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Copper-dependent interaction of glutaredoxin with the N termini of the copper-ATPases (ATP7A and ATP7B) defective in Menkes and Wilson diseases ☆

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Abstract

The P-type ATPases affected in Menkes and Wilson diseases, ATP7A and ATP7B, respectively, are key copper transporters that regulate copper homeostasis. The N termini of these proteins are critical in regulating their function and activity, and contain six copper-binding motifs MxCxxC. In this study, we describe the identification of glutaredoxin (GRX1) as an interacting partner of both ATP7A and ATP7B, confirmed by yeast two-hybrid technology and by co-immunoprecipitation from mammalian cells. The interaction required the presence of copper and intact metal-binding motifs. In addition, the interaction was related to the number of metal-binding domains available. GRX1 catalyses the reduction of disulphide bridges and reverses the glutathionylation of proteins to regulate and/or protect protein activity. We propose that GRX1 is essential for ATPase function and catalyses either the reduction of intramolecular disulphide bonds or the deglutathionylation of the cysteine residues within the CxxC motifs to facilitate copper-binding for subsequent transport.
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The copper-transporting P-type ATPases ATP7A and ATP7B are critical for regulating systemic copper levels as demonstrated by the devastating effects seen in patients with Menkes disease (MD) and Wilson disease (WD), respectively [1]. ATP7A is expressed ubiquitously in all tissues except the liver whereas ATP7B is expressed predominantly in the liver [2,3]. These proteins actively transport copper to enzymes within the lumen of the *trans*-Golgi network (TGN) and when intracellular copper levels are in excess, they traffic to different locations within the cell; ATP7A to the basolateral membrane [4] and ATP7B to subapical vesicles in which copper is sequestered and eliminated from the cell via the biliary canalicular membrane [1].

The N-terminal regions of ATP7A and ATP7B are essential for regulating the catalytic activity and trafficking of these proteins [5,6]. There are six conserved metal-binding domains (MBD) within an approximately 650 amino acid N-terminal region. Each domain is approximately 70 amino acids in length and contains one metal-binding site (MBS, MxCxxC). The six repeating MxCxxC motifs bind six molar equivalents of copper via the conserved cysteines and this binding of copper is the means by which they regulate the copper transport and the copper-responsive redistribution of the ATPases [7,8]. However, the domains are not functionally equivalent [9] and are between 20% and 60% identical in amino acid sequence. The divergence in their sequences could suggest that they have additional distinct roles. In fact most of the metal-binding sites are

☆ *Abbreviations:* WD, Wilson disease; MD, Menkes disease; GRX, glutaredoxin; TGN, *trans*-Golgi network; AD, activation domain; BCS, bathocuproinedisulphonic acid; BD, binding domain; CPRG, chlorophenol red-β-D-galactopyranoside; TCA, trichloroacetic acid; MBS, metal-binding sites; MBD, metal-binding domains.

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not essential for the overall catalytic activity and trafficking of the ATPases [5,6], and the N-terminal regions may have roles in addition to copper-binding [10]. There are also other signals or motifs within the N-terminal region that potentially direct the copper-induced trafficking of ATP7A and ATP7B [11,12]. In addition, three proteins are now known to interact with the N terminus of either or both of the copper ATPases. The copper chaperone ATOX1 interacts with the N termini of both ATP7A and ATP7B in a copper-dependent manner and this interaction is essential for copper delivery to the secretory pathway [13,14]. COMMD1 (MURR1) is mutated in Bedlington terrier dogs to produce a WD-like copper toxicosis [15] and interacts with the N terminus of ATP7B [16] but not with ATOX1 or ATP7A, a finding consistent with its proposed role in copper excretion from the liver. Recently we showed that dynactin subunit p62 interacts specifically within the region between MBS 4 and 6 of the ATP7B N terminus and we proposed that this interaction plays a role in the copper-regulated trafficking of ATP7B [17]. Copper homeostasis is a complex process most likely mediated by a network of protein interactions. Hence, it is likely that many proteins interact with the ATPases to influence and regulate their copper transport and/or trafficking activities, and also represent potential targets for disease-causing mutations.

To further dissect the regulatory potential of the ATP7B N terminus (ATP7B-N), we previously employed yeast two-hybrid screening of a human liver cDNA library using ATP7B-N as a bait to search for additional interacting partners [17]. This report focuses on one of the proteins, glutaredoxin (GRX1), that interacted with both ATP7A and ATP7B, and is likely to have an essential role in maintaining the activity of the copper ATPases. GRX1 is a small cytosolic protein that catalyses the reduction of disulphide bridges and in particular is known to be a specific and efficient catalyst of protein-SSG deglutathionylation [18]. In this study we show that GRX1 interaction with the N terminus of ATP7A and ATP7B required copper and the CxxC motifs. In addition, the extent of the interaction was related to the number of intact metal-binding sites available to bind copper and potentially the extent to which they are saturated with copper. These results suggest that we have identified a new and key component of the copper homeostatic machinery, one that is likely to regulate the copper-binding and transport activity of the ATPases.

