Role of Glutaredoxin1 and Glutathione in Regulating the Activity of the Copper-transporting P-type ATPases, ATP7A and ATP7B

Received for publication, June 14, 2010. Published, JBC Papers in Press, June 21, 2010, DOI 10.1074/jbc.M110.154468

William C. J. Singleton‡, Kelly T. Mclnnes†, Michael A. Cater§, Wendy R. Winnall‡, Ross McKirdy†, Yu Yu†, Philip E. Taylor†, Bi-Xia Ke‡, Des R. Richardson‡, Julian F. B. Mercer‡, and Sharon La Fontaine‡

From the ‡ Strategic Research Centre for Molecular and Medical Research and Centre for Cellular and Molecular Biology, School of Life and Environmental Sciences, Deakin University, Burwood, 3125 Victoria, the ‡ Mental Health Research Institute of Victoria, Parkville, 3052 Victoria, the § Centre for Reproduction and Development, Monash Institute of Medical Research, Clayton, 3168 Victoria, and the † Department of Pathology, University of Sydney, Sydney, 2006 New South Wales, Australia

The copper-transporting P-type ATPases (Cu-ATPases), ATP7A and ATP7B, are essential for the regulation of intracellular copper homeostasis. In this report we describe new roles for glutathione (GSH) and glutaredoxin1 (GRX1) in Cu homeostasis through their regulation of Cu-ATPase activity. GRX1 is a thiol oxidoreductase that catalyzes the reversible reduction of GSH-mixed disulfides to their respective sulfhydryls (deglutathionylation). Here, we demonstrated that glutathionylation of the Cu-ATPases and their interaction with GRX1 were affected by alterations in Cu levels. The data support our hypothesis that the Cu-ATPases serve as substrates for Cu-dependent GRX1-mediated deglutathionylation. This in turn liberates the Cu-ATPase cysteinyl thiol groups for Cu binding and transport. GSH depletion experiments led to reversible inhibition of the Cu-ATPases that correlated with effects on intracellular Cu levels and GRX1 activity. Finally, knockdown of GRX1 expression resulted in an increase in intracellular Cu accumulation. Together, these data directly implicate GSH and GRX1 with important new roles in redox regulation of the Cu-ATPases, through modulation of Cu binding by the Cu-ATPase cysteine motifs.

Copper (Cu) is an essential micronutrient for all organisms because it is an indispensable catalytic and structural cofactor for many essential enzymes and proteins (1). Consequently, organisms have evolved mechanisms that regulate intracellular Cu levels to support physiological requirements while preventing a toxic buildup of Cu that would lead to oxidative damage.

This control is mediated by carrier proteins for the uptake, delivery, and efflux of Cu (2). Dysregulation of Cu homeostasis can lead to a variety of pathologies that include the genetically inherited Cu transport disorders, Menkes and Wilson diseases, respectively, and is also implicated in neurodegenerative diseases such as Alzheimer disease (3, 4).

The Cu transport proteins ATP7A and ATP7B are members of the P1b subfamily of the P-type ATPases and are key regulators of systemic Cu levels in mammals (for review, see 5, 6). Through ATP-dependent catalytic activity they transport Cu across cellular membranes. They comprise eight transmembrane domains and an N terminus with six Cu-binding domains. Each Cu-binding domain is ~70 amino acids in length and contains the highly conserved Cu-binding GMXCXXC motif (where X is any amino acid). The Cu-binding domains can each bind one Cu(I) ion that is coordinated by the thiol groups from the two cysteine residues at each site (7–9). At a cellular level, ATP7A and ATP7B have both biosynthetic and protective roles. They reside at the trans-Golgi network (TGN) for the metallaition of Cu-dependent enzymes of the secretory pathway. With elevated Cu levels they undergo a steady-state shift to vesicular compartments in close proximity to the basolateral (ATP7A) or apical (ATP7B) surface for Cu efflux from the cell (10–12) (for review, see 5). When copper levels are restored to normal, ATP7A and ATP7B recycle back to the TGN. Mutation of the corresponding genes in Menkes disease (ATP7A) (Online Mendelian Inheritance in Man (OMIM) 309400) and Wilson disease (ATP7B) (OMIM 2177900) leads to systemic Cu deficiency and liver Cu toxicity, respectively (3). The expression and activity of ATP7A and ATP7B are regulated by a variety of physiological stimuli in addition to Cu as well as posttranslational modifications and protein-protein interactions (13, 14).

Recently, we showed that glutaredoxin1 (GRX1) interacted with the N terminus of ATP7A and ATP7B, and this interaction required Cu and the CXXC motifs (15). GRX1 is a member of the thiol oxidoreductase family of proteins and specifically

© 2010 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
Glutaredoxin1 and Glutathione Regulate ATP7A and ATP7B

reverses S-glutathionylation of protein substrates (16); that is, it catalyzes the reduction of disulfide bonds formed between protein thiol groups and glutathione (GSH), which are often referred to as protein-SSG mixed disulfides. GRX1 is a 12-kDa protein present within the cytoplasm and the intermembrane space of mitochondria, has a GSH-binding site, and is dependent on GSH for its activity. GRX1 also has two redox-active cysteines in a conserved active site motif, CPYC, and it uses one or both cysteines to catalyze deglutathionylation (for review, see 16–18).

Glutathionylation is a reversible posttranslational modification of cysteinyl thiols that is emerging as an important and widespread means of redox regulation and signaling, comparable with protein phosphorylation (for review, see 19, 20). Under conditions of oxidative stress, glutathionylation of proteins is increased and serves as a protective mechanism to prevent irreversible oxidation of thiol groups (e.g. 21, 22). Glutathionylation thus can alter protein function, either activating or temporarily inactivating proteins. Although mechanisms of deglutathionylation have been well characterized, mechanisms of protein-SSG formation have yet to be resolved and may occur spontaneously or via enzymatic reactions (19).

GRX1 interaction with the cysteine-based, Cu-binding motifs in the N-terminal region of ATP7A/7B implicated glutathionylation of the Cu-ATPases as a regulatory or protective modification. We propose that at least some of the Cu-binding domain cysteines may be glutathionylated and that GRX1 deglutathionylates the cysteines to allow Cu ions to coordinate with the thiol groups for subsequent transport (Fig. 1A). Here, we confirm the interaction between the endogenous Cu-ATPases and GRX1 from mammalian cells and provide support for our hypothesis that glutathionylation and GRX1-mediated deglutathionylation regulate the redox state of the Cu-ATPase cysteines and their access to Cu. This study reveals new roles for GSH and GRX1 in regulating ATP7A/7B and thus Cu homeostasis.

