Intracellular trafficking of the human Wilson protein: the role of the six N-terminal metal-binding sites

Michael A. CATER*, John FORBES†, Sharon LA FONTAINE*, Diane COX† and Julian F. B. MERCER*¹

*Center for Cellular and Molecular Biology, School of Biological and Chemical Sciences, Deakin University, 221 Burwood Highway, Burwood, Vic. 3125, Australia, and †Department of Medical Genetics, University of Alberta, Edmonton, AB, Canada T6G 2H7

The Wilson protein (ATP7B) is a copper-transporting CPx-type ATPase defective in the copper toxicity disorder Wilson disease. In hepatocytes, ATP7B delivers copper to apo-ceruloplasmin and mediates the excretion of excess copper into bile. These distinct functions require the protein to localize at two different subcellular compartments. At the *trans*-Golgi network, ATP7B transports copper for incorporation into apo-ceruloplasmin. When intracellular copper levels are increased, ATP7B traffics to post-Golgi vesicles in close proximity to the canalicular membrane to facilitate biliary copper excretion. In the present study, we investigated the role of the six N-terminal MBSs (metal-binding sites) in the trafficking process. Using site-directed mutagenesis, we mutated or deleted various combinations of the MBSs and assessed the effect of these changes on the localization and trafficking of ATP7B. Results show that the MBSs required for trafficking are the same as those previously found essential for the

copper transport function. Either MBS 5 or MBS 6 alone was sufficient to support the redistribution of ATP7B to vesicular compartments. The first three N-terminal motifs were not required for copper-dependent intracellular trafficking and could not functionally replace sites 4–6 when placed in the same sequence position. Furthermore, the N-terminal region encompassing MBSs 1–5 (amino acids 64–540) was not essential for trafficking, with only one MBS close to the membrane channel, necessary and sufficient to support trafficking. Our findings were similar to those obtained for the closely related ATP7A protein, suggesting similar mechanisms for trafficking between copper-transporting CPx-type ATPases.

Key words: ATP7B, copper, P-type ATPase, trafficking, Wilson disease, WND.

INTRODUCTION

CPx-type ATPases are transmembrane proteins that carry out ATP-driven translocation of metal cations across cellular membranes. Members of this family have been identified in bacteria, yeast, nematodes, mammals and plants, and translocate a variety of metals including copper, silver, zinc, lead and cadmium [1]. Two homologous human proteins, ATP7A (Menkes protein) and ATP7B (Wilson protein), are copper-transporting CPx-type ATPases that have eight predicted transmembrane domains and six N-terminal MBSs (metal-binding sites) [2–6]. Each binding motif contains the signature sequence of heavy metal-binding proteins, GMXCXXC. The N-termini of both ATP7A and ATP7B have been shown to bind copper preferentially (relative to cadmium, cobalt or zinc) with a stoichiometry of one copper atom per binding repeat [7]. Copper binds in its reduced state Cu⁺ to the GMXCXXC motif via the cysteine residues [7,8]. ATP7A and ATP7B share 59 % amino acid sequence identity and differ in their tissue expression patterns. ATP7B is predominantly expressed in the liver [9], whereas ATP7A is expressed in most non-hepatic tissues [10]. The essential role of both CPx-type ATPases in maintaining copper homeostasis is illustrated by the severe nature of the genetic copper disorders Menkes and Wilson diseases [11].

Immunocytochemical analysis of mammalian cell lines expressing either ATP7A or ATP7B has revealed that both proteins reside at the TGN (*trans*-Golgi network) [12–14]. At this location, both proteins translocate copper into the TGN lumen for incorporation into copper-dependent apo-enzymes as they migrate

through the secretory pathway [15,16]. In hepatocytes, an increase in copper levels results in trafficking of ATP7B from the TGN to pericanalicular vesicles and then ATP7B recycles back to the TGN when copper levels are restored [14]. The trafficking of ATP7B represents a transition in function, from a role in biosynthesis of holo-ceruloplasmin at the TGN [15] to copper export/homeostasis, involving post-Golgi vesicular compartments and excretion into bile [14,17]. The steady-state localization of ATP7B depends on copper levels, although, recycling is probably constitutive in the presence of either high or low copper levels (S. La Fontaine and J. F. B. Mercer, unpublished work). In non-hepatic cells, ATP7B traffics to a dispersed population of cytoplasmic vesicles, possibly involved in sequestering excess copper as a means of detoxification [18,19]. Studies on the subcellular localization of ATP7A have shown a similar trafficking behaviour with copper-induced redistribution from the TGN to cytosolic vesicular compartments and the plasma membrane [12,13].

One proposed role for the N-terminal copper-binding sites of ATP7A and ATP7B is that they individually or co-operatively act as a copper sensor, regulating the subcellular localization of the ATPase depending on the copper status of the cell. Several recent findings on ATP7B have supported this proposal [20,21]. Structural analysis of the N-terminus has revealed that both secondary and tertiary structural changes take place after the binding of copper [20]. Furthermore, it was demonstrated that copper coordination induces the phosphorylation of ATP7B, which coincides with the trafficking of the protein to vesicular compartments [21].

Abbreviations used: BME, Eagles basal medium; CHO, Chinese-hamster ovary; ER, endoplasmic reticulum; FCS, foetal calf serum; MBS, metal-binding site; TGN, *trans*-Golgi network; wt, wild-type.

¹ To whom correspondence should be addressed (e-mail jmercer@deakin.edu.au).

The role of the N-terminal domain in the copper transport activity of ATP7B has been investigated using a yeast complementation assay [22]. In *Saccharomyces cerevisiae*, the ATP7B orthologue Ccc2p transports copper to the ferroxidase Fet3p [23], which is analogous to ATP7B-mediated transport of copper to apo-ceruloplasmin in mammalian hepatocytes [15]. ATP7B can complement a yeast strain that lacks *ccc2* expression (*ccc2Δ*) indicating that ATP7B can transport copper to Fet3p [9]. In addition, complementation of the *ccc2Δ* mutant with ATP7B N-terminal variants showed that the copper-binding sites closest to transmembrane domain 1 were more important for the copper-transporting activity than the sites closer to the N-terminus [22]. Therefore it was postulated that copper co-ordination by MBSs closest to the N-terminus may be involved in initiating copper-dependent trafficking of ATP7B to vesicular compartments [22]. The objective of the present study was to test this hypothesis and to clarify the role of the N-terminal MBSs in ATP7B trafficking. We report that there is no difference in the MBS requirement for the copper transport and the copper-induced trafficking activity of ATP7B. Furthermore, trafficking of ATP7B does not depend on co-operative copper binding to the N-terminal domain, with only one MBS close to the channel, necessary and sufficient to support trafficking. In addition, our results now enable a detailed comparison of the role of the N-terminal MBSs between ATP7A and ATP7B and suggests a functional conservation of the MBSs in copper-induced trafficking and copper transport of these CPx-type ATPases.