Materials and methods

Bacterial, yeast, and mammalian cell strains. Escherichia coli strain DH10B (F^-, mcrA, Δ(*mrr-hsd*RMS-*mcr*BC), Φ80*lac*ZΔM15, Δ*lac*X74, deoR, recA1, araD, Δ(*ara*,*leu*)7697, *gal*U, *gal*K, *rps*L, *end*A1, *nup*G) was used for the maintenance and propagation of plasmids, and grown in Luria–Bertani medium (LB) (0.1% (w/v) bactotryptone (Difco), 0.05% (w/v) yeast extract (Difco), and 0.1% (w/v) NaCl) containing 100 μg/ml ampicillin for plasmid selection. *Saccharomyces cerevisiae* strain YGH1 (*ura3-25 his3-200 ade2-101 lys-2,801 trp1-901 leu2-3* Can^r *GAL4-542 gal80-538* LYS2::*gal*1$_{uas}$-*gal*1$_{tata}$-HIS3 *URA3*::*gal1-lacZ*) [19] and transformants thereof were maintained at 30 °C on standard yeast extract–peptone–dextrose (YPD) solid or liquid medium (1% yeast extract (Difco), 1% (w/v) tryptone (Difco), 2% (w/v) glucose) [20], or the appropriate synthetic complete dropout (SD) medium (Q-biogene) for the maintenance of plasmids, respectively. Copper-limited SD medium was prepared using yeast nitrogen base without copper sulphate (Q-biogene), and supplemented with the appropriate concentration of bathocuproinedisulphonic acid (BCS) (Sigma). To deplete cells of copper, cultures were passaged for 48 h in BCS-supplemented medium. When copper supplementation was required, the appropriate concentration of $CuSO_4$ was added and cultures were incubated for a further 24 h.

Human skin fibroblast cells overexpressing ATP7A (A12-H9) or ATP7B (WND-16) [21] were maintained in Eagle's basal medium (BME; Thermo Trace) supplemented with 10% (w/v) fetal calf serum (Thermo Trace), 2 mM L-glutamine, 0.2 mM proline, 20 mM Hepes, 0.2% (w/v) sodium bicarbonate, and Geneticin (400 μg/ml) (Invitrogen). Cultures were incubated at 37 °C in a 5% CO_2 humidified incubator. Copper-limited medium was prepared by supplementing BME with 100 μM BCS and 100 μM D-penicillamine (Sigma).

Generation of plasmid constructs. To generate a bait construct that encoded the N terminus of ATP7B (ATP7B-N), a 1971 bp fragment that encoded amino acids 1 to 657 was amplified by PCR using pCMB278 [5] as a template, the forward primer 5′-ccc**catatg**ATGCCTGAGCAGGA GAGACAG-3′ (hWND30) and the reverse primer 5′-ggg**cagc tg**CGACGTGTCCTTTCTGAAGAAGGTGAC-3′ (hWND8) (*Nde*I and *Sal*I restriction sites in lowercase, boldface type). The resulting PCR product was cloned into the *Nde*I/*Sal*I restriction sites of the pAS2-1 GAL4 DNA-binding domain (BD) vector (Clontech) to create pAS2-1/ATP7B-N (pSLB10). A 1.8 kb fragment of ATP7A that encoded the N terminus from amino acid 1 to 600 was cloned as a *Bam*HI/*Pst*I fragment from pCMB151 [14] into pAS2-1, to create pAS2-1/ATP7A-N (pSLB34).

To create plasmid constructs that encoded mutated or deleted ATP7B N-terminal regions as baits, PCR was carried out using the forward primer hWND30 and the reverse primer hWND8 and the following plasmids as templates [5]: pCMB398 (ATP7B-N with both cysteines within the CxxC motifs of all six of the metal-binding domains mutated to serine, that is SxxS); pCMB404 (ATP7B-N with MBS 1–5 deleted); pCMB405 (ATP7B-N with MBS 4–6 deleted). The PCR products were cloned into the *Nde*I/*Sal*I sites of pAS2-1 to create the following plasmids: pAS2-1/MBS1-6c/s (pSLB96); pAS2-1/MBS1-5del (pSLB97); pAS2-1/MBS4-6del (pSLB98), respectively.

To produce a construct that encoded an N-terminal c-myc epitope-tagged GRX1, the full-length *GRX1* cDNA was amplified from the pACT2/GRX1 library plasmid (pSLB99) using the forward primer 5′-gca**aagctt**GGCATGGAACAAAAACTCATATCAGAAGAAGACCTG GCTCAAGAGTTTGTGAA CTGC-3′ (GRX1-F1) and the reverse primer 5′-tgc**ggatcc**TTACTGCAGAGCTCCAATCTGCTT-3′ (GRX1-R2) (*Hin*dIII and *Bam*HI restriction sites in lowercase, boldface type). The sequence that encoded the c-myc epitope tag (underlined, amino acid sequence EQKLISEEDL) was incorporated into the forward primer. The PCR product was cloned into the pGEM-T Easy vector (Promega). The *GRX1-myc* cDNA was then isolated as a *Hin*dIII/*Bam*HI fragment and cloned into the *Hin*dIII and *Bam*HI sites of the mammalian expression vector pcDNA3 (Invitrogen), to create pcDNA3/*GRX1-myc* (pSLB101).

Yeast two-hybrid screening. A human liver cDNA library constructed in the pACT2 GAL4 activation domain (AD) vector (Clontech) was screened using ATP7B-N as the bait, as previously described [17]. Liquid β-galactosidase assays using chlorophenol red-β-D-galactopyranoside (CPRG) (Sigma) as the substrate were performed by following the Clontech protocol (Yeast Protocols Handbook, Clontech, 2000). For each culture within a single experiment the β-galactosidase activity of triplicate samples was measured. The values obtained represent β-galactosidase activity calculated in Miller units using the average value obtained from at least three independent experiments ± SD.

