ATP7B Mediates Vesicular Sequestration of Copper: Insight Into Biliary Copper Excretion

MICHAEL A. CATER, SHARON LA FONTAINE, KRISTY SHIELD, YOLANDA DEAL, and JULIAN F. B. MERCER
Centre for Cellular and Molecular Biology, School of Biological and Chemical Sciences, Deakin University, Burwood, Australia

Background & Aims: The Wilson protein (ATP7B) regulates levels of systemic copper by excreting excess copper into bile. It is not clear whether ATP7B translocates excess intrahepatic copper directly across the canalicular membrane or sequesters this copper into exocytic vesicles, which subsequently fuse with canalicular membrane to expel their contents into bile. The aim of this study was to clarify the mechanism underlying ATP7B-mediated copper detoxification by investigating endogenous ATP7B localization in the HepG2 hepatoma cell line and its ability to mediate vesicular sequestration of excess intracellular copper.

Methods: Immunofluorescence microscopy was used to investigate the effect of copper concentration on the localization of endogenous ATP7B in HepG2 cells. Copper accumulation studies to determine whether ATP7B can mediate vesicular sequestration of excess intracellular copper were performed using Chinese hamster ovary cells that exogenously expressed wild-type and mutant ATP7B proteins.

Results: In HepG2 cells, elevated copper levels stimulated trafficking of ATP7B to pericanalicular vesicles and not to the canalicular membrane as previously reported. Mutation of an endocytic retrieval signal in ATP7B caused the protein to constitutively localize to vesicles and not to the plasma membrane, suggesting that a vesicular compartment(s) is the final trafficking destination for ATP7B. Expression of wild-type and mutant ATP7B caused Chinese hamster ovary cells to accumulate copper in vesicles, which subsequently undergo exocytosis, releasing copper across the plasma membrane.

Conclusions: This report provides compelling evidence that the primary mechanism of biliary copper excretion involves ATP7B-mediated vesicular sequestration of copper rather than direct copper translocation across the canalicular membrane.

Wilson disease patients have a marked reduction in biliary copper excretion and consequently accumulate toxic levels of copper in liver hepatocytes and the central nervous system. If untreated, patients develop a potentially fatal liver disease and often irreversible neurologic degeneration. The impairment of biliary copper excretion in these patients arises from mutations in the ATP7B gene, which encodes a copper-transporting P-type ATPase. P-type ATPases are transmembrane proteins that utilize energy derived from ATP-hydrolysis (γ-phosphorylation) to translocate cations across cellular membranes. ATP7B contains 8 transmembrane spanning domains, 6 N-terminal copper-binding sites (GMXXXC), and is expressed predominantly in liver hepatocytes but also in regions of the brain, breast, and placenta. In mammals, hepatocytes are the central storage site of copper, and biliary copper excretion is the primary mechanism for elimination of copper from the body. The level of copper in the serum of adults is maintained at approximately 17–27 μmol/L. The 2 main roles of the hepatic ATP7B protein are to deliver copper to the ferroxidase ceruloplasmin and to mediate the excretion of excess systemic copper into bile.

The HepG2 human hepatoma cell line has proven useful for studying the functional properties of hepatocytes because it retains many of the biosynthetic and specialized functions of normal hepatocytes, including secretion of albumin and ceruloplasmin and the synthesis of...
thesis of bile salts.\textsuperscript{14} Biliary canaliculi are also produced between 2 or more juxtaposed cells and form secretory vacuoles reminiscent of the liver bile ductules.\textsuperscript{15,16} In HepG2 cells, ATP7B has been shown to traffic to pericanalicular vesicles and extensively colocalizes with the canaliculus-located MRP2.\textsuperscript{17} Canicular membrane localization has important implications for the mechanism by which ATP7B mediates biliary copper excretion, because it suggests direct translocation of copper by ATP7B into the canaliculus. However, in a previous study ATP7B could not be detected on the canalicular membrane in vitro and in vivo,\textsuperscript{11,12} and in several nonhepatic mammalian cell lines, ATP7B has been shown to confer copper resistance by trafficking to cytosolic vesicles and not to the plasma membrane.\textsuperscript{18–21} Therefore, it seems probable that ATP7B can mediate vesicular sequestration of excess copper as a means of detoxification.

In this report, we provide compelling evidence indicating that the main mechanism for ATP7B-mediated biliary copper excretion involves copper sequestration into exocytic vesicles, rather than direct copper translocation across the canalicular membrane. ATP7B exogenously expressed in CHO-K1 cells can mediate vesicular sequestration of excess copper for detoxification and eventual removal from the cell through vesicular exocytosis.

Investigation of the copper-dependent subcellular localization of endogenous ATP7B in HepG2 cells revealed predominantly vesicular localization consistent with studies by Schaefer et al.\textsuperscript{11,12} rather than extensive canalicular membrane localization as previously reported by Roelofsen et al.\textsuperscript{17} Mutation of an endocytic retrieval signal located in the C-terminus of ATP7B, which was predicted to trap ATP7B molecules at their final trafficking destination, caused the protein to localize constitutively to vesicular compartments and not to the plasma membrane but still permitted copper accumulation to the same extent as the wild-type (wt) protein. Finally, we have shown that the patient mutation 4193ΔC produces structural instability in ATP7B and causes the protein to mislocalize, accounting for the Wilson disease phenotype observed in patients with this mutation. This study provides important new insight into the mechanistic basis of ATP7B-mediated copper detoxification and reveals a fundamental functional difference in comparison with the closely related copper-ATPase ATP7A.