EXPERIMENTAL

Bacterial and yeast strains, cell culture and stable transfections

Escherichia coli DH5α [ϕ 80*clacΔ* ZM15, Δ (lacZYA-argF)U169 *recA endA1 hsdR17* (rK[−], mK⁺) *supE44 λ-thi-1 gyrA relA1*], used for the propagation of plasmids, was cultured in Luria–Bertani medium (0.1 % trypticase peptone, 0.1 % NaCl and 0.05 % yeast extract) containing 100 µg/ml ampicillin for plasmid selection. The *S. cerevisiae* mutant strain, *ccc2Δ* (*MATα*, *his3-200*, *trp-1-101*, *ura3-52*, *leu2*, *ade5*, *CCC2Δ::LEU2*) [24], was cultured on YPD plates [2 % (w/v) trypticase peptone/1 % (w/v) yeast extract/2 % (w/v) glucose/2 % (w/v) agar]. This strain was transformed with plasmid DNA using a lithium acetate method as described previously [25] and transformants were selected on DOB-ura [0.66 % yeast nitrogen base without amino acids, 2 % glucose and 0.08 % CSM (complete supplement mixture)-uracil]. CHO (Chinese-hamster ovary)-K1 cells for transfection and immunofluorescence studies were cultured at 37 °C as monolayers in BME (Eagles basal medium) (Trace BioSciences, Nobel Park, Victoria, Australia), supplemented with 0.2 mM proline, 10 % (v/v) FCS (foetal calf serum), 2 mM L-glutamine, 1.2 mM NaHCO₃ and 20 mM Hepes (Commonwealth Serum Laboratories, Broadmeadows, Victoria, Australia). Transfection of plasmid DNA into CHO-K1 cells was performed using LIPOFECTAMINE™ (Life Technologies, Mount Waverley, Victoria, Australia) according to the manufacturer's instructions. Briefly, CHO-K1 cells were seeded in 25 cm² flasks and allowed to grow to 50 % confluence. Approx. 10 µg of purified plasmid DNA (5 µg linearized with *Bgl*III) was mixed with 20 µl of LIPOFECTAMINE™ (Life Technologies) and brought to 400 µl with FCS-free BME. The DNA/LIPOFECTAMINE™ mixture was incubated at room temperature (20–25 °C) for 30 min, and then made to a total volume of 1.5 ml with FCS-free BME. After 3 h of incubation with the DNA/LIPOFECTAMINE™ mixture, the cells were allowed to recover overnight in BME containing 10 % FCS

and the transfectants were selected using 500 µg/ml G418 (Life Technologies) for 14 days. G418-resistant colonies were pooled and analysed for ATP7B expression by immunofluorescence.

Generation of sheep polyclonal antibodies (NC36) against ATP7B

The amino acids 1–199 (N-terminal), 1309–1315 and 1376–1465 (C-terminal) of ATP7B were expressed as a 36-kDa fusion protein with a His₆ tag at the N-terminus of the protein. To generate the expression construct, nt 1–597 and 4126–4395 of the *ATP7B* cDNA were amplified through PCR. The oligonucleotides 5'-GGGCTCTAGAGGATCCATGCCTGAACAGGAGAGA-3' (hWND#24) and 5'-GGGGCCTCGAGGCTAGCGTCCCTGAGGTCTTCGGGCTG-3' (hWND#23), used to amplify the fragment encoding the N-terminal amino acids, incorporated a *Bam*HI site directly upstream of the start codon and a *Nhe*I site at the 3' end. The oligonucleotides 5'-GGGCTCTAGAGTGGTGCTAGCATTCACCTTCCAGGTATAAGAAGCCTGACCTGGA-GAGGTATGAG-3' (hWND#31) and 5'-GGGCTCTAGAGTCGACTCATCAGATGTACTGCTCCTCATCCCT-3' (hWND#30), used to amplify the fragment encoding the C-terminal region, introduced a *Sal*I site directly downstream of the stop codon and a native *Nhe*I site at the 5' end. The two PCR products were digested appropriately and ligated with *Bam*HI–*Sal*I-digested pProEX HT-b vector (Gibco), generating the expression construct pCMB430. The integrity of the construct was confirmed by sequencing.

Expression was induced in *E. coli* DH5α cells grown to an A₆₀₀ 0.6 using 1 mM isopropyl β-D-thiogalactoside for 4 h at room temperature. The recombinant protein was discovered to be extremely insoluble and was therefore purified by nickel-affinity chromatography under denaturing conditions with a buffer containing 8 M urea (100 mM NaH₂PO₄ · H₂O, 10 mM Tris and 8 M urea). The *E. coli* lysate was incubated with Ni²⁺-nitrilotriacetate–agarose resin (1 ml resin/4 ml lysate) (Qiagen, Hilden, Germany) for 1 h with constant agitation. The protein–resin mixture was applied to a column and washed with 20 vol. of wash buffer (100 mM NaH₂PO₄ · H₂O/10 mM Tris/8 M urea, pH 6.5), before the target protein was eluted with several fractions of elution buffer (250 µM imidazole/100 mM NaH₂PO₄ · H₂O/10 mM Tris/8 M urea, pH 4.5). Purified protein was dialysed against several changes of PBS and resuspended in PBS containing 1 % SDS to overcome problems with hydrophobicity. Protein concentration was measured by the Bradford method (Pierce) and approx. 10 µg was subjected to fractionation by SDS/PAGE (12 % gel). Protein purity was ascertained by Coomassie staining [50 % (v/v) methanol, 10 % (v/v) acetic acid and 0.1 % (w/v) Coomassie dye]. Five 400 µl aliquots, each containing 2.5 mg of dialysed protein, were supplied to the IMVS (Institute of Medical and Veterinary Sciences, University of Adelaide, SA, Australia) for immunization of a sheep. The IgG fraction of the antiserum was ammonium sulphate precipitated as described previously [26]. The antibody was designated NC36.

To determine if both the N- and C-terminal segments of the fusion protein were immunogenic, each region was expressed and purified separately and the ability of NC36 to detect both regions was tested by Western-blot analysis (Figure 2B). To generate the N-terminal (amino acids 1–199) expression construct, pCMB430 was digested with *Nhe*I–*Xba*I to drop out the region encoding the C-terminus, and re-ligated to generate plasmid pCMB431. To create the C-terminal expression construct, nt 3935–3945 and 4126–4395 of *hATP7B* were isolated from pCMB430 by digestion with *Nhe*I–*Sal*I and subcloned into the *Xba*I–*Xho*I sites of pProEX HT-b to generate plasmid pCMB432. The N- and C-terminal proteins, including the translated vector sequence, were

Figure 1 Schematic representation of mutations and deletions introduced into the N-terminus of ATP7B

(A) Shaded boxes denote MBS mutated from CXXC to SXXS, normal MBSs are indicated by white boxes. (B) N-terminal deletions are represented by open rectangles with deleted amino acids indicated. The vertical black bar represents the transmembrane domain 1. Diagrams are orientated N- to C-termini (left to right) and are not to scale.