DNA sequence analysis. Sequencing of plasmid DNA was carried out using ABI PRISM BigDye Terminator chemistry (Applied Biosystems) with reactions analysed on an Applied Biosystems 3730S capillary

sequencer apparatus at Micromon DNA Sequencing Facility, Monash University (Melbourne, Australia). Sequence analysis and basic sequence manipulation were performed using Sequencher (GeneCodes). Electronic database searches were conducted using the BLASTN and BLASTP algorithms on the NCBI website (www.ncbi.nlm.nih.gov/BLAST/).

Immunoblot analysis of S. cerevisiae YGH1 transformants. Total protein extracts from *S. cerevisiae* YGH1 transformants were prepared using a TCA extraction method as described in the Yeast Protocols Handbook (Clontech, 2000). Approximately 50 μg of protein was fractionated by SDS–PAGE. Proteins were transferred to Hybond-C+ nitrocellulose membranes (Amersham) using a Trans-Blot semi-dry electrophoretic transfer apparatus (Bio-Rad) and detected with either sheep-derived, affinity-purified NC36 anti-ATP7B antibody (diluted 1:500 [5]), or rabbit-derived antibodies directed against the GAL4 DNA-BD (final concentration 2 μg/ml) (Sigma) or the GAL4 AD (final concentration 1 μg/ml) (Sigma). Secondary antibodies used for detection were a rabbit anti-goat IgG peroxidase conjugate (Sigma) and a goat anti-rabbit IgG peroxidase conjugate (Chemicon), respectively. Protein bands were detected after incubation in Lumi-Light Chemiluminescence blotting substrate (Roche) and visualised using the Luminescent Image Analyser LAS-3000 (Raytest Isotopenmessgeraete GmbH).

Mammalian cell transfection and co-immunoprecipitation. Transient transfection of plasmid DNA into mammalian fibroblast cell lines was carried out using Lipofectamine (Invitrogen) and recommended protocols. For co-immunoprecipitation of interacting proteins from mammalian cells, the ProFound™ Mammalian Co-Immunoprecipitation Kit (Pierce) was used according to the manufacturer's instructions. Specifically, 200 μg of the affinity-purified NC36 anti-ATP7B antibody [5], 200 μg of the affinity-purified R17-BX anti-ATP7A antibody [22] and, as a control, sheep pre-immune serum were covalently immobilised to the amine-reactive antibody coupling gel. Human Menkes patient fibroblasts that stably expressed either ATP7A (A12-H9) or ATP7B (WND-16) [21] were transiently transfected with pcDNA3/*GRX1-myc* or left untransfected and grown to confluence in 75 cm^2 flasks either in the absence or presence of the copper chelators BCS (100 μM) and D-penicillamine (100 μM). Cells were lysed and applied to the gel. The protein complexes were eluted in 50 μl elution buffer, boiled in sample buffer with added DTT (20 mM final concentration), and fractionated by SDS–PAGE. Proteins were transferred to Hybond-C+ membrane (Amersham) using a Trans-Blot semi-dry electrophoretic transfer apparatus (Bio-Rad) and detected with either the NC36 antibody (diluted 1:500 [5]), the R17-BX antibody (diluted 1:1000 [22]), or a rabbit-derived anti-myc polyclonal antibody (diluted 1:1000 Abcam (ab9106)). The secondary antibodies used for detection were a rabbit anti-goat IgG peroxidase conjugate (Sigma) or a sheep anti-rabbit IgG peroxidase conjugate (Chemicon). Protein bands were detected after incubation in Lumi-Light Chemiluminescence blotting substrate (Roche) and visualised using the Luminescent Image Analyser LAS-3000 (Raytest Isotopenmessgeraete GmbH).

Results

Screening of a human liver cDNA library for interacting partners of the ATP7B N-terminal domain

A human liver cDNA library was previously screened with the entire N-terminal domain of ATP7B from amino acid 1 to 657 [17]. Of 36 different cDNAs identified to encode interacting proteins one encoded the full-length GRX1 protein. Based on the presence of the conserved copper-binding motif, CxxC in GRX1, the well-established role of GRX1 in regulating the redox status of protein thiols, in particular its role in reducing intramolecular disulphide bonds and in deglutathionylating cysteinyl thiols, and the abundance of cysteine residues in the copper ATPases, GRX1 seemed a likely candidate to impact on the activity of the copper ATPases and was selected for further detailed analysis. The plasmid contained an insert of approximately 800 bp of which 321 bp comprised an open reading frame that encoded the full-length GRX1 protein (106 amino acids), 54 bp comprised part of the 5′untranslated region (UTR), and 420 bp comprised part of the 3′UTR to the start of the polyA tail.

GRX1 interacts with the N terminus of ATP7A and ATP7B

Saccharomyces cerevisiae YGH1 co-transformants were generated that expressed the full-length GRX1 protein and either ATP7B-N or ATP7A-N fused in-frame to the DNA-BD of GAL4. The negative control was a transformant that contained pAS2-1/ATP7B-N and pACT2. Using a β-galactosidase activity assay we showed that GRX1 interacted with the N termini of ATP7B and ATP7A, albeit to a slightly lesser extent with ATP7A (Fig. 1A). The negative control showed that the positive β-galactosidase result with ATP7B-N and GRX1 did not arise from interaction with vector-derived proteins. There was also no interaction between GRX1 and the unrelated protein Bcl2 (data not shown). The appropriate fusion proteins were expressed in all co-transformants, so that the lack of β-galactosidase activity in the negative control transformant was not due to lack of protein expression (Fig. 1B).