**Materials and Methods**

**Cells and Antibodies**

The human HepG2 hepatoma cell line was cultured at 37°C in Dulbecco’s modified Eagle medium (high glucose) (Trace BioSciences, Nobel Park, Victoria, Australia) supplemented with 0.2 mmol/L proline, 5% fetal calf serum (FCS), 2 mmol/L L-glutamine, 0.6 mmol/L NaHCO\textsubscript{3}, 20 mmol/L HEPES, 200 μg/mL penicillin, and 200 μg/mL streptomycin (Commonwealth Serum Laboratories, Broadmedows, Victoria, Australia). Chinese hamster ovary (CHO-K1) cells were cultured at 37°C as monolayers in Eagle basal medium (BME) (Trace BioSciences) supplemented with 0.2 mmol/L proline, 10% FCS, 2 mmol/L L-glutamine, 1.2 mmol/L NaHCO\textsubscript{3}, and 20 mmol/L HEPES (Commonwealth Serum Laboratories). Transfection of plasmid DNA into CHO-K1 cells was performed using LIPOFECTAMINE (Invitrogen) and a protocol similar to that recommended by the manufacturer. CHO-K1 cells were seeded in 25 cm\textsuperscript{2} flasks and allowed to grow to 50%–80% confluence. Approximately 10 μg purified plasmid DNA (5 μg linearized with BglII) was mixed with 20 μL LIPOFECTAMINE and brought to 400 μL with FCS-free BME. The DNA/LIPOFECTAMINE mixture was incubated at room temperature for 30 minutes, adjusted to a total volume of 1.5 mL with FCS-free BME, and applied to the cells. Following 3 hours of incubation, the DNA/LIPOFECTAMINE mixture was removed, and the cells were allowed to recover overnight in BME containing 10% FCS. Transfected cells were selected using 500 μg/mL G418 (Life Technologies, Inc, Mount Waverley, Victoria, Australia) for 14 days. G418-resistant colonies were pooled and analyzed for ATP7B expression by immunofluorescence.

The *Saccharomyces cerevisiae* mutant strain, *ace2Δ* (MAT\textalpha, his3-200, trp-1-101, ura3-52, leu2, ade5, CCC2ΔLEU2) was cultured in 2% trypticase peptone, 1% yeast extract, and 2% glucose (YPD) medium or on YPD agar plates (2% trypticase peptone, 1% yeast extract, 2% glucose, and 2% agar) containing 30 μg/mL chloramphenicol to prevent bacterial growth. The *S. cerevisiae* mutant strain *ace2Δ* was transformed with plasmid DNA using a lithium acetate method as previously described,\textsuperscript{22} and transformants were selected on DOB-ura plates (0.66% yeast nitrogen base, no amino acids; 2% glucose; 0.08% CSM-Uracil; and 2% agar) at 30°C for 3 days.

The polyclonal anti-ATP7B antibody (NC36) was generated as previously described.\textsuperscript{23} The mouse monoclonal antibody against the TGN-resident protein p230 (p230) was purchased from Stressgen, and the mouse monoclonal antibody against the canalicular membrane bound P-glycoprotein (C219) was purchased from Calbiochem.

**Generation of ATP7B Mutants**

The mutant ATP7B cDNA constructs generated in this study and the expression vectors used are listed in Table 1. The C-terminal trileucine residues (LLL\textsuperscript{3783–3785}) were changed to alanines using PCR-based mutagenesis of the ATP7B cDNA. The oligonucleotides 5'-CCGGAGATGC-GACTCATCAGATGTACTGCTCCTCATCCTCGCATG-GCCGCCGCCAGCACCATTGTC-3' (hWND#31) and 5'-ATGGGGTGTGGACTGGTTTCT-3' (hWND#11) were used to amplify nucleotides 3634–4395 of ATP7B. The hWND#31 oligo introduced the codon substitutions and a
Sal site directly downstream of 2 stop codons. The sequence encoding the alanines provided a NsiI endonuclease site. The PCR product was digested with NheI (native at bp position 3925) and SaiI to produce a 479-bp fragment. This fragment was then ligated together with an SaiI/NheI fragment isolated from pCMB278 (nucleotides 2153–3925 of ATP7B) into SaiI/SalI-digested pCMB278, creating the final LLL-AAA expression plasmid designated pCMB525.

The aspartate residue167 in the phosphorylation domain was changed to glutamate (D-E) by PCR-based mutagenesis of the ATP7B cDNA. Several preliminary cloning steps were required prior to the mutagenesis. First, the SaiI endonuclease site in the bacterial vector pWSK29 was eliminated by digesting the plasmid with SaiI and blunt ending the linearized product using Klenow polymerase exonuclease activity (Roche, Castle Hill, NSW, Australia) before religation. The modified pWSK29 plasmid designated pCMB410. Next, wt-ATP7B cDNA was cloned into the BamHI site of the modified pWSK29 plasmid to generate pCMB411. The correct orientation of the wt-ATP7B insert was verified by an endonuclease digestion profile. These steps rendered the native SaiI site in ATP7B (bp position 3760) unique, which allowed this site to be used in the generation of the D-E construct. The mutagenesis was accomplished by using oligonucleotides 5′-GACCTGCGCCATGGAGTGAAATGTTACAGTCGTCTTCTA−3′ (hWND#26) and 5′-CCCCATATGCATTGGGAAAAGGCAAAACCTCAG-3′ (hWND#9) to amplify nucleotides 2344–3117 of ATP7B. The oligonucleotide hWND#26 introduced the desired codon change and an ApaI endonuclease site, necessary for screening the ligated inserts. The PCR product was digested with AarII and AscI to produce a 181-bp fragment (nucleotides 2924–3104), which was then ligated together with an AscI/AarII fragment isolated from ATP7B (nucleotides 3105–3764) and an SaiI/SalI fragment isolated from ATP7B (nucleotides 3765–4395). These 3 fragments were ligated first before being inserted into AarII/SaiI-digested pCMB411 (product designated pCMB513). The entire ATP7B cDNA containing the mutation was isolated from pCMB513 using SpeI and SaiI and cloned into the Nhel/SaiI sites of the mammalian expression vector pCMB77 to create the final construct, designated pCMB514.