**EXPERIMENTAL PROCEDURES**

**Bacterial and Mammalian Cell Strains—**Escherichia coli strain DH10B (Invitrogen) was used for cloning, maintenance, and propagation of plasmids according to standard methods (23). Chinese hamster ovary cells stably expressing ATP7B (CHO-K1/ATP7B) and the ATP7A-expressing fibroblast cell line (A12-H9) were cultured at 37 °C in Eagle’s basal medium (ThermoTrace) as previously described (24, 25). The human HepG2 hepatoma cell line was cultured at 37 °C in Dulbecco’s modified Eagle’s medium (high glucose) (ThermoTrace) as previously described (10). M17 human neuroblastoma cells and HEK293T cells were cultured in OptiMEM (Invitrogen) supplemented with 10% (v/v) FBS, at 37 °C in a 5% CO2 humidified incubator. Where indicated, cell growth medium was supplemented with bathocuproine disulfonic acid (BCS; Sigma) and d-penicillamine (d-Pen; Sigma), buthionine sulfoximine (BSO; Sigma), CuCl2, cycloheximide (Sigma), or N-acetylcyesteine (NAC; Sigma).

**Mammalian Cell Transfections and Immunoprecipitation—**Transient transfection of plasmid DNA into mammalian fibroblast cell lines was carried out using FuGENE® HD (Roche) according to the manufacturer’s instructions. For A12-H9 cells, following transfection with ~5 µg of either pcDNA3 (Invitrogen) or pcDNA3/GRX1-myc (pSLB101) (15) and overnight recovery, cells were left untreated, or the medium was supplemented with 0.1 mM BCS (72 h) and 0.1 mM d-Pen (72 h), 1.0 mM BSO (72 h), 0.2 mM CuCl2 (2 h), or 1 mM BSO (72 h) and 0.2 mM CuCl2 (2 h). After a further 48 h cells were harvested for protein extraction. For co-immunoprecipitations, protein G-coupled magnetic beads (Dynabeads® Protein G; Invitrogen) were used according to recommended protocols. Briefly, cells were lysed as described (26), and ~3 mg of total cell protein or lysis buffer alone was applied to Dynabeads prepared with immobilized antibody, either affinity-purified sheep α-ATP7A (~3 µg of R17-BX) (27) or ammonium sulfate-precipitated sheep α-ATP7B (~80 µg of NC36) (24). Protein complexes were eluted by heating at 90 °C for 5 min in sample buffer supplemented with 20 mM dithiothreitol (DTT) and 50 mM tris(2-carboxyethyl)phosphine (Pierce).

To detect GSH bound to ATP7A, either Dynabeads® M-280 Tosylactivated (Invitrogen) immobilized with ATP7A antibodies were used to immunoprecipitate ATP7A according to the manufacturer’s instructions, or Dynabeads® Protein G prepared with immobilized α-GSH antibodies (~3 µg) (Millipore) were used to immunoprecipitate total GSH-bound proteins. The M-280 tosylactivated Dynabeads were used to prevent the co-elution of nonreduced IgG molecules with ATP7A. Briefly, M17 cells were left untreated or were supplemented with 20, 50, or 200 µM CuCl2 (2 h) or 100 µM BCS/d-Pen (72 h). Cells were lysed in the presence of 50 mM N-ethylmaleimide, and ~3 mg of total cell protein was applied to the antibody-bound Dynabeads. Proteins were eluted from the tosylactivated beads in 0.1 M glycine (pH 2.5), neutralized with 1 M Tris-HCl (pH 8.0), and prepared for electrophoresis in nonreducing sample buffer supplemented with 20 mM DTT only where indicated. GSH-bound proteins were eluted from protein G Dynabeads by heating in sample buffer supplemented with 50 mM DTT.

**Western Blotting—**Protein preparations were fractionated by electrophoresis on standard SDS-polyacrylamide gels or on Novex® 10–20% gradient pre-cast gels using the Xcell Surelock™ mini-cell system (Invitrogen) and transferred to nitrocellulose membranes (Amersham Biosciences). Membranes were incubated with the following primary antibodies at 4 °C overnight as indicated: affinity-purified sheep α-ATP7A (R17-BX, 1:1,000 dilution) (27), ammonium sulfate-precipitated sheep α-ATP7A (1:1,000 dilution), ammonium sulfate-precipitated sheep α-ATP7B (NC36, 1:1,000 dilution) (24), rabbit α-GRX1 (Sapphire BioScience, 1:2,000 dilution), mouse monoclonal α-GSH (Millipore, 1:1,000 dilution), mouse monoclonal α-β-actin (Sigma, 1:10,000 dilution), or rabbit α-catenin (1:500 dilution; Santa Cruz Biotechnology Inc.). Following incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (Sigma, 1:4,000; Dako, 1:10,000), proteins were detected with ECL Western blotting Detection Reagent (Amersham Biosciences), and images were captured using the Luminescent Image Analyzer LAS-3000 (Raytest Isotopenmessgeraete GmbH) and analyzed using Multi Gauge software (Fujifilm).
**Glutaredoxin1 and Glutathione Regulate ATP7A and ATP7B**

**Glutaredoxin Assay**—GRX1 activity of A12-H9 cells was assayed by the standard β-hydroxyethylene disulfide assay as previously described (30). Briefly, the assay mixture contained 1 mM GSH, 0.4 mM NADPH, 2 mM EDTA, 0.1 mg/ml BSA, 6 μg/ml glutaredoxin reductase in 0.1 M Tris-Cl (pH 8) in which β-hydroxyethylene disulfide was added to a final concentration of 0.7 mM. A mixed disulfide between GSH and β-hydroxyethylene disulfide is formed within 2 min, after which an equal amount of cell lysate (250 μg) was added to start the assay. Background nonenzymatic reduction of NADPH was also determined as the blank value. Enzyme activity was calculated from the linear net change in 340 nm absorbance per min.