Co-immunoprecipitation experiments were carried out using human Menkes patient fibroblast cell lines that stably expressed either ATP7A (A12-H9) or ATP7B (WND-16) [21]. In this study, these cell lines were transfected with pcDNA3/*GRX1-myc* to transiently express myc-tagged GRX1. In these experiments, the NC36 antibody precipitated both ATP7B and the myc-tagged GRX1 from cell lysates, whereas the pre-immune serum did not precipitate either protein (Fig. 1C). Similarly, the R17-BX antibody precipitated ATP7A and GRX1-myc from the ATP7A-expressing cell line (Fig. 1D). We concluded that the interaction between both copper-ATPases and GRX1 represented true and specific interactions in mammalian cells.

Copper is required for the interaction between GRX1 and the ATPases

We demonstrated that copper is required for the interaction between GRX1 and the ATP7B N terminus. *S. cerevisiae* YGH1 that expressed ATP7B-N and GRX1 was grown for 48 h in medium that was depleted of copper by the addition of various concentrations of the copper chelator BCS. β-Galactosidase activity assays showed that 100 μM BCS was enough to reduce the interaction by four-fold (Fig. 2A), and 500 μM BCS almost completely abolished the interaction. We showed previously that the addition of BCS does not affect the activity of β-galactosidase [17]. Therefore, the reduced interaction in the presence of BCS cannot be attributed to an effect of BCS on β-galactosidase activity. The addition of 50 μM $CuSO_4$ to the

