1/2} = (\ln 2) / \text{fractional decay rate}$$

RESULTS AND DISCUSSION

Two human diseases (Menkes' disease and Wilson's disease) exhibit inborn errors of Cu metabolism which are forms of Cu toxicity [33]. The X-chromosome-linked mouse mutations *brindled* and *blotchy* exhibit changes in copper homeostasis [34], and the murine effects of *brindled* (which bear a close resemblance to those of Menkes' disease [34,35]) have been extensively investigated as a possible animal model of this fatal disorder. LEC rats with abnormal hepatic Cu accumulation and increased hepatitis have also been described [36]. Adult *tx* mice harbour an autosomal recessive mutation which results in liver copper levels 30–40-fold higher than in wild-type animals, along with neurological and hepatic damage [20]. Because of the altered copper retention, and the fact that MT has been shown to be a major hepatic Cu-binding protein in Wilson's disease [37,38], we examined tissue MT levels as well as the rate of decay (K_d) of newly-synthesized MT and non-MT proteins, in *tx* mice and the unaffected wild-type animals from which they were derived.

Adult (3–5-month-old) *tx* mice had greater than 100-fold more MT protein in the 10000 *g* supernatant fraction of liver proteins than wild-type mice in the absence of exogenous inducing agents ($P < 0.0002$) (Table 1). The 10000 *g* precipitate (from livers and kidneys of wild-type and *tx* mice) did not contain MT measurable by the Ag-haem method or a competitive solid-phase radioimmunoassay [30] (results not shown), indicating that assay of soluble proteins was appropriate to follow MT appearance and disappearance. There was no significant difference in liver MT levels between male and female *tx* mice ($P < 0.365$). Hepatic cytosol copper levels were 38-fold higher in *tx* versus wild-type male liver and 130-fold higher in *tx* versus wild-type females. In this case there was a sex-associated difference, with female *tx* copper levels significantly higher than in *tx* males ($P = 0.002$) (results not shown). Liver Zn levels were approximately twice as

Table 1 MT, Cu, Zn and Cd in *tx* and wild-type mice

Results are means $\pm$ S.D. for the numbers of animals given in parentheses. Tissue samples were collected 24 h after treatment with 5 mg/kg Cd ('CdCl₂') or iso-osmotic saline ('Control'). Abbreviation used: N.D., not detected.

Mice	Organ	Treatment	Content (μ g/g of tissue)			
			MT	Cu	Zn	Cd
Wild-type	Liver	Control	10.3 $\pm$ 3.3 (8)	4.8 $\pm$ 1.5 (8)	33.6 $\pm$ 2.7 (8)	N.D. (4)
		CdCl ₂	174 $\pm$ 6 (8)	7.0 $\pm$ 0.3 (8)	44.4 $\pm$ 1.5 (8)	8.4 $\pm$ 1.1 (8)
	Kidney	Control	20.8 $\pm$ 0.4 (7)	4.6 $\pm$ 0.4 (7)	19.1 $\pm$ 0.9 (7)	0.2 $\pm$ 0.1 (7)
		CdCl ₂	25.9 $\pm$ 5.6 (7)	5.7 $\pm$ 0.4 (7)	18.8 $\pm$ 0.9 (7)	13.2 $\pm$ 8.6 (7)
<i>tx</i>	Liver	Control	2215 $\pm$ 127 (12)	325 $\pm$ 116 (12)	71 $\pm$ 6 (12)	0.22 $\pm$ 0.13 (4)
		CdCl ₂	1969 $\pm$ 141 (11)	246 $\pm$ 16 (11)	60 $\pm$ 43 (11)	10.0 $\pm$ 0.2 (11)
	Kidney	Control	35.5 $\pm$ 9.3 (12)	9.0 $\pm$ 0.7 (11)	27.5 $\pm$ 0.4 (11)	0.4 $\pm$ 0.2 (4)
		CdCl ₂	48.0 $\pm$ 10.1 (11)	11.6 $\pm$ 0.8 (11)	20.2 $\pm$ 0.8 (11)	2.7 $\pm$ 0.2 (11)