**Knockdown of GRX1**—The following pairs of oligonucleotides encoding GRX1-targeted hairpin siRNAs (Table 1) were annealed and cloned into the pSilencer 4.1-CMV puro vector (Ambion): GRX1–9(F)/GRX1–10(R) and GRX1–11(F)/GRX1–12(R). Note that the hairpin siRNA encoded by GRX1–11/12 was based on previously reported sequences used for GLRX1–9/10 and GRX1–11/12 separately and in combination. After 72 h, cells were harvested and cell pellets were snap-frozen in liquid nitrogen and stored at −80 °C for RNA extraction, or prepared for off-target effects.

**RNA Extraction and cDNA Synthesis**—Transfected HEK293T cells were lysed in 1 ml TRIzol (Invitrogen), and total RNA was purified by chloroform extraction and treated with DNase to remove genomic DNA using the DNAfree kit (Ambion) according to the manufacturer’s instructions. RNA concentrations were determined using nanodrop spectrophotometry.
Glutaredoxin1 and Glutathione Regulate ATP7A and ATP7B

(Thermo Scientific). Random hexamer (Invitrogen) primed cDNA was synthesized from 1 μg of RNA using the Superscript kit™ (Invitrogen) according to the manufacturer’s instructions. Nonreverse transcribed controls were produced where primers and RNA were subjected to the same synthesis conditions minus reverse transcriptase enzyme. Added to the master mix of each cDNA reaction was an equal amount of an in vitro-transcribed RNA spike. This spike controls for the efficiency of the cDNA reaction and can be used to verify the constant expression of a housekeeping gene. Its construction and use have been described in detail in Ref. 32.

QRT-PCRs—QRT-PCR analysis was performed using the FastStart DNA Master SYBR Green system (Roche Applied Science) and an iQ5 QRT-PCR machine (Bio-Rad). Specific primers for the detection of GRX1 and 18 S rRNA are described in Table 1. Amplification conditions for each primer set consisted of 10-min denaturation at 95 °C followed by 40 repeats of 15 s at 95 °C, 10 s at 60 °C, and 20 s at 72 °C in the presence of 3 mM Mg²⁺. Sample cDNAs were diluted 1:15 in diethylpyrocarbonate-treated water immediately prior to PCRs, making a final dilution of 1:150 cDNA in the PCR. Melting curve data acquisition was conducted from 70 °C to 97 °C to confirm product purity. For all real time PCRs, standard curves were generated using purified PCR products in sequential 10-fold dilutions to calculate the efficiency of the reaction. In initial experiments, PCR product sizes were verified by agarose gel electrophoresis and sequenced using the primers in Table 1 to confirm identity. The absence of contaminating genomic DNA in cDNA samples was confirmed using nonreverse transcribed controls. PCR product purity was routinely monitored by determination of the PCR product melting temperature. To validate the use of 18 S rRNA as a housekeeping gene for normalization, 18 S rRNA levels were measured and normalized using an RNA spike using the gene for normalization, 18 S rRNA levels were measured and monitored by determination of the PCR product melting temperature. To validate the use of 18 S rRNA as a housekeeping gene for normalization, 18 S rRNA levels were measured and normalized using an RNA spike using the ΔΔCt method (33) as previously described (32). 18 S rRNA levels remained stable across the experimental groups (data not shown), making 18 S rRNA an appropriate housekeeping gene for normalization in these studies. GRX1 mRNA levels were therefore normalized to 18 S rRNA levels using the Livak method. The efficiency of each reaction was between 90 and 105%.

Statistical Analysis—Unless otherwise indicated, data are reported as means ± S.E., where n = at least 3. Statistical analysis was carried out using Student’s t test or one-way analysis of variance using GraphPad Prism version 5.01 software (GraphPad Software). A Bartlett’s test for equal variance determined that the data in Fig. 7A required transformation. These data were log-transformed and analyzed by one-way analysis of variance using a Dunnett’s multiple comparison test.

RESULTS

GRX1 Interacts with ATP7A and ATP7B—Previously we showed that myc-tagged GRX1 exogenously expressed in fibroblast cells that also stably expressed ATP7A (A12-H9 cells) or ATP7B (WND#16 cells) could be co-immunoprecipitated with both proteins (15). In the current study, we used antibodies specific for ATP7A (27) and ATP7B (24) to immunoprecipitate the endogenous proteins from M17 and HepG2 cells, respectively and showed that endogenous GRX1 also co-precipitated (Fig. 1B). This result confirmed that GRX1 interacts with both Cu-ATPases.

Interaction between GRX1 and ATP7A Requires and Is Enhanced by Copper—The interaction between ATP7A and GRX1 was analyzed under different conditions. A12-H9 cells that transiently expressed myc-tagged GRX1 or transfected with the empty pcDNA3 vector as a control were treated to alter Cu and GSH levels using BCS/D-Pen and BSO, respectively. BSO is an irreversible inhibitor of γ-glutamylcysteine synthetase, the first enzyme in the GSH biosynthetic pathway, and the concentration used depleted cells of GSH by >90% (see below). Analysis of cell lysates (Fig. 1Ci) showed that the amount of ATP7A was unchanged following the various treatments. GRX1-myc levels also remained unchanged between samples. Approximately equal amounts of ATP7A were immunoprecipitated from each cell lysate (Fig. 1Cii). The amount of GRX1-myc that co-precipitated with ATP7A decreased following Cu depletion with BCS/D-Pen, as previously shown (15), but was increased with the addition of Cu (Fig. 1C, ii and iii). A similar increase was detected in GSH-depleted cells supplemented with Cu (BSO + CuCl₂), but there was no change in the amount of co-precipitated GRX1-myc when cells were depleted of GSH alone (+ BSO). These data confirmed that the GRX1-ATP7A interaction was dependent on Cu and in fact was enhanced by Cu and were consistent with our hypothesis and model (Fig. 1A).