30 and 15 kDa respectively. Expression and purification of both the proteins were performed as described above.

Wild-type (wt) and mutant ATP7B expression constructs

The ATP7B cDNA constructs (with the exception of MBS 1-4 + 6c/s) were generated previously [22]. Briefly, both cysteine residues within each of the metal-binding domains (GMXCXXC) were mutated in separate plasmid constructs to serine. Using native restriction sites, full-length constructs containing different combinations of active and mutated MBSs were generated (Figure 1A). N-terminal domain deletion constructs were also created by PCR (Figure 1B). The ATP7B cDNA constructs were originally generated in the yeast expression vector pG3. Excision of the mutated cDNAs was performed using *Bam*HI and *Sal*I, and the digested fragments were cloned into the *Xho*I–*Sal*I sites of the mammalian expression vector pCMB77 [13]. For several of the mutant constructs, the excised fragments were also subcloned into the *Bam*HI–*Sal*I sites of pWSK29 to enable more convenient generation of other constructs. Final plasmid constructs were designated as shown in Table 1. The ATP7B construct with MBSs 1-4 + 6 mutated (MBS 1-4 + 6c/s, Figure 1A) was created by ligating a 2.6 kb *Nhe*I–*Eco*RI fragment (MBS 5 intact, 6 mutated) isolated from pCMB417, with a 1.3 kb *Eco*RI–*Xba*I fragment (MBS 1-4 mutated) from pCMB414 and *Nhe*I cut pCMB77 to generate pCMB409. The correct orientation of the cDNA construct was verified by restriction digestion profiles and the appropriate metal binding and ligation sites sequenced to confirm integrity.

For expression in *ccc2Δ* yeast, wt ATP7B and mutated constructs were generated in the yeast expression vector pVT-103U

Table 1 Plasmids generated and used in the present study

Plasmid designation	Characteristics	Reference
pWSK29	Bacterial propagation vector	[43]
pCMB77	Mammalian expression vector	[13]
pVT-103U	Yeast expression vector	[27]
pProEX HT	Bacterial expression vector	Gibco
pCMB278	ATP7B-wt in pCMB77	This study
pCMB398	MBS 1-6c/s in pCMB77	This study
pCMB399	MBS 1-5c/s in pCMB77	This study
pCMB400	MBS 1-4c/s in pCMB77	This study
pCMB401	MBS 3-5c/s in pCMB77	This study
pCMB402	MBS 6c/s in pCMB77	This study
pCMB403	MBS 1-6del in pCMB77	This study
pCMB404	MBS 1-5del in pCMB77	This study
pCMB405	MBS 4-6del in pCMB77	This study
pCMB406	MBS 3-5del in pCMB77	This study
pCMB407	MBS 3-6del in pCMB77	This study
pCMB413	MBS 1-6c/s in pWSK29	This study
pCMB414	MBS 1-5c/s in pWSK29	This study
pCMB417	MBS 6c/s in pWSK29	This study
pCMB554	ATP7B-wt in pVT-103U	This study
pCMB555	MBS 1-2c/s in pVT-103U	This study
pCMB557	MBS 1-6c/s in pVT-103U	This study
pCMB558	MBS 1-4 + 6c/s in pVT-103U	This study
pCMB430	NC36 antigen in pProEX HT	This study
pCMB431	N-terminus (N30) in pProEX HT	This study
pCMB432	C-terminus (C15) in pProEX HT	This study

[27]. Nucleotides 1–1937 of *hATP7B* were amplified through PCR using the oligonucleotides 5'-CCCGATAGCGGATCCAT-GCCTGAACAGGAGAGACAGATCACAGCC-3' (hWND#34) and 5'-CCATCTTGTGGTCCAAGAGATGAGC-3' (hWND#7). Two cDNA templates, pCMB413 and pCMB278, were used to generate a PCR fragment with MBSs 1–2 mutated and the corresponding wt fragment respectively. Both PCR fragments were digested with *Bam*HI (introduced upstream ATG) and *Bse*AI (native) to produce a 499 bp fragment, which was then ligated separately with a *Bse*AI–*Bam*HI fragment isolated from pCMB277 into *Bam*HI-digested pVT-103U (wt-ATP7B-pCMB554 and MBS 1-2 c/s-pCMB555). MBS 1-6c/s and MBS 1-4 + 6c/s in pVT-103U were generated by isolating a *Bse*AI–*Sal*I fragment from pCMB413 and pCMB409 respectively, which were used separately to replace the corresponding region in pCMB555 (*Bse*AI–*Xho*I) (MBS 1-6c/s-pCMB557 and MBS 1-4 + 6c/s-pCMB558). The integrity of the constructs was confirmed by sequencing.

Protein preparations and Western blotting

For the analysis of protein expressed in mammalian cells, cells were harvested from confluent 75 cm² flasks and centrifuged at 1000 *g* for 5 min. Cell pellets were washed once with PBS, resuspended in buffer (1 % SDS/10 mM Tris/HCl) and sonicated (Fisher Scientific Model 550 Sonic Dismembrator) using a micro-tip probe, amplitude setting 3, 2 cycles of 30 s followed by 60 s cooling on ice. Yeast protein extracts were prepared using the trichloroacetic acid method as described in the Clontech's Yeast Protocols Handbook (PT3024-1). Whole-cell protein extract (50 µg) was fractionated by SDS/PAGE (7.5 % gel). After electrophoretic separation, proteins were transferred to nitrocellulose (Amersham Biosciences) and immunoblot analysis was performed using the Lumi-Light Chemiluminescence blotting kit (Roche) according to the manufacturer's instructions. ATP7B was detected using the ammonium sulphate-precipitated NC36

antibody (diluted 1/1000), followed by horseradish peroxidase-conjugated donkey anti-sheep IgG (Chemicon, Boronia, Victoria, Australia) (1/4000).

Immunofluorescence microscopy

Transfected cells were seeded on 13 mm glass coverslips and cultured in BME containing 10% FCS. At 50–70% confluence, the growth medium was replaced with either the basal medium (0.5–1 μ M Cu) or a medium supplemented with 200 μ M CuCl₂. After 2 h of incubation, cells were fixed using 4% (w/v) paraformaldehyde in PBS for 10 min, then permeabilized with 0.1% (v/v) Triton X-100 in PBS for 10 min. Cells were blocked in 1% (w/v) BSA and 1% (w/v) gelatin in PBS at 4 °C overnight. Coverslips were incubated with ammonium sulphate-precipitated NC36, diluted 1/10000 in blocking solution for 1 h. After four PBS washes (15 min each), coverslips were incubated with the secondary antibody, donkey anti-sheep IgG Alexa 488 (1/4000) (Chemicon) and again washed with PBS as above. Coverslips were mounted on glass slides using 2.6% (w/v) 1,4-diazadi-cyclo[2.2.2]octane (Sigma) in 90% (v/v) glycerol. Immunolabelled cells were analysed using a $\times 60$ -oil objective with an Olympus PROVIS AX70 microscope.