The human patient mutation 4193ΔC was introduced into ATP7B cDNA by using the oligonucleotides 5′-ATGGGTT-

| Table 1. Plasmids Generated and Used in the Present Study |
|------------------|------------------|------------------|
| Plasmid designation | Characteristics | Reference |
| pWSK29 | Bacterial propagation vector | Vernet et al.23 |
| pCMB410 | pWSK29 (−Sac site) | This study |
| pCMB411 | Wt-ATP7B in pCMB410 | This study |
| pCMB77 | Mammalian expression vector | Petris et al.32 |
| pVT-103U | Yeast expression vector | Vernet et al.47 |
| pCMB278 | Wt-ATP7B in pCMB77 | Catter et al.23 |
| pCMB514 | D-E in pCMB77 | This study |
| pCMB554 | Wt-ATP7B in pVT-103U | Catter et al.23 |
| pCMB525 | LLL-AAA in pCMB77 | This study |
| pCMB606 | LLL-AAA in pVT-103U | This study |
| pCMB562 | 4193ΔC in pCMB77 | This study |
| pCMB609 | 4193ΔC in pVT-103U | This study |

Immunofluorescence Microscopy

HepG2 cells were grown in 75-cm² flasks to >90% confluence before being harvested using 5 mL trypsin solution (0.05% trypsin, 0.02% EDTA) and adjusted to 10 mL with medium. In a 24-well tray, 100 μL of the 10 mL cell suspension was seeded onto flamed 13-mm glass coverslips and cultured for 3 days to allow for canalicular vacuole formation. This duration of culturing has previously been shown to result in the maximum amount of canalicular structures.24 The growth medium was replaced with either basal (0.5–1 μmol/L copper) or CuCl₂-supplemented medium, with treatment conditions as outlined in the Results section. Following treatment, cells were fixed with acetone (−20°C) for 2 minutes and air-dried for 2 minutes and either stored short-term (<1 month) at −80°C or processed straight away. Coverslips were incubated in phosphate-buffered saline (PBS) for 2 minutes to rehydrate cells prior to immunolabeling. To label endogenous ATP7B, cells were incubated overnight at 4°C with ammonium sulphate-precipitated NC36 diluted 1/5000 in 1% donkey serum. Following 4 PBS washes (15 minutes each), coverslips were incubated with the secondary antibody donkey anti-sheep IgG Alexa 488 (1/4000) (Chemicon, Boronia, Victoria, Australia) for 1 hour at room temperature and again washed 4 times with PBS. Coverslips were mounted on glass slides using 2.6% (wt/vol) Dabco (Sigma) in 90% glycerol. Immunolabeled cells were analyzed using a 100× oil objective with an LEICA TCS SP2 confocal microscope.

CHO-K1 cells were grown in 75 cm² flasks to >90% confluence before being harvested, using 5 mL trypsin solution (0.025% trypsin, 0.02% EDTA) and adjusted to 10 mL with medium. Of the cell suspension, 80 μL was seeded onto 13-mm glass coverslips in a 24-well tray and cultured for 16–24 hours. Following treatment with either basal (0.5–1 μmol/L copper) or CuCl₂ supplemented medium, cells were fixed using 4% (wt/vol) paraformaldehyde in PBS for 10
minutes then permeabilized with 0.1% (vol/vol) Triton X-100 in PBS for 10 minutes. Cells were blocked in 1% (wt/vol) BSA, 1% (wt/vol) gelatin in PBS at 4°C overnight. For the detection of ATP7B, coverslips were incubated with ammonium sulfate-precipitated NC36, diluted 1:10,000 in blocking solution for 1 hour. Following 4 PBS washes (15 minutes each), the cells were incubated with the secondary antibody donkey anti-sheep IgG Alexa 488 (1/4000) (Chemicon) and again washed with PBS 4 times. Coverslips were mounted on glass slides using 2.6% (wt/vol) Dabco (Sigma) in 90% glycerol. Immunolabeled cells were analyzed using a 60× oil objective with an Olympus PROVIS AX70 microscope.

**CHO-K1 Cell Copper Accumulation Assay**

Approximately 1.5 × 10^7 cells of the parental and transfected CHO-K1 cell lines were seeded into separate 75-cm^2 flasks and cultured for 24 hours. The medium was then replaced with either fresh basal or 100 μmol/L CuCl_2-supplemented medium, and the cells were further incubated for 24 hours. The cells were then washed with 2 mL trypsin solution (0.025% trypsin, 0.02% EDTA) and harvested using 1.5 mL trypsin left over from the wash combined with the 1.5 mL trypsin used to harvest the cells made approximately 1.7 mL total harvest volume. Of the 1.7 mL, 0.1 mL was used for cell counts, whereas 1.5 mL of the cell suspension was centrifuged at 1000 g for 5 minutes. Cell pellets were washed twice with ice-cold ddH_2O, then resuspended in iron-limited DOB-ura containing 50 mL medium (50 μmol/L CuCl_2), allowed to grow overnight. Cells were then pelleted and washed as before and resuspended in fresh iron-limited medium (50 mL) to a cell density of OD_600 = 0.01. Growth rates of the yeast strains were determined by measuring the OD_{600} (Beckman DUP 530 Spectrophotometer) at 0 hour, then at 2-hour intervals between 10 and 26 hours, and finally at 36 hours. The gradient of the linear growth trend line during the exponential growth phase (between 12 and 20 hours) from at least duplicate experiments (averaged) was taken as the growth rate.