ATP7A Is Glutathionylated—To determine whether GSH could be detected bound to the Cu-ATPases, ATP7A was immunoprecipitated from M17 cells that were treated with N-ethylmaleimide to block free sulfydryls and prevent further thiol/disulfide exchange. ATP7A was detected in the M17 cell lysate as well as in the eluted immunoprecipitated protein samples, and neither N-ethylmaleimide nor DTT affected the amount of protein that was immunoprecipitated (Fig. 2A, left panel). The two bands observed are likely to represent the unglycosylated and mature glycosylated forms of ATP7A (34). When a duplicate membrane was probed with anti-GSH antibodies, GSH-bound proteins were immunoprecipitated from M17 cells that were treated with CuCl₂ (20 and 200 μM, 2 h) or BCS/D-Pen (100 μM, 72 h), then probed with anti-ATP7A antibodies. ATP7A expression levels were not affected by these treatments (Fig. 2B, Lysates). There was a decrease in the amount of immunoprecipitated ATP7A with 200 μM Cu but no obvious change following exposure to 20 μM Cu or BCS/ D-Pen (Fig. 2Bi). When the experiment was repeated to quantify the GSH-bound ATP7A, there was a significant decrease in immunoprecipitated ATP7A following exposure to 50 and 200 μM CuCl₂ (Fig. 2Bii). We concluded that Cu levels can affect the extent to which the Cu-ATPases are glutathionylated.
GSH Depletion Impairs the Cu-regulated Trafficking of ATP7A and ATP7B—To investigate the effect of depleting cellular GSH on Cu-ATPase trafficking, A12-H9 (ATP7A) and CHO-K1 cells stably expressing ATP7B (CHO-K1/ATP7B) (24) were analyzed for Cu-induced trafficking of ATP7A/7B following treatment with BSO and CuCl2, alone and in combination. The viability of A12-H9 and CHO-K1/ATP7B cells was maintained at 80–100% relative to untreated controls, following treatment with up to 1 mM BSO for 72 h. Under these conditions, cellular GSH was reduced by 90% in both cell lines, consistent with previous reports (35). The expression levels of ATP7A and ATP7B were not affected (data not shown). For all subsequent experiments, a concentration of 0.5 mM BSO over 72 h was used.

Cells treated with CuCl2, BSO, or both were analyzed by immunofluorescence microscopy for effects on ATP7A and ATP7B trafficking. Under basal conditions, ATP7A and ATP7B were located predominantly at the TGN, indicated by co-localization with the TGN marker proteins p230 and syntaxin6, and this localization was not affected by BSO (Fig. 3, A and B, 1st and 3rd rows).

Following exposure to 200 μM Cu for 2 h, a significant proportion of ATP7A molecules trafficked toward the cell periphery (calculated as a 66% decrease (p < 0.0001) in co-localization with p230, compared with that in basal medium) (Fig. 3 A, 2nd row). ATP7B trafficked to a vesicular compartment (Fig. 3 B, 2nd row). These results were consistent with similar previous observations in these cell lines (24, 25). However, when cells were exposed to elevated Cu levels for the last 2 h of the 72-h BSO treatment, the Cu-induced redistribution of ATP7A and ATP7B was not observed (the amount of co-localization between ATP7A and p230 was not significantly different from untreated cells) (Fig. 3, A and B, 4th rows). Similar effects of BSO were observed with endogenously expressed ATP7A in M17 cells (data not shown).
CuCl₂ supplementation and then returned to basal medium for ing and impaired Cu efflux.

Asterisks indicated the antibodies were immunoprecipitated from cells treated with CuCl₂ after normalization with mean immunoprecipitated calnexin from the same lysate. Data represent the error bars and were derived from three independent experiments. Asterisks indicate values that are significantly different from the control: *, p < 0.05; **, p < 0.005.

FIGURE 2. ATP7A is glutathionylated. A, detection of GSH bound to ATP7A. Immunoprecipitation (IP) of ATP7A from M17 cell lysates was carried out using immobilized ATP7A antibodies. Lysis buffer alone was added to the antibody (Ab)-bound beads as a negative control. Duplicate samples of total protein (~50 µg) and the immunoprecipitated proteins without or with 20 mM DTT, as indicated, were fractionated and then immuno-blotted with α-ATP7A or α-GSH antibodies. B, glutathionylation of ATP7A was decreased following exposure to elevated Cu concentrations. i, total GSH-bound proteins were immunoprecipitated from cells treated with CuCl₂ or BCS/O-Pen as indicated. Lysis buffer alone was added to the antibody-bound beads as a negative control. The immunoprecipitated proteins and total cell protein (~50 µg) were immuno-blotted with α-ATP7A (top panels) or α-calnexin antibodies (bottom left panel). ii, densitometric quantitation of immunoprecipitated ATP7A protein from cells treated with CuCl₂ after normalization with immunoprecipitated calnexin from the same lysate. Data represent the mean ± S.E. (error bars) and were derived from three independent experiments. Asterisks indicate values that are significantly different from the control; *, p < 0.05; **, p < 0.005.

not shown). We concluded that the Cu-regulated trafficking of ATP7A and ATP7B is impaired when GSH levels are depleted.

**GSH Depletion Increases Cellular Cu Levels**—The effect of BSO-mediated disruption of Cu-ATPase trafficking on Cu accumulation in cells was assessed by measuring total cellular Cu. When A12-H9 and CHO-K1/ATP7B cells were supplemented with 0.1 mM CuCl₂ alone for 24 h, there was a modest increase in the amount of Cu that accumulated in cells. However, following supplementation with CuCl₂ for the last 24 h of the 72-h BSO treatment, cells accumulated ~4-fold and 8-fold more Cu, respectively, than cells treated with CuCl₂ alone (Fig. 4, Ai and Bi). This result was consistent with defective trafficking and impaired Cu efflux.

When cells were treated as above with BSO followed by CuCl₂ supplementation and then returned to basal medium for 8 or 24 h, the amount of Cu that accumulated in cells gradually decreased (Fig. 4, Ai and Bi), suggesting that the ability of cells to export the excess Cu was slowly being restored. The distribution of ATP7A and ATP7B in the cells at various time points after return to basal medium was determined by immunofluorescence microscopy. At the “0” time point (maximal Cu accumulation), both ATP7A/7B had a tight perinuclear location and remained there for up to 8 h when some trafficking could be detected. By 24 h, the majority of both proteins had relocated to cytoplasmic vesicular compartments and the cell periphery, consistent with the high levels of Cu still remaining within cells at this time point (Fig. 4, Aii and Bii). Therefore, recovery of Cu export coincided with restoration of trafficking activity. We concluded that the BSO-mediated Cu accumulation was directly linked to the inability of the Cu-ATPases to undergo Cu-induced trafficking and/or to transport Cu under these conditions. Treatment of BSO/Cu-treated cells with the protein synthesis inhibitor, cycloheximide during the recovery period in basal medium, showed that the recovery of trafficking and Cu transport activity was largely due to preexisting Cu-ATPase proteins. GRX1 levels also were largely unaffected (data not shown).