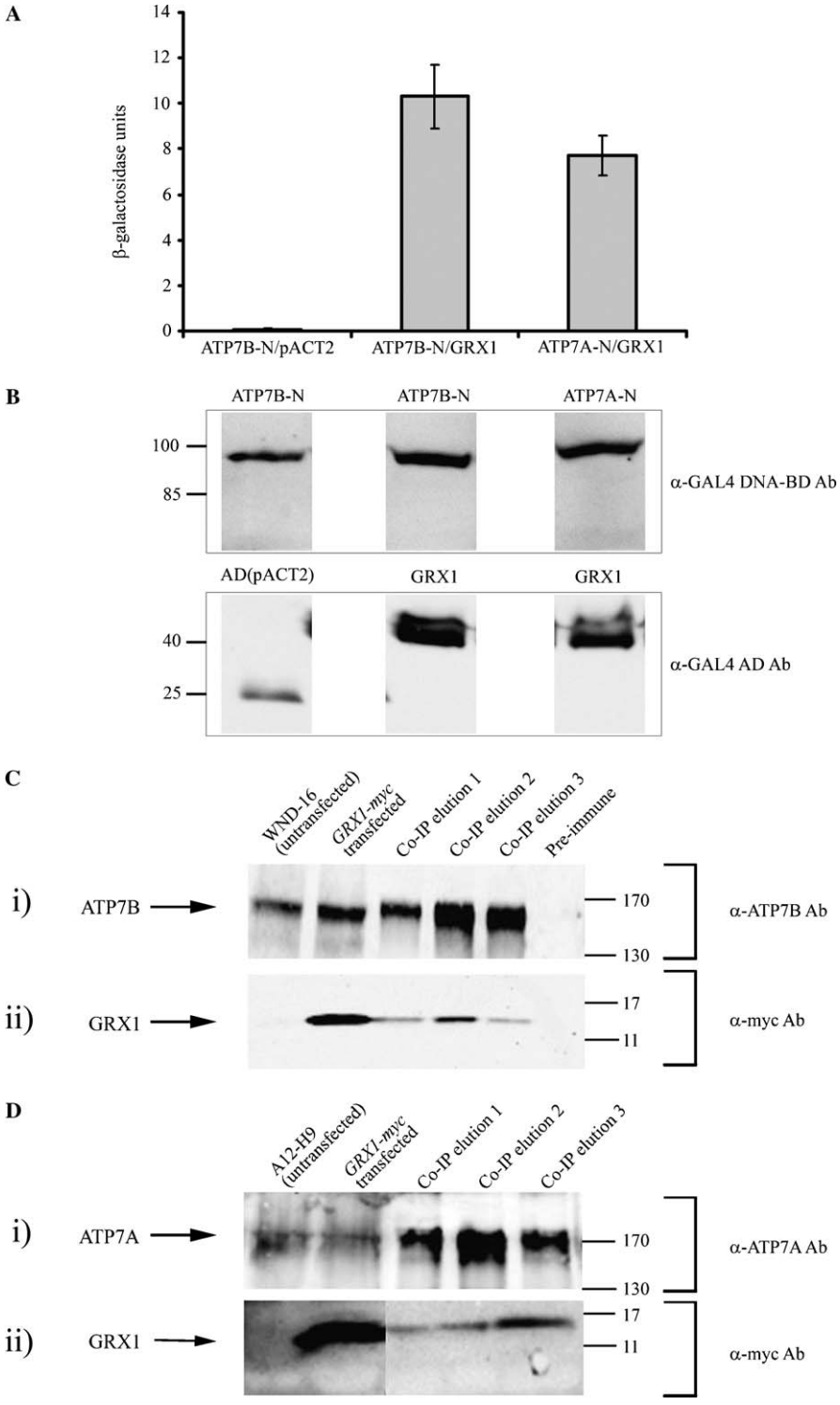

Fig. 1. Interaction of GRX1 with the N terminus of ATP7A and ATP7B. (A) β-Galactosidase activity of *S. cerevisiae* YGH1 co-transformants is shown in Miller units and using the average value obtained from at least three independent experiments ± SD. (B) Expression of GAL4 DNA-BD and AD fusion proteins. Panels show the GAL4 DNA-BD and AD fusion proteins (indicated above each lane) that are expressed in each co-transformant as detected with affinity-purified GAL4 DNA-BD and AD antibodies (Sigma), respectively. The position of protein molecular weight markers (Fermentas and Bio-Rad) are shown on the left in kilodaltons (kDa). (C,D) Co-immunoprecipitation of ATP7B or ATP7A and GRX1 from human fibroblast cell lines, WND16 and A12-H9 [21], respectively, transiently transfected with pcDNA3/*GRX1-myc*. Samples from the first three elutions (Co-IP elution 1, 2, and 3) were fractionated on SDS–PAGE gels. As controls for protein size and expression, aliquots of untransfected, and pcDNA3/*GRX1-myc*-transfected cell lysates also were loaded on each gel. A sample of the eluate from a column prepared with immobilised serum was also loaded and served as a negative control. Proteins were detected with: (C) (i) NC36 (anti-ATP7B, diluted 1:500) or (ii) ab9106, (anti-c-myc, diluted 1:1000, Abcam), and (D) (i) R17-BX (anti-ATP7A, diluted 1:1000) or (ii) ab9106 (diluted 1:1000). The sizes of prestained protein molecular weight markers (Fermentas and Bio-Rad) are shown on the right in kilodaltons (kDa).