high in *tx* versus wild-type mice (Table 1), with no significant differences associated with sex (results not shown). Kidney cytosolic MT and whole-tissue Cu levels were also elevated in *tx* mice, but to a lesser degree than in liver. Therefore it was evident that the *tx* mutation in mice resulted in extremely high hepatic MT protein accumulation with concomitant increased accumulation of Cu (to a major degree) and Zn (to a lesser degree). In order to determine whether the increased protein accumulation was the result of increased MT-gene transcription, combined MT-1 and MT-2 mRNA accumulation was assessed. There was no evidence of elevated total MT mRNA in association with high constitutive MT protein levels in liver (Figure 1 and Table 2). It is of interest that MT mRNA levels in human patients with Wilson's disease have also been reported to be variable and unrelated to liver copper [38]. This differs from the elevated MT-1 mRNA accumulation reported by Mercer et al. [39] in a limited number (two males) of adult *tx* mice. No corresponding MT-2 mRNA accumulation or MT protein measurements were reported in that study. We observed no increased MT mRNA accumulation in a larger number of adult *tx* animals of both sexes ($n = 25$), for which both MT-1 plus MT-2 and MT protein accumulation were measured. We suggest, therefore, that high constitutive liver metallothionein levels in *tx* mice were not the result of increased MT mRNA accumulation. Increased MT must have been the result of post-transcriptional events.

Treatment with metal salts (Cd or Zn) induced MT mRNA accumulation in liver and kidney of both *tx* and wild-type mice, similar to that seen in other mouse strains [22,40] (Table 2 and Figure 1). After metal induction there was no significant difference in the level of induced total MT mRNA between *tx* and wild-type mice ($P = 0.4$), nor were there sex-related differences in the MT mRNA level in control *tx* ($P = 0.4$) or wild-type ($P = 0.2$) mice (results not shown). The size of the MT mRNA from induced *tx* mutant mice was identical with that seen in wild-type liver RNA after hybridization to the radiolabelled MT-1/MT-2 probe (Figure 1). MT protein from *tx* mouse livers was associated with Cu and Zn, and separable on a Sephadex G-75 column in a manner indistinguishable from purified MT isolated from normal mouse livers (Figure 2). Therefore it is unlikely that constitutive MT mRNA levels were underestimated in *tx* livers because of gross mutations in MT genes, which could render transcribed MT mRNA unhybridizable with the MT-1/MT-2 probe. Thus MT gene control elements (for MT-1, MT-2 or both) were responsive to metal induction in both *tx* and

Figure 1 MT mRNA accumulation in wild-type and *tx* mouse livers

Total (MT-1 + MT-2) MT mRNA accumulation in livers of individual wild-type and *tx* mice. Animals were treated with metal salts or iso-osmotic saline, and total liver RNA was isolated and hybridized to a radiolabelled mouse MT-1/MT-2 genomic DNA probe and a mouse β -actin cDNA probe by Northern blotting as described in the Experimental section. Lanes 1 and 4, iso-osmotic-saline-treated mice; lanes 2 and 5, ZnCl₂ (20 mg of Zn/kg)-treated mice; lanes 3 and 6, CdCl₂ (5 mg of Cd/kg)-treated mice.

Table 2 MT mRNA accumulation in wild-type and *tx* mice

Organ	Treatment	MT mRNA/ β -actin mRNA*	
		Wild-type	<i>tx</i>
Liver	Control	0.3 $\pm$ 0.1 (8)	0.25 $\pm$ 0.004 (6)
	CdCl ₂ †	2.8 $\pm$ 0.3 (4)	2.5 $\pm$ 0.2 (6)
	ZnCl ₂ ‡	1.9 $\pm$ 0.2 (6)	2.0 $\pm$ 0.2 (5)
Kidney	Control	0.20 $\pm$ 0.02 (5)	0.20 $\pm$ 0.04 (5)
	CdCl ₂ †	1.9 $\pm$ 0.2 (6)	2.2 $\pm$ 0.3 (5)
	ZnCl ₂ ‡	0.9 $\pm$ 0.3 (5)	1.2 $\pm$ 0.1 (6)

* From densitometry of autoradiographs of total tissue RNA dot blots hybridized to MT and β -actin cDNA probes; results are means $\pm$ S.E.M. for the numbers of animals given in parentheses; MT/ β -actin mRNA ratios were measured in six individual RNA dots for each tissue sample.

† Animals treated with 1 mg of Cd/kg as described in the Experimental section.

‡ Animals treated with 20 mg of Zn/kg as described in the Experimental section.

wild-type mice, resulting in increased gene transcription and elevated mRNA accumulation.

Cd treatment also resulted in increased MT protein accumulation in kidneys of both wild type and *tx* mice, and in livers of

Figure 2 Cu, Zn and Cd content of MT in *tx* mouse liver

In vivo association of *tx*-mouse liver MT with Cu, Zn and Cd 24 h after treatment with iso-osmotic saline (a) and 5 mg/kg Cd (as CdCl₂) (b). Liver cytosols were prepared and separated by using a Sephadex G-75 column, and Zn (—), copper (---) and cadmium (·····) content of fractions determined as described in the Experimental section. The arrow indicates the position at which purified rat liver MT-2 [41] and rabbit liver MT-1 and MT-2 (Sigma) was eluted from the column and the peak of Cd content in liver cytosol isolated from wild-type mice 24 h after treatment with 5 mg/kg Cd.