**Increasing GSH Levels Enhances Cu Export**—The rescue of Cu export in GSH-depleted cells as shown above, was potentially due to new synthesis of GSH. To determine the GSH content under these conditions and whether this rescue effect could be further enhanced by replenishing GSH levels, the experiment was repeated with recovery for 8 h in basal medium or in basal medium supplemented with NAC. NAC, a synthetic derivative of L-cysteine, is readily absorbed by cells and is rapidly hydrolyzed to cysteine, the rate-limiting component in GSH synthesis. The cellular GSH content was inversely related to Cu levels (Fig. 5). During the recovery period, GSH levels were slightly increased as Cu levels decreased. However, enhanced GSH levels (by NAC addition) corresponded to a further significant decrease in cellular Cu levels (Fig. 5). The increase in GSH levels was a direct effect of NAC addition because NAC in the presence of BSO has little effect (data not shown).

**GRX1 Has a Role in Maintaining Intracellular Cu Levels**—Because GRX1 is dependent upon GSH for its activity, it was possible that the effects of GSH depletion on Cu-ATPase activity were manifested through reduced GRX1 activity under these conditions. GRX1 activity was reduced by 10% (p < 0.005) following Cu supplementation and by 15% (p < 0.005) with BSO + CuCl₂ treatment, relative to untreated cells (Fig. 6). GRX1 activity was restored to normal levels only following recovery in basal medium for 24 h, in the absence and presence of NAC (Fig. 6).

To analyze the contribution of GRX1 to Cu homeostasis directly, GRX1 expression levels were knocked down using plasmid-encoded hairpin siRNAs (GRX1–9/10 and GRX1–11/12), alone and in combination. GRX1–11/12 was more effective than GRX1–9/10, reducing GRX1 mRNA expression levels by ~60% relative to the control siRNA (Fig. 7A). The knock-down experiment was repeated using just the GRX1–11/12 and control siRNA plasmids in duplicate sets, where one set was left untreated and one was Cu-treated. Here, GRX1 expression was reduced by ~90% in the GRX1–11/12-transfected cells (data not shown). There was little difference in Cu levels between control- and GRX1–11/12-transfected cells that were not treated with Cu. However, following Cu supplementation,
there was a significant (~30%) increase in the amount of Cu that accumulated in the GRX1–11/12-transfected cells compared with the control siRNA-transfected cells (Fig. 7B). Therefore, knockdown of GRX1 reduced the ability of HEK293T cells to export excess Cu.

**DISCUSSION**

The data presented demonstrate new key roles for GSH and GRX1 in regulating the activity of ATP7A and ATP7B, and hence Cu homeostasis. This regulation is through posttranslational mechanisms, glutathionylation/deglutathionylation that are involved in the regulation of an increasing number of proteins but for the first time have been implicated in the regulation of Cu-trafficking proteins. The model under consideration in this study was based on the demonstrated interaction between GRX1 and the Cu-binding N terminus of ATP7A and ATP7B (Ref. 15 and this study). According to this model, at least some of the thiol groups of the Cu-binding cysteines within the CXXC motifs of ATP7A/7B are glutathionylated and consequently, unavailable to bind Cu. GRX1 would deglutathionylate the ATP7A/7B-SSG mixed disulfide, releasing the reduced ATP7A/7B-SH protein that can then coordinate Cu(I) ions for subsequent transport (Fig. 1A). The results from this study support this model.

Cu(I) binding is favored by sulfur donor ligands such as the cysteine thiolate and Cu coordination to cysteinyl thiols has been well documented among Cu-sensing and Cu-trafficking proteins (36, 37). Thus, cytosolic Cu is trafficked and maintained as Cu(I) complexes such as cysteinyl thiols such as CXXC. Because of high levels of small thiol-containing molecules such as GSH, overall, the eukaryotic cytosol is a reducing environment in which the cysteine residues of Cu-trafficking proteins are thought to be reduced and available to bind Cu(I) (36). However, the susceptibility of thiol groups to oxidative modifications makes them prime candidates for posttranslational modifications such as glutathionylation, either for redox regulation and signaling or for protection during conditions of oxidative stress (19, 38). Ion translocators are among the many proteins regulated by thiol oxidation (39, 40). Despite the reducing environment of the cytosol, glutathionylation of some proteins persists, whereas others require a stimulus (19, 20). In particular, the cysteine residues of some redox-sensitive proteins such as enzymes and receptors may exist as the thiolate anion that is particularly reactive and susceptible to glutathionylation (41).

We demonstrated an interaction between endogenous GRX1 and the Cu-ATPase proteins, which suggested some basal degree of glutathionylation of the Cu-ATPases under normal conditions, as observed with other proteins (38, 39). Despite the high expression level of endogenous GRX1 detected in M17 and HepG2 cells, the amount of co-precipitated GRX1 was consistently low and difficult to detect (Fig. 1B). This observation was not surprising because GRX1 has a widespread role in the cell and is likely to be associated with a large number of cellular proteins in addition to the Cu-ATPases. This result also was consistent with the rapid, transient nature of glutathionylation/deglutathionylation (38) that would permit constitutive Cu translocation by the Cu-ATPases, for example into the TGN lumen for metallation of Cu-dependent enzymes.

The increased ATP7A-GRX1 interaction with increased Cu levels and the decreased interaction with Cu depletion were consistent with our model and suggested that GRX1 interaction with the Cu-ATPases was tied to the need for Cu binding and
Glutaredoxin1 and Glutathione Regulate ATP7A and ATP7B

The increased GRX1 co-precipitation when elevated Cu levels were combined with depleted GSH levels was unexpected (Fig. 1Cii). However, a similar observation was made for apoptosis signal regulating kinase1 (ASK1) where the dissociation of GRX1 from ASK1 was inhibited by BSO under conditions of oxidative stress and hence was dependent on GSH (42). In the current study, conceivably, with elevated Cu levels GRX1 was recruited to ATP7A, but with depleted GSH levels, GRX1 was ineffective at deglutathionylating ATP7A and was unable to dissociate from it. This nonproductive interaction of GRX1 with ATP7A was consistent with the disrupted Cu-ATPase trafficking and Cu export under these conditions, which led to increased Cu accumulation in cells (Fig. 4A).