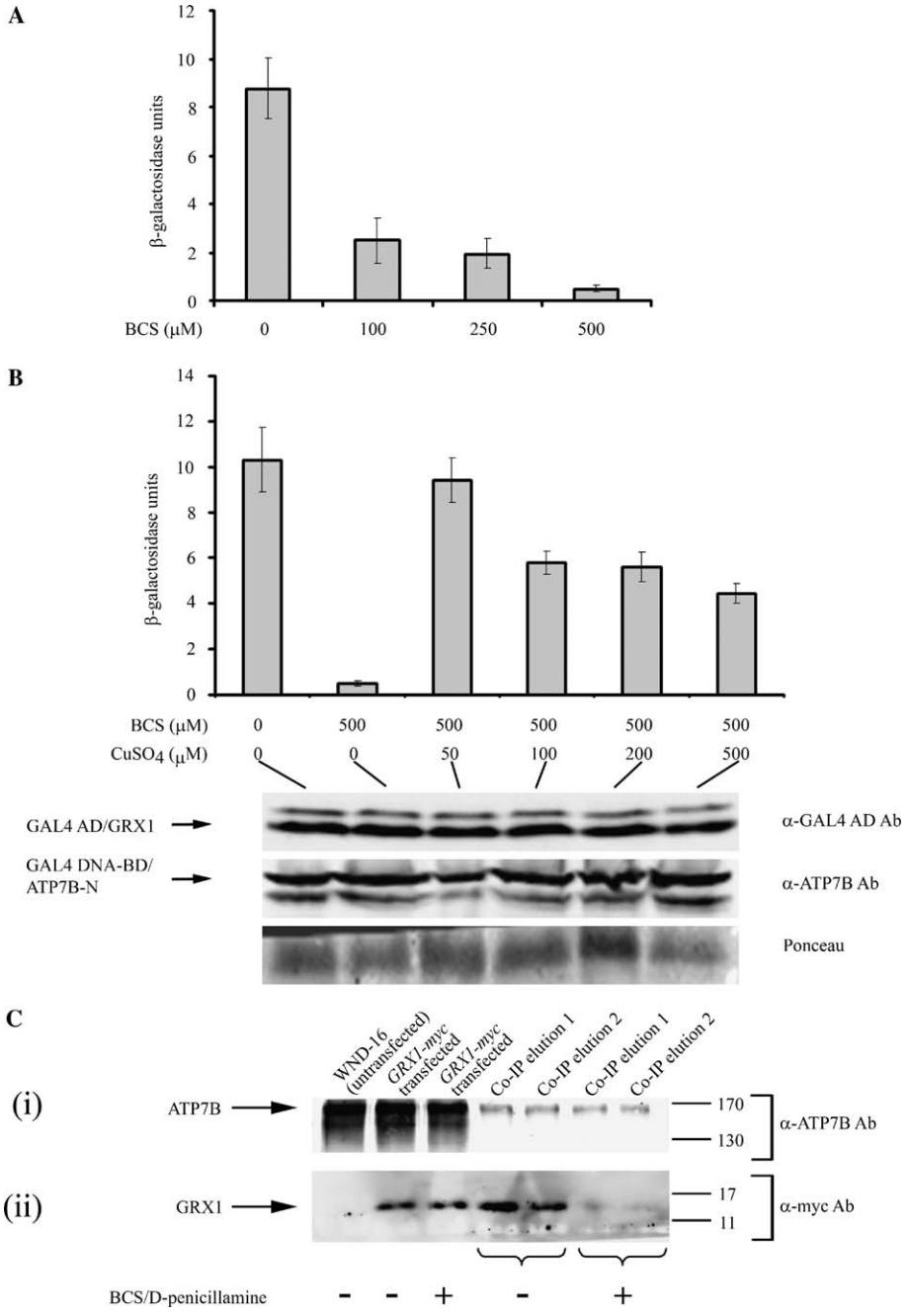

Fig. 2. Copper is required for the interaction between GRX1 and the ATP7B N terminus. Cultures of *S. cerevisiae* YGH1 co-transformants that expressed ATP7B-N and GRX1 were passaged for 48 h in (A) medium supplemented with the copper chelator BCS, or (B) medium supplemented with 500 μM BCS and $CuSO_4$. β-Galactosidase activity was measured in three independent experiments and values shown represent means ± SD. Panels below the graph show immunoblots of the GAL4 DNA-BD/ATP7B-N and GAL4 AD/GRX1 fusion proteins that are expressed in each culture represented in the graph, detected with the GAL4 AD antibody (final concentration 1 μg/ml) (Sigma) and NC36 (anti-ATP7B, diluted 1:500 [5]). An image of a portion of the membrane stained with Ponceau Red demonstrates approximately equivalent protein loading in each lane. (C) The ATP7B/GRX1 interaction is dependent on copper in mammalian cells. WND-16 fibroblast cells [21] were transiently transfected with pcDNA3/*GRX1-myc* and then grown for 72 h in the absence or presence of 100 μM BCS and 100 μM D-penicillamine. Proteins from treated and untreated cell lysates were co-immunoprecipitated from a column prepared with immobilised NC36 antibodies [5]. Samples (20 μl) from the first two elutions (Co-IP elutions 1 and 2) were fractionated on SDS–PAGE gels. As controls for protein size and expression, aliquots of treated and untreated cell lysates also were loaded on each gel. Proteins were detected with: (i) NC36 (diluted 1:500 [5]) or (ii) ab9106 (anti-c-myc, diluted 1:1000, Abcam). The sizes of prestained protein molecular weight markers (Fermentas) are shown on the right in kilodaltons (kDa).

BCS-supplemented medium restored the interaction to normal levels (Fig. 2B) but there was a notable reduction in the extent of the interaction with $\geqslant 100$ μM $CuSO_4$. Similar results were obtained when these experiments were repeated with the ATP7A N terminus (data not shown). Note that protein levels were not affected by the variations in

medium copper levels, so that changes in the extent of the interaction were not a reflection of copper-induced changes in protein expression (Fig. 2B).

Co-immunoprecipitation experiments confirmed that copper was required for the ATP7B/GRX1 interaction to occur. WND-16 cells transfected with pcDNA3/*GRX1-myc* to transiently express myc-tagged GRX1 and grown either in the absence or in the presence of 100 μM BCS plus 100 μM D-penicillamine for 72 h. The NC36 antibody precipitated both ATP7B and myc-tagged GRX1 from the cells grown in normal medium (Fig. 2C(i)). However, when cells were grown in copper-depleted medium there was a substantial reduction in the amount of GRX1-myc that was co-precipitated with ATP7B (Fig. 2C(ii)). There was no difference in the amount of GRX1-myc or ATP7B protein in each of the lysates indicating that the BCS/D-penicillamine treatment did not affect the expression levels of these proteins. We concluded that the reduction in the amount of GRX1 that was co-precipitated with ATP7B was due to copper depletion, confirming that copper was required for the ATP7B/GRX1 interaction in mammalian cells. Note that concentrations of BCS between 50 μM and 500 μM have previously been shown to reverse or disrupt the copper-induced trafficking of the ATPases [11,23].

The interaction between ATP7B and GRX1 is dependent upon the CxxC motifs

To investigate the requirement for the CxxC motifs and the involvement of the different MBS within the ATP7B N terminus in the interaction with GRX1, GAL4 DNA-BD fusion constructs were generated in which the cysteines in all six motifs were altered to serines (SxxS) (MBS1-6c/s), or which contained only metal-binding domain 6 (deleting amino acids 64–540) (MBS1-5del) or metal-binding domains 1–3 (deleting amino acids 300–599) (MBS4-6del) (Fig. 3A). *S. cerevisiae* co-transformants that expressed these fusion proteins and GRX1 were assayed for β-galactosidase activity (Fig. 3B). The interaction was negligible when all six CxxC motifs were mutated to SxxS, clearly demonstrating a requirement for these motifs for the interaction to occur. There was an approximately twofold reduction in the interaction between GRX1 and MBS4-6del compared with the wild-type N terminus, suggesting that the extent of the interaction might be related to the number of MBDs available for interaction. However, unexpectedly, there was no detectable interaction between GRX1 and MBS1-5del. Again, immunoblot analysis of co-transformants demonstrated expression of all fusion proteins (Fig. 3C).

A possible explanation for the lack of interaction with MBS1-5del is that there was sufficient copper in normal medium to efficiently saturate the single MBD in all copies of this truncated protein, but that the yeast two-hybrid system was not sufficiently sensitive to detect the initial interaction with GRX1 that would allow copper to bind to this single site. To test this hypothesis the MBS1-5del/GRX1-expressing co-transformant was grown in medium containing 500 μM BCS for 48 h to deplete cells of copper, followed by supplementation with a range of copper concentrations (0–500 μM) for 24 h, and then β-galactosidase activity was measured (Fig. 3D). In cells grown in normal medium, medium with only BCS, and medium containing BCS and 5 μM copper, the interaction was negligible. But as the copper content of the BCS-containing medium increased, the extent of the interaction increased reaching a maximum level (at 50 μM $CuSO_4$) which was approximately one-sixth that of the wild-type ATP7B-N/GRX1 interaction. The interaction decreased with the addition of 100 μM $CuSO_4$ and was reduced significantly with 500 μM $CuSO_4$. We concluded that GRX1 does interact with MBS1-5del but that this interaction is only detectable at a certain level of intracellular copper.