Yeast complementation assay

Complementation of *ccc2 Δ* yeast using the constructs MBS1-6c/s, MBS1-4 + 6c/s and wt-ATP7B (Table 1) was performed by a method adapted from previously described procedures [9,28]. Briefly, 10 ml of DOB-ura was inoculated (in duplicate) with the appropriate yeast strain and grown at 30 °C overnight. Cells were pelleted by centrifugation (10 min, 3500 g), washed twice with ice-cold double distilled H₂O, then resuspended in iron-limited DOB-ura (50 mM Mes/250 μ M ferrozine in DOB-ura) and again allowed to grow overnight. Cells were pelleted and washed as before and resuspended in fresh iron-limited media (50 ml) to a cell density of A_{600} 0.01. Growth rates of the yeast strains were analysed spectroscopically and measurements (A_{600}) were taken at times 0, 10–24 (2 h intervals) and 36 h. The linear exponential growth phase between 12 and 20 h from duplicate experiments (averaged) was used to determine the growth rate, which was the gradient of the trend line. The copper transport activity of MBS1-4 + 6c/s was also assessed by growth on iron-limited plates as described previously [28].

RESULTS

Generation of ATP7B constructs and anti-ATP7B antibody (NC36)

The role of the N-terminal MBSs of ATP7B in intracellular trafficking was investigated using site-directed and deletion mutagenesis. Constructs encoding ATP7B with different combinations of wt (CXXC) and mutated (SXXS) MBSs (Figure 1A), or harbouring N-terminal deletions (Figure 1B), were expressed in CHO-K1 cells. To enable detection of the expressed proteins, a polyclonal antibody (NC36) was raised in sheep against a 36 kDa fusion protein comprising amino acids 1–199 (N-terminus), 1309–1315 and 1376–1465 (C-terminus) of ATP7B (as described in the Experimental section). The specificity of the antibody was tested by Western-blot analysis (Figure 2A). The antibody specifically detected a single 165 kDa protein in lysates from HepG2 and PMC-42 cell lines (Figure 2A), which have been previously shown to express ATP7B [14]. No protein was detected in lysate from CHO-K1 cells (Figure 2A), nor in HepG2 or PMC-42 cell lines when immunoblots were incubated with preimmune serum

Figure 2 Characterization of anti-ATP7B antibody (NC36) by Western-blot analysis

(A) Total protein (50 μ g) from the cell types indicated was separated by SDS/PAGE (7.5% gel) and transferred on to a nitrocellulose membrane. Protein was immunolabelled with ammonium sulphate-precipitated NC36 (diluted 1/1000), followed by horseradish peroxidase-conjugated donkey anti-sheep IgG (diluted 1/4000) and analysed by chemiluminescence. HepG2, human hepatoma cell line; PMC-42, human breast carcinoma cell line. (B) The N- (N30) and C-terminal (C15) segments of the ATP7B antigen (NC36) used to generate the antibody were expressed and purified separately to test immunogenicity. Each lane contained approx. 1 μ g of purified protein and was detected using the above conditions. The position of the molecular-mass standards (Bio-Rad) are indicated on the left in kDa.

or secondary antibody alone (results not shown). To demonstrate that antibodies were raised against both the N- and C-terminal segments of the ATP7B fusion protein, each region was expressed and purified separately and detected by Western-blot analysis (Figure 2B).

Expression of wt, mutated and truncated ATP7B proteins in CHO-K1 cells

Immunofluorescence microscopy was performed on CHO-K1 cells stably expressing wt and mutated forms of ATP7B. Initially, several of the constructs were transiently transfected into CHO-K1 cells; however, the results were difficult to interpret because expression levels were extremely variable, ranging from barely detectable to overexpression causing mislocalization (results not shown). Therefore stable cell lines were generated for all constructs, allowing trafficking to be assessed in cells expressing consistent levels of the target protein. In all cell lines generated, except as indicated below, a single protein band of the expected mass was detected by the NC36 antibody (Figure 3A). Under basal copper conditions (0.5–1 μ M Cu), cells expressing wt ATP7B displayed fluorescent staining within the perinuclear region (Figure 4C), which is consistent with previously described TGN localization of ATP7B [14,29–31]. Intracellular trafficking of ATP7B was induced by incubating the cells for 2 h in media supplemented with 200 μ M CuCl₂ (Figure 4D). As a result of copper treatment, the intensity of the perinuclear staining decreased, with a predominant vesicular-like staining throughout the cytoplasm (Figure 4D). NC36 antibody used on CHO-K1 cells transfected with the mammalian expression vector alone (pCMB77) showed minimal cross-reactivity with endogenous proteins (Figures 4A, 4B and 5A, 5B).

Effect of N-terminal MBS mutations (CXXC-SXXS) on the trafficking of ATP7B

Mutation of the cysteines in all six MBSs to serines (MBS 1-6c/s) abrogated the copper-induced redistribution of ATP7B,

Figure 3 Western-blot analysis of CHO-K1 cells and *ccc2Δ* yeast expressing ATP7B variants

(A, B) Total cellular protein (50 μ g) was separated by SDS/PAGE (7.5 % gel) and transferred on to a nitrocellulose membrane. Protein was immunolabelled with ammonium sulphate-precipitated NC36 (diluted 1/1000), followed by horseradish peroxidase-conjugated donkey anti-sheep IgG (diluted 1/4000) and analysed by chemiluminescence. Western blots comparing the expression levels of the indicated ATP7B constructs stably expressed in CHO-K1 cells (A) and expressed in *ccc2Δ* yeast (B).

with perinuclear staining observed in the presence of both basal and increased levels of copper (Figures 4E and 4F). Constructs encoding ATP7B with different combinations of wt (CXXC) and mutated (SXXS) MBSs were used to establish the MBSs required for copper-induced trafficking. All of the mutated proteins localized to the TGN in cells were cultured in basal media (Figure 4, basal). When cells were incubated in 200 μ M CuCl₂ for 2 h, redistribution of ATP7B occurred in lines expressing MBS 1-5c/s, MBS 3-5c/s and MBS 6c/s, with a cytosolic vesicular pattern indistinguishable from that of wt ATP7B (Figures 4H, 4L and 4N). In contrast, cells expressing MBS 4-6c/s maintained the perinuclear staining in increased levels of copper (Figure 4J), indicating that MBSs 1-3 were not sufficient for relocation of ATP7B. These results suggested that some or all three metal-binding motifs closest to transmembrane domain 1 (MBS 4-6) are required to support the trafficking activity. MBS 6 alone was able to support the relocation of ATP7B (MBS 1-5c/s) (Figure 4H), but since ATP7B with MBS 6 mutated (MBS 6c/s) was still capable of trafficking (Figure 4N), this indicated that MBS 6 was not essential. To determine if MBS 5 alone could also support copper-induced trafficking, the construct MBS 1-4 + 6c/s (5 alone intact) was generated (Figure 1A). MBS 1-4 + 6c/s had a TGN localization in cells cultured in basal media (Figure 4O) and a cytosolic vesicular localization indistinguishable from that of wt ATP7B in media supplemented with 200 μ M CuCl₂ (Figure 4P). The redistribution of MBS 1-4 + 6c/s illustrated that the presence of MBS 5 alone is sufficient to allow the trafficking of ATP7B.