Consistent with our model, ATP7A glutathionylation was decreased following exposure of cells to elevated Cu levels. This observation was likely due to enhanced deglutathionylation by GRX1 to allow Cu binding and was consistent with the increased GRX1 interaction seen with Cu excess. Physiological Cu levels are in the range of 1–20 μM (43).

The Cu-loading conditions used in this study were chosen to represent near physiological levels (20 and 50 μM CuCl₂) or Cu excess (200 μM), the latter designed to obtain maximal responses of the Cu-ATPases. For both ATP7A and ATP7B, trafficking has been demonstrated at high Cu levels (>200 μM) and at physiological Cu levels (even as low as 1 μM Cu) by ours and other groups (10, 12, 44).

Cu is a redox active metal that by its nature can generate oxidative stress at greater than physiological concentrations. A precise and key role of the Cu-ATPases is to expel excess Cu to prevent and/or alleviate Cu-mediated oxidative stress, and they achieve this by trafficking to the cell periphery. This study reveals that the Cu-ATPases are redox-regulated in a Cu-dependent manner; that is, that the level of Cu-ATPase glutathionylation progressively decreases with increasing Cu, and this deglutathionylation allows Cu binding to and activation of the Cu-ATPases to traffic to expel the excess Cu. So whether at physiological Cu levels or in Cu excess, the trafficking of the Cu-ATPases is not a nonspecific effect of oxidative stress, but rather, an elegant and specific mechanism regulated by their

FIGURE 4. Effect of GSH depletion on Cu accumulation in A12-H9 and CHO-K1/ATP7B. Cells of A12-H9 (A) and CHO-K1/ATP7B (B) were cultured in basal medium (0.5–1.0 μM CuCl₂) or in basal medium containing BSO (72 h), CuCl₂ (24 h), or both. Cells were also treated with CuCl₂ or CuCl₂ + BSO followed by incubation in basal medium for 8 h or 24 h. Ai and Bi, Cu content of cell pellets was measured in triplicate by atomic absorption spectrophotometry. Data are expressed as the mean ± S.E. (error bars; n = 3) and were consistent over three independent experiments. Asterisks indicate values that are significantly different. *p < 0.05 versus CuCl₂ + BSO; †p < 0.05 versus CuCl₂ + BSO. Aii and Bii, cells treated with CuCl₂ + BSO as above were transferred to basal medium for different times (0, 1, 2, 8, and 24 h) before being immunostained with α-ATP7A (Aii) or α-ATP7B (Bii) antibodies. Immunolabeled cells were analyzed using a 60 × oil objective lens with an Olympus PROVIS AX70 microscope. Only cells at the 0, 8, and 24 h time points are shown because cells at the 1 and 2 h time points were similar to those at 0 h.

transport. The increased GRX1 co-precipitation when elevated Cu levels were combined with depleted GSH levels was unexpected (Fig. 1Cii). However, a similar observation was made for apoptosis signal regulating kinase1 (ASK1) where the dissociation of GRX1 from ASK1 was inhibited by BSO under conditions of oxidative stress and hence was dependent on GSH (42). In the current study, conceivably, with elevated Cu levels GRX1 was recruited to ATP7A, but with depleted GSH levels, GRX1 was ineffective at deglutathionylating ATP7A and was unable to dissociate from it. This nonproductive interaction of GRX1 with ATP7A was consistent with the disrupted Cu-ATPase trafficking and Cu export under these conditions, which led to increased Cu accumulation in cells (Fig. 4A).

Consistent with our model, ATP7A glutathionylation was decreased following exposure of cells to elevated Cu levels. This observation was likely due to enhanced deglutathionylation by GRX1 to allow Cu binding and was consistent with the increased GRX1 interaction seen with Cu excess. Physiological Cu levels are in the range of 1–20 μM (43).

The Cu-loading conditions used in this study were chosen to represent near physiological levels (20 and 50 μM CuCl₂) or Cu excess (200 μM), the latter designed to obtain maximal responses of the Cu-ATPases. For both ATP7A and ATP7B, trafficking has been demonstrated at high Cu levels (>200 μM) and at physiological Cu levels (even as low as 1 μM Cu) by ours and other groups (10, 12, 44).

Cu is a redox active metal that by its nature can generate oxidative stress at greater than physiological concentrations. A precise and key role of the Cu-ATPases is to expel excess Cu to prevent and/or alleviate Cu-mediated oxidative stress, and they achieve this by trafficking to the cell periphery. This study reveals that the Cu-ATPases are redox-regulated in a Cu-dependent manner; that is, that the level of Cu-ATPase glutathionylation progressively decreases with increasing Cu, and this deglutathionylation allows Cu binding to and activation of the Cu-ATPases to traffic to expel the excess Cu. So whether at physiological Cu levels or in Cu excess, the trafficking of the Cu-ATPases is not a nonspecific effect of oxidative stress, but rather, an elegant and specific mechanism regulated by their
redox state, which in turn is determined mechanistically (rather than nonspecifically) by Cu-dependent GRX1-mediated deglutathionylation. Up to a point, the greater the Cu load, the more pronounced are these effects.

GSH depletion and subsequent recovery experiments linked GSH with the ability of the Cu-ATPases to traffic and mediate Cu export. A recent report that BSO-mediated GSH depletion down-regulated the Cu importer CTR1 (45) suggested that the Cu accumulation seen here in +Cu/+BSO was not due to enhanced Cu uptake. There are three possible explanations for our observations that may not be mutually exclusive.

First, the lack of Cu-ATPase trafficking and Cu accumulation may represent the effects of GSH depletion on other proteins required for Cu-ATPase activity, such as the Cu chaperone ATOX1 and/or other trafficking proteins (5, 6). The formation of a yeast Cu(I)-bridged Atx1 dimer of high affinity for Cu(I) required GSH (47), prompting the suggestion that the Cu(I)-GSH complex is the major source of Cu(I) for Atx1. Hence, it is plausible that GSH depletion disrupted ATOX1-mediated Cu delivery to the Cu-ATPases.