**Results**

**ATP7B Traffics From the TGN to Pericanalicular Vesicles and not to the Canalicular Membrane in HepG2 Cells**

In HepG2 cells, the trafficking of ATP7B from the TGN has previously been shown to be stimulated by physiologically relevant levels of copper (1–20 μmol/L), with the degree of redistribution copper-dose dependent. To demonstrate that ATP7B resides at the TGN under low copper conditions as previously shown, the subcellular localization of ATP7B was compared with that of the TGN resident protein p230. The p230 protein localizes to the cytosolic-facing peripheral membrane of the TGN and is believed to be impor-
tant for the biogenesis of nonclathrin-coated vesicles. In HepG2 cells cultured in medium containing the copper chelator BSC (100 μmol/L) or supplemented with the indicated concentration of copper. The cells were fixed with cold acetone (−20°C) and double stained for ATP7B and the TGN-resident p230 protein. Detection of ATP7B was performed using ammonium sulphate-precipitated NC36 (diluted 1 in 5000) and donkey anti-sheep IgG Alexa 488 (diluted 1 in 4000) (Chemicon). P230 was detected with a mouse monoclonal antibody (diluted 1 in 500) (BD Biosciences) and donkey anti-mouse IgG Cy5 (diluted 1 in 100) (Chemicon). The nuclei were stained with ethidium bromide (10 μg/mL solution made in PBS) by incubating the cells for 5 minutes immediately following secondary antibody incubation. The color of stained nuclei was changed to blue to reduce interference with green/red overlay. Photographs were taken using a 100× oil objective with a Leica TCS SP2 confocal microscope. Scales are shown.

Figure 1. ATP7B localizes to the TGN in HepG2 cells. HepG2 cells grown on coverslips (72 hours) were cultured for 4 hours with medium containing the copper chelator BSC (100 μmol/L) or supplemented with the indicated concentration of copper. The cells were fixed with cold acetone (−20°C) and double stained for ATP7B and the TGN-resident p230 protein. Detection of ATP7B was performed using ammonium sulphate-precipitated NC36 (diluted 1 in 5000) and donkey anti-sheep IgG Alexa 488 (diluted 1 in 4000) (Chemicon). P230 was detected with a mouse monoclonal antibody (diluted 1 in 500) (BD Biosciences) and donkey anti-mouse IgG Cy5 (diluted 1 in 100) (Chemicon). The nuclei were stained with ethidium bromide (10 μg/mL solution made in PBS) by incubating the cells for 5 minutes immediately following secondary antibody incubation. The color of stained nuclei was changed to blue to reduce interference with green/red overlay. Photographs were taken using a 100× oil objective with a Leica TCS SP2 confocal microscope. Scales are shown.

In HepG2 cells cultured in medium containing the copper chelator bathocuproinedisulfoinic acid (BCS) and in basal medium, both ATP7B and p230 were found to be closely associated in the perinuclear region (Figure 1). The close proximity, rather than complete overlap between ATP7B and p230, suggests that both proteins reside in distinct subcompartments of the TGN. In cells incubated with 20 or 40 μmol/L copper, ATP7B clearly dispersed away from the perinuclear region to cytosolic vesicular compartments (Figure 1).

To investigate whether ATP7B traffics to the canalicular membrane, HepG2 cells that had been cultured for 72 hours were treated with various copper concentrations, and the subcellular localization of ATP7B and P-glycoprotein were compared (Figure 2). The P-glyco-
HepG2 cells were incubated with 20–100 μmol/L copper and became more pronounced in 20 or 40 μmol/L copper. In addition, when cells were treated with 20 or 40 μmol/L copper, the vesicular compartments containing ATP7B often clustered around canalicular vacuoles. However, clear colocalization between ATP7B and P-glycoprotein was not observed, and ATP7B-laden vesicles that accumulated around the periphery of a vacuole always appeared distinct from the P-glycoprotein stained membrane (Figure 2). Increasing copper levels to 100 μmol/L copper (Figure 2), or prolonged incubations with 20 or 40 μmol/L copper for up to 16 hours (not shown), did not result in canalicular localization for ATP7B. Line profile histograms constructed by the Leica software (LSC version 2.5, build 1347) indicated that there were possibly a small number of ATP7B molecules that colocalized with P-glycoprotein (MDR1 and MDR3) when HepG2 cells were incubated with 20–100 μmol/L copper (not shown). Nevertheless, such a minor amount of ATP7B possibly residing at the canalicular membrane suggested that ATP7B-mediated efflux of copper directly into the bile is unlikely to be the main mechanism of biliary copper excretion.

To demonstrate that ATP7B redistribution is reversible upon the removal of copper, cells were exposed to 40 μmol/L copper for 4 hours and then cultured in basal medium supplemented with the copper chelator BCS (100 μmol/L) for 4 hours (Figure 2). Decreasing the available copper caused ATP7B to relocalize to the perinuclear region. These results demonstrated that ATP7B cycles between the TGN and pericanalicular vesicles, with the predominant subcellular localization dependent on the level of medium copper and, hence, intracellular copper.

A C-Terminal Trileucine Sequence Is Required for the TGN Localization of ATP7B

Mutation of the C-terminal leucine repeat (LLL1454–1456) in ATP7B was predicted to inhibit the TGN-retrieval of ATP7B molecules from their final trafficking destination. This hypothesis was based on our previous findings with the closely related Menkes protein (ATP7A) in which mutation of the corresponding dileucine motif (LL1487–1488) inhibited endocytic retrieval of ATP7A from the plasma membrane for recycling back to the TGN. The effect of mutating the C-terminal LLL1454–1456 sequence to alanines (LLL-AAA) on the subcellular localization of ATP7B was investigated in CHO-K1 cells (Figure 3A) rather than HepG2 cells because, in HepG2 cells, the mutant protein could not be distinguished from endogenous ATP7B. The trafficking behavior of ATP7B in CHO-K1 cells has been extensively studied. In cells cultured in both basal medium and medium supplemented with 200 μmol/L CuCl2, the ATP7B (LLL-AAA) mutant localized to cytosolic vesicles (Figure 3A) with a staining pattern indistinguishable from that observed with wt-ATP7B under elevated copper conditions (Figure 3A). These results indicate that, as with ATP7A, the C-terminal leucine-based sequence is necessary for the TGN localization of ATP7B in basal medium. We propose that this sequence has a role in the recycling of trafficked ATP7B molecules back to the TGN and, therefore, that ATP7B enters its endocytic route from vesicles and not from the plasma membrane.