Effect of N-terminal deletions on the trafficking of ATP7B

To establish if the amino acids surrounding the MBSs (GMX-CXXC) are also required for copper-induced redistribution, the trafficking ability of ATP7B constructs with large N-terminal deletions (Figure 1B) was assessed. In CHO-K1 cells, several of the truncated proteins exhibited an alternative subcellular localization in comparison with wt ATP7B. Deletion of MBSs

Figure 4 Immunofluorescence microscopy of CHO-K1 cells expressing ATP7B wt and N-terminal MBS mutants

CHO-K1 cells transfected with the indicated construct were cultured in basal media (0.5–1 μ M Cu) or in media supplemented with 200 μ M CuCl₂. Immunofluorescence detection was performed by incubation with ammonium sulphate-precipitated NC36 (diluted 1/10 000) and donkey anti-sheep IgG Alexa 488 (diluted 1/4000).

1-6 (MBS 1-6del), 3-5 (MBS 3-5del) and 3-6 (MBS 3-6del) resulted in proteins that were distributed in a reticulated pattern throughout the cytoplasm (Figures 5C, 5D and 5I–5L). This phenotype was observed in all of the transfected cells irrespective of expression level and the addition of 200 μ M CuCl₂ to the growth media had no effect on the localization. The truncated ATP7B in cells expressing MBS 3-5del and MBS 3-6del could not be detected by Western blot and only marginal amounts of intact protein were detected in cells expressing MBS 1-6del (Figure 3A). This result was consistent with a decrease in the number of cells expressing these mutant proteins and with the decrease

Figure 5 Immunofluorescence microscopy of CHO-K1 cells expressing ATP7B N-terminal deletion mutants

CHO-K1 cells transfected with the indicated construct were cultured in basal media (0.5–1 μ M Cu) or media supplemented with 200 μ M CuCl_2 . Immunofluorescence detection was performed by incubation with ammonium sulphate-precipitated NC36 (diluted 1/10 000) and donkey anti-sheep IgG Alexa 488 (diluted 1/4000).

in immunofluorescence labelling of cells that did express these mutant proteins. These results suggested that the truncated proteins MBS 1-6del, MBS 3-5del and MBS 3-6del are unstable and localized to the ER (endoplasmic reticulum), typical of other misfolded ATP7B variants [18,29]. MBS 1-6del co-localized with the ER protein, protein disulphide isomerase (results not shown).

Two deletion mutants (MBS 1-5del and MBS 4-6del) did preserve a perinuclear distribution indistinguishable from the localization of wt ATP7B under basal copper conditions (Figures 5E and 5G). When copper levels were increased, however, only MBS 1-5del redistributed to post-Golgi vesicular compartments (Figure 5F), indicating that the amino acids surrounding MBS 6 were sufficient for trafficking activity. The inability of MBS 4-6del to undergo copper-induced trafficking, demonstrated that the first three MBSs are not equivalent to and cannot replace sites 4–6, even when placed in the same sequence position.

MBS 5 alone supports copper transport

Forbes et al. [22] established that MBS 6 alone is sufficient for ATP7B to transport copper to Fet3p in *ccc2* Δ yeast. In the

Table 2 Comparison of the transport and trafficking ability of ATP7B N-terminal variants

The ability of ATP7B N-terminal mutants to transport copper was assessed through *ccc2* Δ complementation. The growth rate of selected yeast strains was quantified and is shown as the percentage of growth rate compared with wt-ATP7B. The linear exponential growth phase between 12 and 20 h from duplicate experiments was used to determine the growth rate, which was the gradient of the trend line. The localization of the same mutant proteins in CHO-K1 cells exposed to both basal (0.5–1 μ M) and increased levels (200 μ M) of copper is shown. Nd, not determined.

Yeast strain/construct	Transport activity		Localization in CHO-K1 cells*	
	Plate growth assay†	Growth rate (%)	Basal	+ Cu
pVT-103U	Inactive	$2.9 \pm 2.23^*$	Nd	Nd
Wt-ATP7B	Active	$100 \pm 0.000^*$	TGN	Vesicles
MBS 1-6c/s	Inactive	$3.3 \pm 0.92^*$	TGN	TGN
MBS 1-5c/s	Active	$101.5 \pm 0.046^\dagger$	TGN	Vesicles
MBS 4-6c/s	Inactive	Nd	TGN	TGN
MBS 3-5c/s	Active	Nd	TGN	Vesicles
MBS 6c/s	Active	Nd	TGN	Vesicles
MBS 1-4 + 6c/s	Active*	$72.5 \pm 0.49^*$	TGN	Vesicles
MBS 1-6del	Inactive	Nd	Diffuse	Diffuse
MBS 1-5del	Active	$95 \pm 0.037^\dagger$	TGN	Vesicles
MBS 4-6del	Inactive	Nd	TGN	TGN
MBS 3-5del	Inactive	Nd	Diffuse	Diffuse
MBS 3-6del	Inactive	Nd	Diffuse	Diffuse

* This study.

† Results from Forbes et al. [22].

present study, we demonstrated that MBS 5 or MBS 6 alone can support trafficking of ATP7B and postulated that this is also the case for copper transport. To test this hypothesis, the ATP7B construct with MBS 5 intact and all others mutated (MBS 1-4 + 6c/s) was expressed in *ccc2* Δ yeast and evaluated for its copper transport ability by assessing the growth of the transformed yeast on solid iron-limited media and by measuring the growth rate of the same transformant in iron-limited liquid media (Table 2). Mutant (*ccc2* Δ) yeast carrying the empty expression vector (pVT-103U) or expressing MBS 1-6c/s were unable to grow in the iron-limited media, consistent with previous results [22]. Yeast strain viability was confirmed on iron-sufficient culture plates (results not shown). Wt-ATP7B supported copper delivery to Fet3p, allowing *ccc2* Δ yeast to grow on iron-depleted media. MBS 1-4 + 6c/s could also complement *ccc2* Δ yeast; however, the growth rate was slightly lower in comparison with wt-ATP7B (approx. 72.5%), suggesting that the copper transport ability of this molecule was somewhat reduced. Western-blot analysis (Figure 3B) showed that the reduced growth rate of MBS 1-4 + 6c/s was not due to a decreased protein level, with MBS 1-4 + 6c/s showing greater amounts of protein when compared with the wt-ATP7B.