Figure 3 ³⁵S radiolabel is incorporated into protein in mouse liver

Isolation of high-molecular-mass-associated ³⁵S label. Soluble hepatic protein extract was prepared from *tx* mice uninjured with Cd 24 h after [³⁵S]cysteine treatment and separated on a Sephadex G-25 column as described in the Experimental section. The elution profile of protein from one representative mouse is shown. High-numbered fractions were of low molecular mass and were retarded on the column. Low-numbered fractions contained high-molecular-mass molecules. The horizontal bar indicates the fractions pooled for further analysis. Radioactivity was measured by liquid-scintillation counting, and protein concentration was measured by the differential u.v. absorbance method of Warburg and Christian [56].

wild-type mice. However, no significant increase in *tx* mouse liver MT protein was detectable (Table 1). This could be because: (1) MT mRNA synthesized in response to Cd induction might not be translated into MT protein in *tx* mice or (2) increases in hepatic MT resulting from MT-gene transcription might be too

Figure 4 Separation and identification of radiolabelled hepatic MT

Soluble hepatic protein fractions from one representative *tx* mouse 24 h after [³⁵S]cysteine treatment (lane 3) or a wild-type mouse 24 h after [³⁵S]cysteine treatment and 30 h after Cd induction (1 mg/kg of mouse) (lane 4) were separated on a polyacrylamide gel, stained (b) and radiolabel was measured in protein fractions as described in the Experimental section. Radiolabel in proteins of increasing molecular mass (*M*) is depicted in (c). MT protein was measured by radioimmunoassay in pooled fractions 1–8, 9–16 and 17–24 and the results are shown in (a). Commercial rabbit MT-1 (lane 1) and MT-2 (lane 2) were run as standards; the indistinct bands between 18.5 and 27.5 kDa are due to contaminating non-MT protein in the commercial preparations.

small to be apparent within measurement error associated with the extremely high constitutive hepatic MT protein levels in *tx* mice (Table 1). We hypothesized that MT protein in mutant mice was degraded at a lower rate than in wild-type mice, resulting in the observed enhanced accumulation. To test this possibility, the decay rate (*K_a*) of MT before and after induction with Cd was measured in both *tx* and wild-type mice. After separation of unincorporated [³⁵S]cysteine from radioactivity incorporated into protein, fractions containing the majority of radioactivity also contained the high-molecular-mass (non-free cysteine) protein fraction (Figure 3), indicating that [³⁵S]cysteine had been incorporated into protein. Greater than 90 % of radiolabel was associated with protein by 24 h following radiolabelled cysteine injection. This was true for all samples, from both wild-type and affected mice and, at all times from 24 to 120 h. In addition, unlabelled L-cysteine was injected to produce an excess of at least 10⁶-fold non-radioactive as compared with radioactive amino acid, radically diminish the specific radioactivity of non-protein cysteine and prevent significant re-utilization of radioactive amino acid released from protein [41,42]. Cellular free cysteine is also actively degraded by cysteine deoxygenase [43], further diminishing radioactive cysteine available for re-incorporation. Thus very little free radiolabelled cysteine (which could have complicated measurement of MT half-life owing to re-incorporation of radioactivity into MT synthesized during the time period of decay observation) was available in liver. When separated by SDS/PAGE, radiolabel was found to be incorporated into both the high-molecular-mass (non-MT) and low-molecular-mass (MT) fractions (Figure 4). The presumed MT band was of appropriate molecular mass (< 10 kDa), competed for binding to rabbit anti-(rat MT-2) antibody (Figure 4) and increased in intensity in soluble protein fractions from Cd-induced wild-type-mouse liver and control and Cd-induced *tx*-adult-mouse liver (Figure 5). Control wild-type-mouse liver soluble proteins had low MT levels (Figure 5). Interestingly a soluble heat-stable protein of approx. 30 kDa was present in increased amounts in wild-type and *tx* mouse liver after Cd induction and in control *tx* mouse liver. Characterization of that protein is underway.

Since the loss of radiolabel in MT with time was an indication of MT stability, the natural logarithm of MT-associated

Figure 5 MT protein is elevated in control *tx* and Cd-induced wild-type mouse liver

Wild-type and *tx* mice were induced with Cd (1 mg/kg) or iso-osmotic saline for 30 h and injected with [³⁵S]cysteine 24 h prior to sampling. Proteins were separated by electrophoresis as described in the Experimental section and stained with Coomassie Blue. Results from one representative mouse liver is depicted in each lane. Lane 1, wild-type Cd-induced; lane 2, wild-type control; lane 3: *tx* Cd-induced; lane 4, *tx* control.