Discussion

The identification of interacting partners of the copper ATPases is important in helping to explain the molecular and biochemical basis for disease-causing mutations and for full understanding of the complex mechanisms involved in copper homeostasis. We have identified an additional interacting partner of both ATP7A and ATP7B. GRX1 was chosen as a candidate likely to be implicated in regulating ATPase activity based on the presence of the conserved copper-binding CxxC motif within its amino acid sequence, its role within cells in modulating the redox status of protein thiols, and the abundance of cysteine residues within the ATPases. The interaction between GRX1 and the ATPases required copper to be present, and mutation of the cysteines to serines in all six CxxC motifs of the metal-binding domains disrupted the interaction. In the full-length ATP7B, this mutation disrupted the copper-regulated redistribution of the protein [5] and its copper transport activity demonstrating the functional importance of these cysteines [9]. We concluded that the ATPase/GRX1 interaction occurs only when copper is present and the CxxC motifs are available to coordinate the copper.

Glutaredoxins (GRX) (also called thioltransferases) are members of the thiol oxidoreductase family of proteins that catalyse the reduction of protein disulphides to their respective sulphydryls [24]. Glutaredoxins contain three functional conserved regions: the active site (CPYC), the glutathione-binding site sequence, and the hydrophobic surface area [25]. With their two redox-active cysteines (CxxC motif), these small proteins are able to catalyse the reduction of intramolecular disulphide bonds and using GSH as a cofactor, they can also specifically reverse protein-*S*-glutathionylation (protein-SSG formation) [18,24]. Glutathionylation of cysteine residues within proteins results in the post-translational formation of a mixed disulphide between glutathione and the thiol group of cysteine. This reversible modification is becoming recognised as a potential regulatory mechanism as well as a protective mechanism to prevent the oxidation of cysteine residues