DISCUSSION

In cells exposed to increased levels of copper, ATP7B rapidly shifts its steady-state localization from the TGN to cytoplasmic vesicles and recycles back to the TGN when basal copper levels are restored [19,30,32]. The ability of ATP7B to respond to changes in cellular copper concentration has been proposed to involve the six MBSs in the N-terminal region [7,22]. In this report, we investigated the trafficking ability of ATP7B variants containing different combinations of active (CXXC) and mutated (SXXS) MBSs (Figure 4) or bearing N-terminal truncations

(Figure 5). Mutation of the cysteine residues in all six MBSs (GMXCXXC) to serine residues (GMXSXXS) completely abrogated ATP7B redistribution (Figures 4E and 4F), demonstrating the necessity of copper binding to the N-terminal domain for regulating the trafficking process. MBSs closest to the transduction channel were more important for copper-induced trafficking than the more N-terminal motifs. Metal binding sites 5 or 6 alone were sufficient for the redistribution of ATP7B in increased levels of copper (Figures 4G, 4H, 4O and 4P), whereas the first three MBSs could not support the copper-mediated response (Figures 4I and 4J). Taken together, these results demonstrate that the trafficking of ATP7B is not dependent on the presence of multiple MBSs or co-operative binding of copper to the N-terminal domain and suggests a functional difference between the first three MBSs and those closer to the transduction channel.

A series of N-terminal deletion constructs were analysed for trafficking activity to investigate whether amino acids other than the cysteine residues in the MBSs (Figure 5) are essential. Several of the truncated proteins (MBS 1-6del, MBS 3-5del and MBS 3-6del) when expressed in CHO-K1 cells displayed a cytosolic reticulated pattern (Figures 5C, 5D and 5I–5L) and had little (MBS 1-6del) or no (MBS 3-5del and MBS 3-6del) intact protein when evaluated by Western-blot analysis (Figure 3). We suggest that the mislocalization of these mutants when expressed in CHO-K1 cells was due to folding irregularities, whereas the inability to detect MBS 3-5del and MBS 3-6del by Western blot was due to low transfection rates coupled with the probable rapid degradation of the expressed misfolding proteins. When these same constructs were previously transformed in yeast however [22], they were expressed to a level similar to that of wt ATP7B. This discrepancy may be explained, in part, by previously published observations that ATP7B variants that misfold and localize to the ER in CHO-K1 cells, are often stable when expressed in yeast [18]. In addition, the stable CHO-K1 cell lines expressing these constructs had lower transfection rates when compared with all other cell lines, limiting the amount of these mutant proteins.

Nevertheless, two truncated proteins MBS 1-5del (Figures 5E and 5F) and MBS 4-6del (Figures 5G and 5H) did provide considerable insight into the N-terminal regions required for trafficking. MBS 1-5del exhibited trafficking behaviour indistinguishable from that of wt ATP7B, indicating that the deleted region (amino acids 64–540) did not contain any sequence essential for retention at the TGN or targeting to post-Golgi vesicular compartments. This result also confirmed that only one MBS close to the transmembrane channel is required for trafficking of ATP7B. The inability of MBS 4-6del to undergo copper-mediated trafficking illustrates that the first three MBSs are not functionally equivalent to and cannot replace sites 4–6 when situated in the same sequence position. This result is particularly important since it further suggests that the sequence, or part thereof, surrounding MBS 6 is specifically required for trafficking and cannot be replaced by the other metal-binding domains. Recently, we have shown that a putative targeting motif exists in the region of MBS 6, which directs ATP7B to a vesicular compartment [33]. We further suggest that this motif while important in targeting ATP7B to vesicles may also be involved in initiating the trafficking process itself, since its deletion perturbs the whole process.

Previously, Forbes et al. [22] have used functional complementation of *S. cerevisiae ccc2Δ* to determine the role of the N-terminal metal-binding domain in ATP7B-mediated copper transport to Fet3p. The ATP7B constructs analysed in this study have all been tested for their ability to transport copper to Fet3p and thereby correct the high-affinity iron uptake deficiency of the *ccc2Δ* strain (Table 2). These results suggest that MBS 6 alone is capable of supporting normal copper transport ability of

ATP7B. The deletion of amino acids 63–540 (MBS 1-5del) did not significantly impede the ability of ATP7B to complement the *ccc2Δ* strain, indicating that most of the N-terminal domain is not essential for copper transport. When MBS 5 alone is left intact (MBS 1-4 + 6c/s), the growth rate is reduced to approx. 72% when compared with that of *ccc2Δ* expressing wt-ATP7B. This result is consistent with the recent results published by Huster and Lutsenko [34], which showed that the catalytic phosphorylation of ATP7B is decreased to 70% of the normal level when MBS 6 is mutated. Therefore it appears that although both MBSs 5 and 6 are capable of supporting copper transport, MBS 6 has a more critical role, which possibly relates to its proximity to the transduction channel.

Table 2 summarizes the trafficking behaviour of ATP7B N-terminal mutants and relates this to their copper transport ability. The trafficking behaviour of the various mutants corresponded to their ability to transport copper. It is clear, as we have observed with ATP7A [33], that ATP7B variants that are defective in copper transport also cannot traffic. This observation is consistent with our recent results, which demonstrated that the acyl-phosphate intermediate of ATP7A is the form of the molecule that undergoes trafficking [35]. Mutations that prevent the formation of the acyl-phosphate intermediate inhibit movement from the TGN, whereas mutation of the phosphatase domain traps the protein in the acyl-phosphate form, which results in a constitutive plasma membrane location for ATP7A and vesicular location for ATP7B [35]. Therefore any mutation that prevents catalytic activation (formation of the acyl-phosphate) will also prevent copper-induced trafficking. This explains why the toxic milk mouse mutation (M1356V) [31], the brindled mouse mutation (Δ A799L800) [36] and a missense mutation (A1362V) that causes mild Menkes disease [37] result in proteins that are located in the TGN and unable to traffic. Presumably, a specific conformation associated with this acyl-phosphate intermediate is required for trafficking [35], possibly by exposing an exocytic signal or occluding a TGN retention sequence (not mutually exclusive). The shift in the steady-state localization from the TGN can be explained by an increase in the number of molecules that are catalytically activated (acyl-phosphorylated) and proceed through the reaction cycle.