ATP7B Mediates Vesicular Sequestration of Excess Intracellular Copper

To investigate whether ATP7B mediates vesicular sequestration of excess intracellular copper, copper accumulation was measured in CHO-K1 cell lines that stably expressed either wt-ATP7B, the LLL-AAA mutant, or an inactive ATP7B mutant harboring a mutation in the phosphorylation domain (D1027E) (Figure 3C). Cell lines that expressed similar levels of the wt and mutated ATP7B proteins, as determined by Western blot analysis, were chosen for the copper accumulation assay (Figure 3B). It is important to note that CHO-K1 cells express endogenous Atp7A (hamster orthologue of ATP7A) and therefore have the capacity to efflux excess intracellular copper directly across the plasma membrane. This copper efflux capacity can be increased with exogenous expression of human wt-ATP7A and a CHO-K1 cell line over expressing wt-ATP7A was used in the copper accumulation assay to illustrate this point. The CHO-K1 cell lines that expressed the ATP7A and ATP7B proteins were cultured in either basal or copper-supplemented medium (100 μmol/L Cu) for 24 hours, and their resultant intracellular copper levels were measured by atomic absorption spectrophotometry (AAS). When cultured in basal copper conditions, there was no significant difference in intracellular copper levels between the cell lines (Figure 3C). However, when cultured in medium supplemented with 100 μmol/L copper (Figure 3C), the exogenous expression of either wt-ATP7A or wt and mutant ATP7B proteins had a marked effect on copper levels in CHO-K1 cells. Cells that express...
Figure 2. Effect of copper on the localization of ATP7B. HepG2 cells grown on coverslips (72 hours) were cultured for 4 hours with medium containing the copper chelator BSC (100 μmol/L) or supplemented with the indicated concentration of copper. The bottom panel shows cells treated first with 40 μmol/L copper (4 hours) then cultured in medium supplemented with the copper chelator BSC (100 μmol/L) for 4 hours. The cells were fixed with cold acetone (−20°C) and double stained for ATP7B and the canalicular membrane located P-glycoprotein (MDR1 and MDR3). Detection of ATP7B was performed using ammonium sulphate-precipitated NC36 (diluted 1 in 5000) and donkey anti-sheep IgG Alexa 488 (diluted 1 in 4000) (Chemicon). P-glycoprotein was detected with a mouse monoclonal antibody (C219) (diluted 1 in 500) (Calbiochem) and donkey anti-mouse IgG Cy5 (diluted 1 in 100) (Chemicon). The nuclei were stained with ethidium bromide (10 μg/mL solution made in PBS) by incubating the cells for 5 minutes immediately following secondary antibody incubation. The color of stained nuclei was changed to blue to reduce interference with green/red overlay. Photographs were taken using a 100× oil objective with a Leica TCS SP2 confocal microscope. Scales are shown.
ATP7A accumulate less than half the amount of copper in comparison to the parental cell line. This result is consistent with earlier reports that demonstrated that overexpression of wt-ATP7A increases the copper efflux capacity of CHO-K1 cells. CHO-K1 cells that exogenously expressed the inactive ATP7B (D1027E) mutant showed a copper accumulation similar to parental CHO-K1 cells. In contrast, exogenous expression of either wt-ATP7B or the ATP7B (LLL-AAA) mutant resulted in a 6.5- and 5.5-fold increase in copper accumulation compared with the parental cell line, respectively.

To demonstrate that the copper that is sequestered inside cells, probably within vesicles, is subsequently eliminated across the plasma membrane, cells were first exposed to 100 μmol/L copper and then cultured in basal medium for 8 hours (Figure 3C). In all cell lines, the

Figure 3. ATP7B mediates vesicular sequestration of excess intracellular copper. (A) CHO-K1 cell lines transfected with either wt-ATP7B, ATP7B with the C-terminal leucine repeat mutated (LLL-AAA), ATP7B with the phosphorylation domain mutated (D–E), or wt-ATP7A were cultured in basal (0.5–1 μmol/L Cu) or 200 μmol/L CuCl₂ supplemented medium. Detection of ATP7B was performed using ammonium sulphate-precipitated NC36 (diluted 1 in 10,000) and donkey anti-sheep IgG Alexa 488 (diluted 1 in 4000) (Chemicon). Photographs were taken using a 60× oil objective with an Olympus PROVIS AX70 microscope. (B) Western blot comparing the expression levels of the indicated ATP7B proteins stably expressed in CHO-K1 cells. Total cellular protein (50 μg) was subjected to electrophoresis by SDS-PAGE (7.5% polyacrylamide) and transferred to nitrocellulose. ATP7B was immunolabelled with ammonium sulphate-precipitated NC36 (diluted 1/1000), followed by horseradish peroxidase-conjugated donkey anti-sheep IgG (diluted 1/4000) and analyzed by chemiluminescence. The nitrocellulose was then stripped using Re-Blot Plus-Mild (Chemicon) and rebotted with mouse anti-β-actin monoclonal antibody (diluted 1/5000) (Sigma) and sheep anti-mouse IgG (diluted 1/4000). The positions of the molecular weight markers (Bio-Rad) are indicated on the left in kilodaltons. (C) The intracellular copper content of the CHO-K1 cells exogenously expressing the indicated ATP7A or ATP7B proteins as measured by AAS. The cells were seeded into 75 cm² flasks and were cultured for 24 hours in basal medium (0.5–1 μmol/L copper) or copper-supplemented medium (100 μmol/L copper). Cells were also exposed to 100 μmol/L copper for 24 hours then incubated in basal medium (8 hours) to demonstrate cellular copper efflux. Results represent the normalized mean ± STDEV (bar) of triplicate determinations for each cell line cultured in the indicated copper conditions. Results shown as copper (ng) per 10⁶ cells.
intracellular copper levels returned to those observed when cells were cultured under basal copper conditions only.