Figure 6 Degradation rate and half-life of hepatic MT

tx (○) and wild-type (●) mice were treated with (b) Cd (1 mg of Cd/kg body wt. 6 h prior to radiolabelling) or (a) iso-osmotic saline. MT-associated radiolabel in total protein separated on polyacrylamide gels was measured as described in the Experimental section and plotted at various times after [³⁵S]cysteine injection. Decay constants (K_d) and half-life ($t_{1/2}$) were calculated as described in the text and are reported there. Correlation coefficients (r^2) were calculated for each regression line. Each point represents results from four to seven mice.

radioactivity/g soluble protein was plotted against time after [³⁵S]cysteine injection (Figure 6). Assuming single-exponential decay kinetics (see below), the stability constant of MT (K_d) was the slope of the line defined by the plot. Hepatic metallothionein in *tx* mice decayed significantly more slowly than in wild-type

animals: K_d (*tx* liver) = -0.019 ± 0.002 ($r^2 = 0.89$) and K_d (wild-type liver) = -0.034 ± 0.007 ($r^2 = 0.87$) (errors were calculated as the S.E.M. of the estimate of the slope) (Figure 6b). The error in K_d for wild-type mouse liver was greater than for *tx* animals because of the greater-than-10-fold lower level of MT-associated radiolabel; wild-type-mouse hepatic MT concentrations (without Cd induction) were considerably lower than in *tx* animals (Table 1 and Figure 5). When both *tx* and wild-type mice were treated with 1 mg/kg Cd, these significant differences in decay constants were preserved: K_d (*tx* liver, Cd-induced) = -0.024 ± 0.003 ($r^2 = 0.94$) and K_d (wild-type liver, Cd-induced) = -0.042 ± 0.004 ($r^2 = 0.91$) (Figure 6a). Curve-fit analysis of plots of the fraction of initial ³⁵S label associated with MT versus time (SigmaPlot, Jandel Scientific) indicated a uni-exponential rate of decay rather than the bi-exponential decay. Computer-fitted curves of time versus fraction of initial ³⁵S yielded better fits for a uni-exponential function [$f(x) = ae^{-bx}$] than a bi-exponential function [$f(x) = ae^{-bx} + ce^{-dx}$] in the time period after 24 h following radiolabelled-cysteine injection. In addition, parameter dependencies approached 1.0 using a double-exponential decay equation (Scientific Graph System Curve Fitting program, Jandel Scientific), suggesting that single-exponential decay more closely described the kinetics of radiolabelled MT disappearance. This may indicate a different situation from that reported by Steinebach and Wolterbeek [42] in cultured rat cells, where higher-order decay fitted the data better. However, their data indicated that the steeper portion of the decay curve (indicative of the influence of a second parameter) was observed only in the 0–10 h period after labelled cysteine administration; further loss proceeded according to uni-exponential kinetics. Since we measured MT levels beginning 24 h following radiolabelled cysteine injection, it is quite possible that we observed differences in long-term decay that followed single-exponential kinetics, entirely consistent with the results reported by Steinebach and Wolterbeek [42].

MT-associated radiolabel 24 h after [³⁵S]cysteine treatment in Cd-induced wild-type mice was greater than 50-fold higher than in uninduced animals, but Cd induction increased MT-associated radioactivity in *tx* mice only 14-fold, suggesting a greater induced increase in hepatic MT protein synthesis in wild-type mice in response to heavy-metal induction. When MT half-lives ($t_{1/2}$) were calculated, wild-type mouse liver had a $t_{1/2}$ of 20.4 ± 3.1 h (no Cd induction) and 16.4 ± 4.1 h (Cd induction). These values were in agreement with the 15–20 h half-life reported for Cu-MT and Zn-MT [44,45]. *tx*-mouse liver MT had a $t_{1/2}$ of 36.5 ± 1.5 h (no Cd induction) and 29.2 ± 3.8 h (Cd induction): significantly more stable than wild-type with or without heavy-metal treatment. The difference in MT half-life between wild-type and *tx* after Cd induction (77%) was not significantly different from that of animals untreated with Cd (79%). The difference in the rate of disappearance of MT between *tx* and wild-type mice was not evident in non-MT proteins. The half-life of non-MT *tx*-mouse liver proteins was 70.0 ± 9.3 h compared with 76.2 ± 11.1 h for wild-type mouse liver (Figure 7b). Cd treatment decreased the half-life of non-MT proteins by approx. 10% in *tx* mice and 17% in wild-type mice, but introduced no significant difference in degradation rate between the two (Figure 7a). Thus decreased loss of MT in *tx*-mouse liver appears to be specific to MT, or a small fraction of hepatic proteins of which MT is a part. Increased stability of MT protein may account for the high MT level observed in adult *tx* mice in the absence of increased MT mRNA. Whether the stability is due to structural differences in MT resulting from increased association with Cu (similar to the approx. 4-fold increase in Cd-MT half-life in comparison with Cu,Zn-MT [45–50]), altered MT-gene structure or decreased