The Menkes protein (ATP7A or MNK) is highly similar to ATP7B with respect to amino acid sequence, structure and cell biology. Analogous to ATP7B, most of the N-terminus of ATP7A (amino acids 8–485) is not required for either copper transport [33], or trafficking [38], with MBS 5 or MBS 6 sufficient for both processes. Thus, for both human CPx-type ATPases, the same MBSs are important for their copper transport and copper-induced trafficking activities. The role of the first four MBSs in both ATP7A and ATP7B is not clear, although it has been postulated that multiple metal-binding repeats may increase the efficacy of cytoplasmic copper scavenging or copper delivery to the transduction channel [38]. Conceivably, the first four MBSs when bound to copper may also prolong the acyl-phosphorylated state of the ATPase, increasing the likelihood for redistribution in the face of increased intracellular copper levels.

The requirement for the copper-chaperone Atox1 in the trafficking of ATP7A and in the control of intracellular copper levels has been established [39], and by inference the chaperone may also have a critical role in ATP7B trafficking. Earlier studies have demonstrated a copper-dependent interaction between Atox1 and both ATP7A and ATP7B N-terminal domains [40]. For ATP7B, it has been demonstrated that the transfer of copper from Cu-Atox1 to ATP7B stimulates catalytic phosphorylation, but that copper binding to all MBSs is not necessary to support the catalytic activity of ATP7B [41,42], consistent with the results presented in the present study.

This study forms a part of a series of investigations, aimed at elucidating the role of the six N-terminal MBSs in the copper transport and copper-induced trafficking function of the human CPx-type ATPases (ATP7A and ATP7B). From the results presented in this and other recent studies, a picture of the molecular components and interactions necessary for the maintenance of intracellular copper levels emerges, whereby cellular copper is bound by Atox1, delivered and transferred to the MBSs of ATP7A or ATP7B; conformational changes in the ATPases stimulate catalytic phosphorylation and expose a targeting signal leading to initiation of trafficking to their respective destinations within the cell. Furthermore, the MBSs closest to the first transmembrane domain are required for this process. Taken together, these studies suggest that the mechanism for copper translocation and trafficking are coupled and are highly conserved between the copper CPx-type ATPases. Further studies are now aimed at elucidating the precise molecular signals in the vicinity of MBSs 5 and 6, which govern the targeting of these two CPx-type ATPases.

We thank S. Firth for guidance in generating the antibody (NC36) and M. Howie for helpful discussions and critical evaluation of the paper. S. La F. is supported by a National Health and Medical Research Council R. Douglas Wright Fellowship. This work was supported in part by the National Health and Medical Research Council of Australia, the Australian Research Council and the International Copper Association.