Second, potentially GSH depletion directly impacted GRX1-mediated deglutathionylation because GRX1 requires GSH for activity. Under the same conditions, GRX1 activity was reduced, and its recovery on return to normal growth conditions corresponded with enhanced GSH levels, restoration of Cu-ATPase trafficking, and Cu export. Whether the magnitude of the effect on GRX1 activity was sufficient to account for the effects on Cu-ATPase activity is not clear. With reduced GSH, GRX1 may use other cellular reducing agents for its deglutathionylating activity (48, 49). A direct involvement of GRX1 in facilitating Cu export was confirmed by GRX1 knockdown, which led to an increase in Cu accumulation following Cu supplementation.

Finally, under physiological conditions, reversible S-glutathionylation of protein thiols plays a role in redox signaling and regulates protein activity and function. During moderate oxidative stress, protein glutathionylation increases globally and is
Glutaredoxin1 and Glutathione Regulate ATP7A and ATP7B

A.

![Graph A](image1)

B.

![Graph B](image2)

**FIGURE 7. Cu accumulation in HEK293T cells following siRNA-mediated knockdown of GRX1.** A. Quantitation of GRX1 knockdown assessed by QRT-PCR. HEK293T cells were transiently transfected with the control siRNA plasmid (pSilencer 4.1-CMV puro) or plasmid-encoded GRX1 siRNA (GRX1-9/10, GRX1-11/12, and GRX1-9/10 + 11/12). GRX1 mRNA levels in each culture were compared using normalization to 18 S rRNA levels by the ΔΔCt method. Data are expressed as the mean ± S.E. (error bars; n = 6) and were consistent over two independent experiments. Asterisks indicate values that are significantly different: *, p < 0.01; **, p < 0.001 versus control siRNA. B. GRX1 knockdown results in Cu accumulation in Cu-supplemented cells. HEK293T cells were transiently transfected with either the siRNA control or the plasmid-encoded GRX1-11/12 siRNA. Transfected cells were left untreated or were supplemented with CuCl2 (24 h) and then harvested for Cu analysis by ICP-MS (four replicates) or QRT-PCR analysis (one replicate to confirm knockdown in this experiment). Data are expressed as the mean ± S.E. (error bars; n = 6). Asterisks indicate values that are significantly different: *, p < 0.05 versus control siRNA.

associated with reduced GSH levels. This represents a storage mechanism of GSH inside the cell and a means of protecting sensitive protein thiols against irreversible oxidation (often at the expense of temporary loss of protein function) (38). If the + BSO/+ Cu conditions in this study constituted moderate oxidative stress (because the effects were recoverable), then increased glutathionylation of the Cu-ATPases with associated temporary loss of function may explain the lack of trafficking and Cu accumulation. It also would explain the inverse relationship between Cu and GSH (Fig. 5) and the recovery of Cu export as GSH levels increase, likely due to restoration of Cu-ATPase function following the reversal of glutathionylation.

GSH creates a redox buffer in cells and is the most abundant intracellular antioxidant. Inside the cell, Cu is bound by GSH and transferred to the metallothioneins for storage and detoxification (50–52). The Cu chaperones (ATOX1 and COX17) and GSH also may form an exchangeable Cu-binding pool (53). In the current study, with depleted GSH, the location and the form of the accumulated Cu are not clear. Potentially, metallothioneins may be up-regulated (46) and bind and sequester some of the Cu.

The observations reported in this study are likely to arise from complex interactions among Cu, GSH, and GRX1 as well as other factors in the cell. However, the data clearly show that GSH and GRX1 and hence glutathionylation and deglutathionylation are important in regulating Cu-ATPase activity in a manner that is dependent on intracellular Cu levels. This study, therefore, provides important new insight into the expanding cellular role of GRX1 and has identified a new key player in intracellular Cu homeostasis.

Acknowledgments—We thank Stephanie Materia and Sean Matthews (Deakin University, Australia) for assistance with glutathionylation experiments, Alison Blake for technical assistance, Lidiana Lee (Mental Health Research Institute of Victoria) for assistance with copper analysis by ICP-MS, and Svetlana Lutsenko for critical reading of the manuscript.

REFERENCES

1. Bertini, I., Cavallaro, G., and McGrevey, K. S. (2010) Coord. Chem. Rev. 254, 506–524
2. Kaplan, J. H., and Lutsenko, S. (2009) J. Biol. Chem. 284, 25461–25465
3. Danks, D. M. (1995) in The Molecular and Metabolic Basis of Inherited Disease (Scriber, C. R., Beaudet, A. L., Sly, W. M., and Valle, D., eds) pp. 2211–2235, 7th Ed., McGraw-Hill, New York
4. Hung, Y. H., Bush, A. L., and Cherny, R. A. (2010) J. Biol. Inorg. Chem. 15, 61–76
5. La Fontaine, S., and Mercer, J. F. B. (2007) Arch. Biochem. Biophys. 463, 149–167
6. Lutsenko, S., Barnes, N. L., Bartee, M. Y., and Dmitriev, O. Y. (2007) Physiol. Rev. 87, 1011–1046
7. Achila, D., Banci, L., Bertini, I., Bunce, J., Ciofi-Baffoni, S., and Huffman, D. L. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 5729–5734
8. Lutsenko, S., Petrukhin, K., Cooper, M. J., Gilliam, C. T., and Kaplan, J. H. (1997) J. Biol. Chem. 272, 18939–18944
9. Yatsunyk, L. A., and Rosenzweig, A. C. (2007) J. Biol. Chem. 282, 8622–8631
10. Cater, M. A., La Fontaine, S., Shield, K., Deal, Y., and Mercer, J. F. (2006) Gastroenterology 130, 493–506
11. Guo, Y., Nyasea, L., Braiterman, L. T., and Hubbard, A. L. (2005) Am. J. Physiol. Gastrointest. Liver Physiol. 289, G904–G916
12. Nyasea, L., Bustos, R., Braiterman, L., Epper, B., and Hubbard, A. (2007) Am. J. Physiol. Gastrointest. Liver Physiol. 292, G1181–G1194
13. van den Berghe, P. V., and Klomp, L. W. (2010) J. Biol. Inorg. Chem. 15, 37–46
14. La Fontaine, S., Ackland, M. L., and Mercer, J. F. B. (2010) Int. J. Biochem. Cell Biol. 42, 206–209
15. Lim, C. M., Cater, M. A., Mercer, J. F., and La Fontaine, S. (2006) Biochim. Biophys. Res. Commun. 348, 423–426
16. Lillig, C. H., Berndt, C., and Holmgren, A. (2008) Biochim. Biophys. Acta 1780, 1304–1317
17. Herrero, E., and de la Torre-Ruiz, M. A. (2007) Cell Mol. Life Sci. 64, 1518–1530
18. Gallogly, M. M., Starke, D. W., and Mieyal, J. J. (2009) Antioxid. Redox Signal. 11, 1059–1081
19. Mieyal, J. J., Gallogly, M. M., Qunungo, S., Sabens, E. A., and Shelton, M. D. (2008) Antioxid. Redox Signal. 10, 1941–1988
20. Shelton, M. D., and Mieyal, J. J. (2008) Mol. Cells 25, 332–346
21. Gilge, J. L., Fisher, M., and Chai, Y.-C. (2008) PLoS ONE 3, e9015
22. Eaton, P., Wright, N., Hearse, D. J., and Shattock, M. J. (2002) J. Mol. Cell. Cardiol. 34, 1549–1560
23. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold

27120 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 285 • NUMBER 35 • AUGUST 27, 2010
Glutaredoxin1 and Glutathione Regulate ATP7A and ATP7B

Circ. Res. 105, 185–193

24. Cater, M. A., Forbes, J., La Fontaine, S., Cox, D., and Mercer, J. F. B. (2004) Biochem. J. 380, 805–813

25. La Fontaine, S. L., Firth, S. D., Camakaris, J., Englezou, A., Theophilos, M. B., Petris, M. J., Howie, M., Lockhart, P. J., Greenough, M., Brooks, H., Reddel, R. R., and Mercer, J. F. (1998) J. Biol. Chem. 273, 31375–31380

26. Tao, T. Y., Liu, F., Klomp, L., Wijmenga, C., and Gitlin, J. D. (2003) J. Biol. Chem. 278, 41593–41596

27. Ke, B.-X., Llanos, R. M., Wright, M., Deal, Y., and Mercer, J. F. (2006) Am. J. Physiol. Regul. Integr. Comp. Physiol. 290, R1460–R1467

28. Costes, S. V., Daelemans, D., Cho, E. H., Dobbin, Z., Pavlakis, G., and Lockett, S. (2004) Biophys. J. 86, 3993–4003

29. Dringen, R., Pfeiffer, B., and Hamprecht, B. (1999) J. Neurosci. 19, 562–569

30. Holmgren, A., and Aslund, F. (1995) Methods Enzymol. 252, 283–292

31. Saeed, U., Durgadoss, L., Valli, R. K., Joshi, D. C., Joshi, P. G., and Ravindranath, V. (2008) PLoS ONE 3, e2459

32. Craythorn, R. G., Girling, J. E., Hedger, M. P., Rogers, P. A., and Winnall, W. R. (2009) Mol. Hum. Reprod. 15, 757–761

33. Livak, K. J., and Schmittgen, T. D. (2001) Methods 25, 402–408

34. Yamaguchi, Y., Heiny, M. E., Suzuki, M., and Gitlin, J. D. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 14030–14035

35. Hall, A. G. (1999) Eur. J. Clin. Invest. 29, 238–245

36. Davis, A. V., and O’Halloran, T. V. (2008) Nat. Chem. Biol. 4, 148–151

37. Huffman, D. L., and O’Halloran, T. V. (2001) Annu. Rev. Biochem. 70, 677–701

38. Dalle-Donne, I., Rossi, R., Giustarini, D., Colombo, R., and Milzani, A. (2007) Free Rad. Biol. Med. 43, 883–898

39. Figtree, G. A., Liu, C.-C., Bibert, S., Hamilton, E. J., Garcia, A., White, C. N., Chia, K. M., Cornelius, F., Geering, K., and Rasmussen, H. H. (2009) Biochem. J. 380, 805–813

40. Wang, W., Oliva, C., Li, G., Holmgren, A., Lillig, C. H., and Kirk, K. L. (2005) J. Gen. Physiol. 125, 127–141

41. Poole, L. B., Karplus, P. A., and Claiborne, A. (2004) Annu. Rev. Pharmacol. Toxicol. 44, 325–347

42. Song, J. I., and Lee, Y. J. (2003) Biochem. J. 373, 845–853

43. Linder, M. C. (1991) in Biochemistry of Copper, pp. 1–13, Plenum Press, New York

44. Roelofsen, H., Wolters, H., Van Luyn, M. J., Muiro, N., Kuipers, F., and Vork, R. J. (2000) Gastroenterology 119, 782–793

45. Chen, H. H., Song, I.-S., Hossain, A., Choi, M.-K., Yamane, Y., Liang, Z. D., Lu, J., Wu, L. Y., Siddik, Z. H., Klomp, L. W. J., Savaraj, N., and Kuo, M. T. (2008) Mol. Pharmacol. 74, 697–704

46. Mattie, M. D., and Freedman, J. H. (2004) Am. J. Physiol. Cell Physiol. 286, C293–C301

47. Miras, R., Morin, I., Jacquin, O., Cuillel, M., Guillain, F., and Mintz, E. (2008) J. Biol. Inorg. Chem. 13, 195–205

48. Raghavachari, N., Krysan, K., Xing, K., and Lou, M. F. (2001) Invest. Ophthalmol. Vis. Sci. 42, 1002–1008

49. Terada, T., Hara, T., Yazawa, H., and Mizoguchi, T. (1994) Biochem. Mol. Biol. Int. 32, 239–244

50. Freedman, J. H., Ciriolo, M. R., and Peisach, J. (1989) J. Biol. Chem. 264, 5598–5605

51. White, A. R., and Cappai, R. (2003) J. Neurosci. Res. 71, 889–897

52. Freedman, J. H., and Peisach, J. (1989) Biochem. Biophys. Res. Commun. 164, 134–140

53. Banci, L., Bertini, I., Ciofi-Baffoni, S., Kozyreva, T., Zovo, K., and Palumaa, P. (2010) Nature 465, 645–648