Figure 7 Degradation rate and half-life of non-MT hepatic proteins

tx (○) and wild-type (●) mice were treated with (a) Cd (1 mg of Cd/kg body wt. 6 h prior to radiolabelling) or (b) iso-osmotic saline. Radiolabel associated with non-MT proteins separated on the same polyacrylamide gels as those described in the legend to Figure 6 was measured as described in the Experimental section and plotted at various times after [³⁵S]cysteine injection. Each point represents results from four to seven mice.

activity of proteinase(s) responsible for MT degradation is not addressed by these experiments.

Metallothionein isolated from *tx*-mouse liver contained the majority (83%) of cytosolic Cu present in that organ and approx. 53% of hepatic cytosolic zinc (Figure 2a). Therefore MT was the major Cu storage protein in *tx* mice. Treatment with Cd (1 mg/kg for 24 h) depleted the cytosolic MT-containing protein fraction of both Zn (decreased 49%) and Cu (decreased 11%). Cu and Zn in the high-molecular-mass non-MT fraction were changed only slightly: Zn content was decreased 7% and Cu content was increased 5% (Figure 2b). Cd treatment increased hepatic cytosolic Cd levels from undetectable to 4.0 p.p.m., almost exclusively within the MT-containing column fraction (Figure 2b). Therefore hepatic metallothionein in *tx* mice was capable of binding administered Cd, presumably by displacing MT-bound zinc (to a major degree) and copper (to a minor degree) in a manner similar to that observed in neonatal rats [49].

In summary, we found that a consequence of the *tx* mutation in mice was a large (100-fold) accumulation of MT protein in

liver without concomitant increased accumulation of MT mRNA, in spite of the fact that *tx* mouse MT genes were responsive to metal induction. We suggest that altered mRNA translation and/or altered proteolysis of MT (either by heritable changes in MT structure or proteolytic activity in liver) must account for the high constitutive levels. In fact, the half-life of MT was 79 and 77% longer (before and after induction with Cd salts, respectively) in *tx* versus wild-type mouse liver. Thus increased MT accumulation in affected mice could involve reduced levels of protein degradation. The underlying metabolic defect(s) leading to reduced proteolysis remains undefined. Increased MT half-life in *tx* animals does not imply that altered MT regulation is the underlying specific defect leading to pathological changes in *tx* mice. Changes in degradation of other proteins, or in transcription, translation, or degradation of MT mRNA or other mRNAs could also contribute to the *tx* phenotype. However, these data suggest that altered proteolysis (possibly through the cathepsin B-mediated pathway suggested to be responsible for MT degradation [51]) could be a basis of the *tx* defect.

The similarities between human Wilson's disease and the consequences of the *tx* mutation in mice (excessive hepatic Cu accumulation, low caeruloplasmin levels, reduced rate of incorporation of Cu into caeruloplasmin [34], high hepatic MT levels [38] with a lack of correlation between MT mRNA accumulation and hepatic Cu level [38], and toxic changes in livers in both *tx* mice and certain patients with Wilson's disease [52,53]) make these mice a possible model with which to study perturbed Cu metabolism. It is not yet clear whether increased MT and Cu accumulation in the human diseases is caused by MT induction by the excess Cu, MT expression induced by toxic stress effects [1,54] co-incident with, or caused by, excess Cu or altered post-transcriptional treatment of MT mRNA or stability of MT protein. We suggest that the increased MT protein stability reported here in *tx* animals may be an appropriate area of investigation for human disorders (Wilson's disease, for example) involving altered Cu metabolism.

It is intriguing that the *tx* defect leads to both Cu deficiency *in utero* and Cu toxicosis *ex utero* in the same *tx* genotype [20]. The fact that the pattern of human and mouse MT expression is similarly developmentally regulated [8,9,55] suggests that this observation may be relevant to human development. The study of *tx* mice may be informative in understanding diseases involving Cu toxicity, the cellular role of MT (heavy-metal and drug resistance, Zn and Cu homeostasis) and the physiological consequences of chronic high MT accumulation.

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