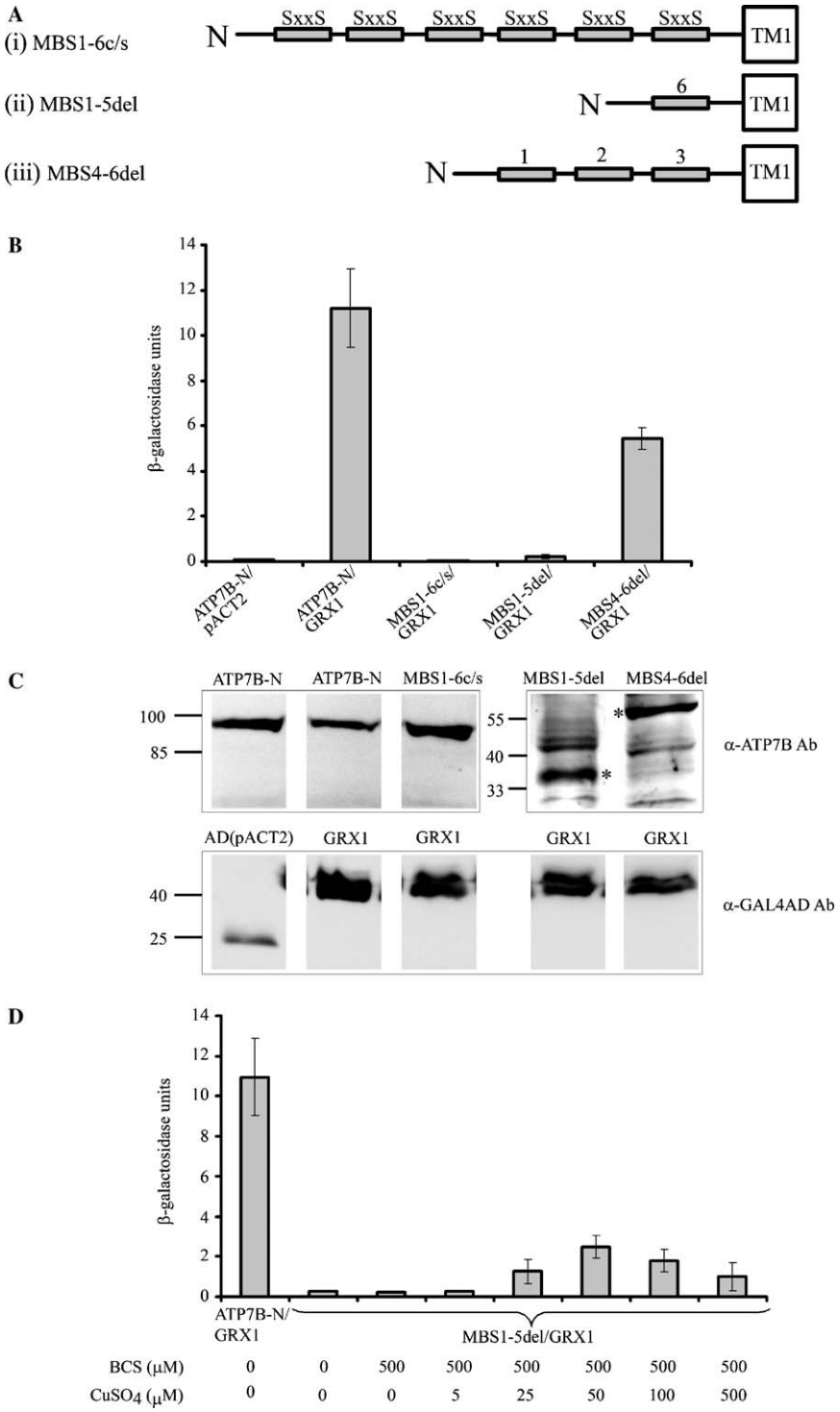

Fig. 3. The ATP7B/GRX1 interaction is related to the number of MBS available and requires the CxxC motifs of ATP7B. (A) Schematic illustration of the ATP7B mutant proteins used in this study. The N-terminal metal-binding domains are shown as filled boxes. (i) MBS1-6c/s, all of the cysteines in the CxxC metal-binding motifs altered to serines (SxxS); (ii) MBS1-5del, the first five MBDs deleted; (iii) MBS4-6del, the three most C-terminal MBDs deleted (B) *S. cerevisiae* YGH1 co-transformants that expressed GRX1 and fusion proteins that contained these mutations were analysed for β-galactosidase activity in three independent experiments. Values shown represent means ± SD. Panels below the graph show immunoblots of the fusion proteins that are expressed in each co-transformant. In the top-right panel the expected protein bands are indicated by an asterisk (∗). Proteins were detected with the GAL4 AD antibody (final concentration 1 μg/ml) (Sigma) and NC36 (anti-ATP7B, diluted 1:500 [5]). The position of protein molecular weight markers (Fermentas and Bio-Rad) is shown on the left in kilodaltons (kDa). (C) The interaction between GRX1 and MBS1-5del is detectable in the presence of a narrow range of medium copper levels. Cultures of *S. cerevisiae* YGH1 co-transformants that expressed (i) ATP7B-N and GRX1 and (ii) MBS1-5del and GRX1 were grown in normal medium. The MBS1-5del/GRX1 co-transformant also was passaged for 48 h in medium supplemented with 500 μM BCS and $CuSO_4$. β-Galactosidase activity was measured in three independent experiments and values shown represent means ± SD.

[18,26]. The oxidoreductions of GRX can either be dithiol reactions where intramolecular protein disulphides are reduced by GRX, or monothiol reductions of mixed disulphides with GSH. With its glutathione-binding site GRX preferentially reduces mixed disulphides [27].

The metal-binding sites of ATP7A need to be in a reduced state for copper to coordinate with the sulphur atoms on the cysteine residues [28]. The intracellular milieu is usually a reducing environment due to high concentrations of GSH but reactive oxygen species can alter the redox balance. Cysteine residues are easily oxidised resulting in protein cross-linking and enzyme inactivation [29]. These irreversible oxidation events can be prevented by protein glutathionylation [30]. With the many cysteine residues within the ATPase N termini and their critical role in co-ordinating copper as an integral part of ATPase function, it is feasible that these cysteines are glutathionylated for protection against irreversible oxidation. Interaction with GRX1 then would serve to deglutathionylate the cysteines when copper is present so that the copper can be coordinated by the thiol groups. Alternatively, GRX may act to reduce intramolecular disulphide bonds formed by the cysteine residues to allow copper coordination by the thiol groups, prior to copper translocation by the ATPases.

The extent of the interaction with GRX1 was related to the number of MBS available and possibly their level of copper saturation. The interaction was reduced by approximately twofold when three MBS (MBS4-6del) were present, alluding to the possibility that the interaction between individual metal-binding sites and GRX1 is functionally equivalent.

Initially and unexpectedly there was no detectable interaction between the MBS 1-5del mutant protein and GRX1 in cells grown in normal medium. However, when copper was depleted from the medium then replenished in increasing amounts, an interaction became apparent and peaked at a level that was approximately one-sixth that of the wild-type ATP7B-N/GRX1 interaction. As the copper content of the medium was increased further ($\geqslant$100 μM), the interaction decreased. A similar pattern of interaction was evident with the wild-type ATP7B-N/GRX1 interaction that also was substantially reduced with the addition of excess copper ($\geqslant$100 μM). The reason for this decrease in the interaction with excess copper is not clear but one possibility is that there is a saturation effect so that once copper is bound by the MBS, in this system there is subsequently a reduced need for interaction with GRX1. Note that the MBS1-5del mutation in the full-length protein could still traffic as wild-type ATP7B [5] and transport copper [9], consistent with its ability to bind copper.

Together these results suggest that the interaction with GRX1 is not specific to any particular metal-binding domain, but that each MBS is equivalent with respect to its interaction with GRX1. This suggestion is consistent with the general role of GRX1 in the cell in regulating the redox status of cysteinyl thiols as part of a protective mechanism and/or to regulate protein activity. In this context it is feasible that GRX1 could also interact with other cysteine-containing copper-binding proteins such as ATOX1, COX17, CCS, and SOD1.

We propose that the ATPase/GRX1 interaction occurs based on copper levels and need. When intracellular copper levels are low, the cysteine residues of the ATPase CxxC motifs are protected from irreversible oxidation either by intramolecular disulphide bond formation or by glutathionylation. In this state, there is little need for GRX1 interaction. When intracellular copper levels increase then the CxxC motifs need to be in a reduced state to accept the metal. GRX1 then interacts with the CxxC motifs when copper is present to deglutathionylate the cysteines or to reduce intramolecular disulphide bonds, thus allowing copper to bind to the ATPases for subsequent transport. When intracellular copper is sufficient or in excess and the MBS of the ATPases are fully occupied by copper, again the need for interaction with GRX1 is eliminated. This model is consistent with our results that demonstrated a requirement for copper and intact cysteine residues in order for the GRX1/ATPase interaction to be detected.

Copper binding to the ATPase N termini is essential to their function in translocating copper across cell membranes [9,31]. Therefore, it is feasible that GRX1 is essential for their activity in its capacity to facilitate copper binding by the CxxC motifs. This possibility is supported by the fact that both ATPases interact with GRX1 to a similar extent and that these interactions require copper. The identification of GRX1 as an interacting partner of the ATPases represents a significant discovery of a molecule whose function is likely to prove integral to the activity of the ATPases, and will provide further insight into the molecular mechanisms that regulate cellular copper homeostasis.

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