**Patient Mutation 4193ΔC Causes Structural Instability in ATP7B**

Wilson disease patients from 7 different Saudi Arabian tribes were found to have a single nucleotide deletion at base pair position 4193 in the ATP7B coding sequence. This deletion causes a frame shift and the subsequent introduction of 5 different amino acids before a premature termination codon, resulting in the truncation of the last 61 amino acids, including the trileucine sequence of the ATP7B protein. Patients with this mutation display the hallmarks of Wilson disease, suggesting that ATP7B-mediated biliary excretion of copper is perturbed. The trileucine sequence is the only region known to be important in the 61 amino acids that are missing in the 4193ΔC mutant protein. Mutation of the trileucine motif caused ATP7B to localize constitutively at vesicles (Figure 3A) but had no impact on copper-translocation activity (Figure 3C). Therefore, if the mechanism for biliary copper excretion involves the exocytosis of copper loaded vesicles, then it was not clear why patients with the 4193ΔC mutation accumulate copper in hepatocytes.

The effect of the 4193ΔC mutation on the copper-translocation activity of ATP7B was assessed using the αα2Δ yeast complementation assay (Figure 4A and B). This assay is based on the ability of ATP7B to replace its yeast orthologue (Ccc2) and transport copper to the ferroxidase Fer3, which is required for growth in iron-limited medium. Initially, 10 transformants were screened for ATP7B (4193ΔC) expression; however, only 2 displayed detectable levels of the mutant protein (not shown). Attempts to isolate transformants with higher levels of 4193ΔC expression failed. The 2 strains in which 4193ΔC expression was detected displayed similar levels of expression, and therefore, only 1 of the yeast strains was used in the yeast complementation assay (Figure 4A and B). Wt-ATP7B and the LLL-AAA mutants were assayed and complemented αα2Δ yeast, allowing growth under iron-limited conditions (Figure 4B). The growth curves for yeast strains expressing LLL-AAA and wt-ATP7B were very similar, suggesting that the LLL-AAA mutant could complement the iron-deficiency phenotype of the αα2Δ yeast with the same capacity as wt-ATP7B. Caα2Δ yeast that expressed 4193ΔC was able to grow in iron-limited medium, despite the low expression levels of the mutant protein. The linear exponential growth phase between 12 and 20 hours was used to determine the growth rate for each yeast strain (gradient of the trend line). The rate of growth for the strain that expressed 4193ΔC was 49.8% in comparison with αα2Δ yeast that expressed wt-ATP7B. However, the reduced growth rate is likely to be due to the lower expression level of the mutant protein rather than a specific effect of the mutation itself. These results suggested that the 4193ΔC mutation does not affect the copper-translocation activity of ATP7B per se, but may cause structural instability, which is consistent with the difficulty in isolating transformants expressing equivalent levels of protein to that obtained with wt-ATP7B.

The effect of the 4193ΔC mutation/truncation on the subcellular localization of ATP7B was investigated in CHO-K1 cells. However, among numerous coverslips examined for cells expressing 4193ΔC, there were very few cells in which the 4193ΔC protein was detected, and these cells displayed only weak immunofluorescence staining (not shown). In the few cells in which 4193ΔC expression was identified, the protein localized aberrantly throughout the cytosol of the cell in a reticulated pattern. This staining pattern indicated endoplasmic reticulum (ER) localization, which is common for misfolded ATP7B variants. To determine whether the localization of the 4193ΔC protein could be corrected by allowing the protein to fold at a reduced temperature, cells that expressed this protein were incubated for 3 days at 30°C before being analyzed by immunofluorescence microscopy (Figure 4C). The reduction in temperature dramatically increased the number of cells in which 4193ΔC was detected (not shown) but had no effect on the subcellular localization of the mutant protein (Figure 4C). The inability of 4193ΔC to fold correctly and localize to the TGN at 30°C suggests that this particular mutation/truncation severely destabilizes ATP7B, although Western blot analysis demonstrated that culturing the cells at 30°C did result in more intact/stable 4193ΔC protein (Figure 4D). Taken together, these results suggest that the 4193ΔC mutation causes ATP7B to misfold and consequently mislocalize in vivo.

**Discussion**

There have been contradictory reports in the literature regarding the subcellular localization of ATP7B and the effect of copper on its localization. Clarification of this issue is essential to elucidate the mechanism of copper efflux from hepatocytes into the biliary canalicus (bile). Here, we studied the copper-dependent subcellular localization of endogenous ATP7B in the HepG2 human hepatoma cell line as a model of hepatocytes. HepG2 cells retain the biosynthetic capability and the specialized functions of hepatocytes, in-
cluding the production of bile canaliculi between 2 or more juxtaposed cells, making them an excellent model cell for the study of ATP7B localization. In HepG2 cells cultured in basal or copper-depleted conditions, ATP7B was found in perinuclear compartments close to but not overlapping the TGN-resident protein p230. The close proximity between ATP7B and p230 and their “nucleus-capping” staining pattern suggested that ATP7B is located in a subcompartment of the TGN distinct from p230. The distribution of ATP7B often favored 1 side of