REFERENCES

- Solizio, M. and Vulpe, C. (1996) CPX-type ATPases: a class of P-type ATPases that pump heavy metals. *Trends Biochem. Sci.* **21**, 237–241
- Chelly, J., Tumer, Z., Tonnesen, P., Petterson, A., Ishikawa-Brush, Y., Tommerup, N., Horn, N. and Monaco, A. P. (1993) Isolation of a candidate gene for Menkes disease that encodes a potential heavy metal binding protein. *Nat. Genet.* **3**, 14–19
- Vulpe, C., Levinson, B., Whitney, S., Packman, S. and Gitschier, J. (1993) Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase. *Nat. Genet.* **3**, 7–13
- Mercer, J. F. B., Livingston, J., Hall, B. K., Paynter, J. A., Begy, C., Chandrasekharappa, S., Lockhart, P., Grimes, A., Bhawe, M., Siemenack, D. et al. (1993) Isolation of a partial candidate gene for Menkes disease by positional cloning. *Nat. Genet.* **3**, 20–25
- Petrukhin, K., Lutsenko, S., Chernov, I., Ross, B. M., Kaplan, J. H. and Gilliam, T. C. (1994) Characterization of the Wilson disease gene encoding a copper transporting ATPase: genomic organization, alternative splicing, and structure/function predictions. *Hum. Mol. Genet.* **9**, 1647–1656
- Yamaguchi, Y., Heiny, M. E. and Gitlin, J. D. (1993) Isolation and characterization of a human liver cDNA as a candidate gene for Wilson disease. *Biochem. Biophys. Res. Commun.* **197**, 271–277
- Lutsenko, S., Petrukhin, K., Cooper, M. J., Gilliam, C. T. and Kaplan, J. H. (1997) N-terminal domains of human copper-transporting adenosine triphosphatases (the Wilson's and Menkes disease proteins) bind copper selectively *in vivo* and *in vitro* with stoichiometry of one copper per metal-binding repeat. *J. Biol. Chem.* **272**, 18939–18944
- DiDonato, M., Narindrasorasak, S., Forbes, J. R., Cox, D. W. and Sarkar, B. (1997) Expression, purification, and metal binding properties of the N-terminal domain from the Wilson disease putative copper-transporting ATPase (ATP7B). *J. Biol. Chem.* **272**, 33279–33282
- Hung, I. H., Suzuki, M., Yamaguchi, Y., Yuan, D. S., Klausner, R. D. and Gitlin, J. D. (1997) Biochemical characterization of the Wilson disease protein and functional expression in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.* **272**, 21461–21466
- Paynter, J. A., Grimes, A., Lockhart, P. and Mercer, J. F. B. (1994) Expression of the Menkes gene homologue in mouse tissues; lack of effect of copper on the mRNA levels. *FEBS Lett.* **351**, 186–190
- Mercer, J. F. B. and Camakaris, J. (1997) Menkes' and Wilson's disease: genetic disorder of copper transport. In *Metal Ions in Gene Regulation* (Silver, S. and Walden, W., eds.), pp. 250–276. Chapman & Hall, New York
- Yamaguchi, Y., Heiny, M. E., Suzuki, M. and Gitlin, J. D. (1997) Biochemical characterization and intracellular localization of the Menkes disease protein. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 14030–14035
- Petris, M. J., Mercer, J. F. B., Culvenor, J. G., Lockhart, P., Gleeson, P. A. and Camakaris, J. (1996) Ligand-regulated transport of the Menkes copper P-type ATPase efflux pump from the Golgi apparatus to the plasma membrane: a novel mechanism of regulated trafficking. *EMBO J.* **15**, 6084–6095
- Schaefer, M., Hopkins, R. G., Failla, M. A. and Gitlin, J. D. (1999) Hepatocyte-specific localization and copper-dependent trafficking of the Wilson's disease protein in the liver. *Am. J. Physiol.* **276**, G639–G646
- Terada, K., Nakako, T., Yang, X.-L., Iida, M., Aiba, N., Minamiya, Y., Nakai, M., Sakaki, T., Miura, N. and Sugiyama, T. (1998) Restoration of holoceruloplasmin synthesis in LEC rat after infusion of recombinant adenovirus bearing WND cDNA. *J. Biol. Chem.* **273**, 1815–1820
- Petris, M. J., Strausak, D. and Mercer, J. F. B. (2000) The Menkes copper transporter is required for the activation of tyrosinase. *Hum. Mol. Genet.* **9**, 2845–2851
- Terada, K., Aiba, N., Yang, X.-L., Iida, M., Nakai, M., Miura, N. and Sugiyama, T. (1999) Biliary excretion of copper in LEC rat after introduction of copper transporting P-type ATPase, ATP7B. *FEBS Lett.* **448**, 53–56
- Forbes, J. R. and Cox, D. W. (2000) Copper-dependent trafficking of Wilson disease mutant ATP7B proteins. *Hum. Mol. Genet.* **9**, 1927–1935
- Lockhart, P. and Mercer, J. F. (2001) Functional analysis of the sheep Wilson disease protein (sATP7B) in CHO cells. *Eur. J. Cell Biol.* **80**, 349–357
- DiDonato, M., Hsu, H. F., Narindrasorasak, S., Que, Jr, L. and Sarkar, B. (2000) Copper-induced conformational changes in the N-terminal domain of the Wilson disease copper-transporting ATPase. *Biochemistry* **39**, 1890–1896
- Vanderwerf, S. M., Cooper, M. J., Stetsenko, I. V. and Lutsenko, S. (2001) Copper specifically regulates intracellular phosphorylation of the Wilson's disease protein, a human copper-transporting ATPase. *J. Biol. Chem.* **276**, 36289–36294
- Forbes, J. R., Hsi, G. and Cox, D. W. (1999) Role of the copper-binding domain in the copper transport function of ATP7B, the P-type ATPase defective in Wilson disease. *J. Biol. Chem.* **274**, 12408–12413
- Yuan, D. S., Dancis, A. and Klausner, R. D. (1997) Restriction of copper export in *Saccharomyces cerevisiae* to a late Golgi or post-Golgi compartment in the secretory pathway. *J. Biol. Chem.* **272**, 25787–25793
- Fu, D., Beeler, T. and Dunn, T. (1995) Sequence, mapping and disruption of CCC2, a gene that cross-complements the Ca^{2+} -sensitive phenotype of *csg1* mutants and encodes a P-type ATPase belonging to the Cu^{2+} -ATPase subfamily. *Yeast* **11**, 283–292
- Elble, R. (1992) A simple and efficient procedure for transformation of yeasts. *Biotechniques* **13**, 18–20
- Harlow, E. and Lane, D. (1998) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Plainview, NY
- Vernet, T., Dignard, D. and Thomas, D. Y. (1987) A family of yeast expression vectors containing the phage f1 intergenic region. *Gene* **52**, 225–233
- Forbes, J. R. and Cox, D. C. (1998) Functional characterization of missense mutations in ATP7B: Wilson disease mutation or normal variant. *Am. J. Hum. Genet.* **63**, 1663–1674
- Payne, A. S., Kelly, E. J. and Gitlin, J. D. (1998) Functional expression of the Wilson disease protein reveals mislocalization and impaired copper-dependent trafficking of the common H1069Q mutation. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 10854–10859
- Schaefer, M., Roelofsens, H., Wolters, H., Hofmann, W. J., Müller, M., Kuipers, F., Stremmel, W. and Vonk, R. J. (1999) Localization of the Wilson's disease protein in human liver. *Gastroenterology* **117**, 1380–1385
- La Fontaine, S., Theophilos, M. B., Firth, S. D., Gould, R., Parton, R. G. and Mercer, J. F. B. (2001) Effect of the toxic milk mutation (tx) on the function and intracellular localization of Wnd, the murine homologue of the Wilson copper ATPase. *Hum. Mol. Genet.* **10**, 361–370
- Roelofsens, H., Wolters, H., Luyn, M. J. A. V., Miura, N., Kuipers, F. and Vonk, R. J. (2000) Copper-induced apical trafficking of ATP7B in polarized hepatoma cells provides a mechanism for biliary copper excretion. *Gastroenterology* **119**, 782–793
- Mercer, J. F. B., Barnes, N., Steverson, J., Strausak, D. and Llanos, R. (2003) Copper-induced trafficking of the Cu-ATPases: a key mechanism for copper homeostasis. *Biomaterials* **16**, 175–184
- Huster, D. and Lutsenko, S. (2003) The distinct roles of the N-terminal copper-binding sites in regulation of catalytic activity of the Wilson's disease protein. *J. Biol. Chem.* **278**, 32212–32218
- Petris, M., Voskoboinik, I., Cater, M., Smith, K., Kim, B., Llanos, R., Strausak, D., Camakaris, J. and Mercer, J. F. B. (2002) Copper-regulated trafficking of the Menkes disease copper ATPase is associated with formation of a phosphorylated catalytic intermediate. *J. Biol. Chem.* **277**, 46736–46742
- La Fontaine, S., Firth, S. D., Lockhart, P. J., Brooks, H., Camakaris, J. and Mercer, J. F. B. (1999) Intracellular localization and loss of copper-responsiveness of Mnk, the murine homologue of the Menkes protein, in cells from blotchy (*Mo^{blu}*) and brindled (*Mo^{br}*) mouse mutants. *Hum. Mol. Genet.* **8**, 1069–1075
- Ambrosini, L. and Mercer, J. F. B. (1999) Defective copper-induced trafficking and localization of the Menkes protein in patients with mild and copper-treated classical Menkes disease. *Hum. Mol. Genet.* **8**, 1547–1555

-
- 38 Strausak, D., La Fontaine, S., Hill, J., Firth, S. D., Lockhart, P. J. and Mercer, J. F. B. (1999) The role of GMXCXC metal binding sites in the copper-induced redistribution of the Menkes protein. *J. Biol. Chem.* **274**, 11170–11177
- 39 Hamza, I., Prohaska, J. and Gitlin, J. (2003) Essential role for Atox1 in the copper-mediated intracellular trafficking of the Menkes ATPase. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 1215–1220
- 40 Hamza, I., Schaefer, M., Klomp, L. W. J. and Gitlin, J. (1999) Interaction of the copper chaperone HAH1 with the Wilson disease protein is essential for copper homeostasis. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 13363–13368
- 41 Walker, J., Tsivkovskii, R. and Lutsenko, S. (2002) Metallochaperone Atox1 transfers copper to the NH₂-terminal domain of the Wilson's disease protein and regulates its catalytic activity. *J. Biol. Chem.* **277**, 27953–27959
- 42 Lutsenko, S., Tsivkovskii, R. and Walker, J. (2003) Functional properties of the human copper-transporting ATPase ATP7B (the Wilson's disease protein) and regulation by metallochaperone Atox1. *Ann. N.Y. Acad. Sci.* **986**, 204–211
- 43 Wang, R. F. and Kushner, S. R. (1991) Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. *Gene* **100**, 195–199
-

Received 25 November 2003/10 February 2004; accepted 4 March 2004

Published as BJ Immediate Publication 4 March 2004, DOI 10.1042/BJ20031804