Figure 4. The effect of the patient mutation 4193ΔC on copper-translocation activity and subcellular localization of ATP7B in CHO-K1 cells. (A) The ccc2Δ yeast strains transformed with either wt-ATP7B, 4193ΔC, or the LLL-AAA mutant were analyzed for ATP7B expression by Western blot analysis. Total cellular protein (30 μg) was subjected to electrophoresis by SDS-PAGE (7.5% polyacrylamide) and transferred to nitrocellulose. ATP7B was immunolabeled with ammonium sulphate-precipitated NC36 (diluted 1/1000), followed by horseradish peroxidase-conjugated donkey anti-sheep IgG (diluted 1/4000) and analyzed by chemiluminescence. The positions of the molecular weight markers (Bio-Rad) are indicated on the left in kilodaltons. (B) Line graph showing the growth patterns of ccc2Δ yeast transformed with either wt-ATP7B (■), 4193ΔC (X), LLL-AAA (▲), or expression vector alone (●) in iron-depleted liquid medium. Growth rates of the yeast strains were analyzed spectroscopically with the OD600 recorded at times 0, 10–26 (2-hour intervals), and 36 hours. Only the growth pattern over the exponential phase (12–20 hours) is shown. STDEVs not shown (largest STDEV was 0.045). (C) Subcellular localization of the 4193ΔC mutant protein in CHO-K1 cells that were cultured at 30°C (72 hours) then incubated for 8 hours (at 30°C) with basal medium or medium supplemented with 200 μM CuCl2. Detection of 4193ΔC was performed using ammonium sulphate-precipitated NC36 (diluted 1 in 10,000) and donkey anti-sheep IgG Alexa 488 (diluted 1 in 4000) (Chemicon). Photographs were taken using a 60x oil objective with an Olympus PROVIS AX70 microscope. (D) Western blot analysis comparing the amount of intact/stable 4193ΔC protein in CHO-K1 cells cultured for 3 days at either 37°C or 30°C. Total cellular homogenates (50 μg) were subjected to electrophoresis by SDS-PAGE (7.5% polyacrylamide) and transferred to nitrocellulose. The membrane was incubated with ammonium sulphate-precipitated NC36 (diluted 1/1000), followed by horseradish peroxidase-conjugated donkey anti-sheep IgG (diluted 1/4000) and analyzed by chemiluminescence. The nitrocellulose was then stripped using Re-Blot Plus-Mild (Chemicon) and rebotted with mouse anti-β-actin monoclonal antibody (diluted 1/5000) (Sigma) and horseradish peroxidase–conjugated sheep anti-mouse IgG (diluted 1/4000). The positions of the molecular weight markers (Bio-Rad) are indicated on the left in kilodaltons.
the nucleus, which is consistent with the typical arrangement of the TGN in polarized HepG2 cells.

As previously demonstrated, increasing copper concentration in the culture medium induced redistribution of ATP7B to cytosolic vesicular compartments (Figures 2 and 3). An observable copper-induced trafficking response could be stimulated with as little as 5 μmol/L added copper but was more evident with 20–40 μmol/L copper. Importantly, the trafficking of ATP7B in HepG2 cells could be induced with modest increases in medium copper within physiologic range. The responsiveness of ATP7B in hepatic cells is consistent with the important role of these cells in copper homeostasis and possibly reflects a high expression of the copper importer hCtrl. The trafficking of ATP7B in hepatic and non-hepatic cell types has been demonstrated by several groups both in vitro and in vivo. However, Harada et al reported that copper does not stimulate intracellular redistribution of endogenous ATP7B in Huh7 hepatocytes or GFP-tagged ATP7B in several other cell types. The reason for this discrepancy is unknown. In polarized hepatocytes (HepG2 cells) used in this study, in which polarization was defined by the formation of a canalicular vacuole, the vesicular compartments containing ATP7B targeted the canalicular (apical) membrane and often accumulated around the entire circumference of a canalicular vacuole. However, an obvious overlap between ATP7B and a canalicular membrane marker could not be detected. This result contrasts to previous observations made by Roelofsen et al. These authors found that ATP7B in HepG2 cells was stimulated by copper to traffic first to large vesicles and more slowly to the canalicular membrane. An electron microscopy image showed ATP7B labelling of a canalicular vacuole. The results presented in this current report indicate that very few ATP7B molecules appear to localize to the canalicular membrane, whereas the vast majority reside at distinct pericanalicular vesicles. This difference is important for understanding the main mechanism of ATP7B function. Based on our findings, the primary mechanism for ATP7B-mediated biliary copper excretion is not likely to involve copper transport by ATP7B directly across the canalicular membrane.

Dileucine (or leucine-isoleucine)-based endocytic signals have been shown to mediate the constitutive TGN-retrograde of several proteins that cycle between the plasma membrane and the TGN, including furin, GLUT 4, and ATP7A. Mutation of the trileucine sequence in ATP7B (LLL-AAA) caused the protein to localize constitutively at cytosolic vesicular compartments in CHO-K1 cells and not at the plasma membrane as previously found with ATP7A. Therefore, the trileucine sequence (or a dileucine motif contained within) is involved in the TGN retrieval of trafficked ATP7B molecules from vesicles rather than the plasma membrane, suggesting that these vesicles are the final trafficking destination for ATP7B in response to copper. ATP7A and ATP7B have different final trafficking destinations within cells and therefore different mechanisms for mediating copper efflux. This is reflected by our observations that CHO-K1 cells expressing either ATP7A or ATP7B display opposite copper phenotypes; ATP7A reduced intracellular copper, whereas ATP7B temporarily increased cellular copper levels. The localization of the LLL-AAA mutant to vesicular compartments in CHO-K1 cells and the fact that it retained normal copper-translocation activity allowed direct assessment of the ability of ATP7B to mediate vesicular sequestration of copper. CHO-K1 cells that exogenously expressed the LLL-AAA mutant or wt-ATP7B accumulated 5.5- and 6.5-fold more copper than the parental cell line, respectively. In contrast, CHO-K1 cells that expressed a nonfunctional ATP7B mutant (D1027E) accumulated copper to a similar extent as the parental cells. Therefore, copper retained by CHO-K1 cells as a consequence of wt-ATP7B or LLL-AAA expression is attributable to copper-translocation activity and not to mere copper coordination by ATP7B molecules. ATP7B can therefore mediate vesicular sequestration of excess copper, and we further demonstrated that the copper sequestered in vesicles by ATP7B is ultimately exocytosed by cells, returning intracellular copper to basal levels. Conversely, ATP7A-expressing cells accumulated less than 50% of copper compared with the parental cells, demonstrating that direct efflux of copper across the plasma membrane is a significantly more efficient means of copper removal than vesicular sequestration and exocytosis. However, hepatic exposure to excess copper in rodents results in a rapid increase in biliary copper excretion after several minutes, consistent with the vesicular process. Although it is known that rodents have much more efficient biliary copper excretory mechanisms than humans, this large increase in excretion of copper could indeed be limited to hepatocytes with intact canalicular function and/or may reflect differences in ATP7B between humans and rodents that affect its function and cell biology. The difference in the efficiency of copper excretion in vivo between ATP7A and ATP7B would be difficult to assess.

Three disease-causing mutations affecting the cytosolic C-terminal region of ATP7B have been identified. Two are missense mutations, 4135C→T and 4301C→T, which result in the substitution of a proline for a serine (P1379S) and a threonine for a methionine (T1434M), respectively.
The other is the 4193ΔC mutation, which causes the truncation of the last 61 amino acids, including the trileucine sequence, of the ATP7B protein. Hsi et al. recently established that the cytosolic C-terminal region of ATP7B is important for protein stability because removal of this region leads to reduced protein levels in yeast. These authors also demonstrated that truncating the last 61 amino acids (Δ4126) did not affect the steady-state level of ATP7B. However, in this study, the 4193ΔC patient mutation, which results in 5 different amino acids (QVSVH-RSVCT) before truncation of the last 61 amino acids, caused a reduction in expression levels in yeast and in CHO-K1 cells mislocalized to the ER. Retention in the ER is common for misfolded ATP7B variants, and reducing the growth temperature often corrects the subcellular localization of such ATP7B variants. However, temperature reduction had no effect on the subcellular localization of the 4193ΔC mutant, indicating that this particular mutation/truncation causes severe misfolding of ATP7B and subsequent retention by the ER. These observations are consistent with reduced expression of the 4193ΔC mutant in Δα2Δ yeast. However, in Δα2Δ yeast, a limited number of 4193ΔC molecules passed the ER protein quality control and were able to complement the iron-deficiency phenotype. Taken together, these results suggest that the 4193ΔC mutant would mislocalize in vivo and consequently would be unable to participate in hepatic copper homeostasis, accounting for the Wilson disease phenotype observed in patients with this mutation.

Conceivably, the other 2 patient mutations, P1379S and T1434M, which affect the extreme C-terminus of ATP7B, could also obstruct the correct folding of the molecule as these changes alter polarity (hydrophobic proline to neutral serine and neutral threonine to hydrophobic methionine, respectively). These mutations do not affect ATP7B expression or the copper-translocation activity of ATP7B in yeast. However, mutations in ATP7B that affect folding are commonly temperature sensitive and unless they severely disturb the native structure are only of consequence when biosynthesis and folding occurs at 37°C. Therefore, further analysis of these mutants at 37°C, for instance in mammalian cells, is necessary to determine the effect of these mutations on ATP7B function.

This study has provided significant insight into mechanisms underlying ATP7B-mediated copper efflux from hepatocytes into bile. We have shown that ATP7B can mediate vesicular sequestration of excess copper for detoxification and eventual efflux through exocytosis. The predominant vesicular localization for ATP7B in polarized hepatocytes (HepG2 cells) is consistent with vesicular exocytosis being the primary copper excretory mechanism into bile. This work also further highlights a fundamental difference between the closely related copper-ATPases (ATP7A and ATP7B) in their mechanism of eliminating copper from cells. Possibly, ATP7B-mediated vesicular copper sequestration may allow temporary copper storage or enable hepatocytes to detoxify copper more rapidly when facing high dietary uptake. This is consistent with the role of the liver in maintaining and regulating the overall copper status of the body. In contrast, the overriding priority for nonhepatic cells in which ATP7A is expressed may be to eliminate excess copper by the most efficient means available, and, therefore, ATP7A traffics to and directly effluxes copper across the plasma membrane.

Our results are consistent with the biliary copper excretion model proposed by Schaefer et al. in which ATP7B mediates vesicular copper sequestration when intrahepatic copper levels rise. Upon the elimination of excess cytosolic copper into vesicles, ATP7B recycles back to the TGN, with this step dependent on recognition of the C-terminal leucine motif by the endocytic-sorting machinery of the cell. The copper containing vesicles (devoid of ATP7B) then move to and fuse with the canalicular membrane to expel the excess copper into bile. Recently, the MURR1 protein has been implicated to play a role in biliary copper excretion and has been shown to interact directly with ATP7B. Perturbed MURR1 expression is believed to be responsible for the massive hepatic copper accumulation and decreased biliary copper excretion observed in Bedlington terriers. It is possible that MURR1 is involved in vesicular sequestration of copper or in the exocytosis of copper-loaded vesicles, consistent with the protein’s intracellular vesicular localization. Future studies will address the regulatory mechanism(s) governing the exocytosis of copper-loaded vesicles and establish whether copper contained in these vesicles is coordinated or ionic.

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Address requests for reprints to: Julian F. B. Mercer, MD, Centre for Cellular and Molecular Biology, School of Biological and Chemical Sciences, Deakin University-Melbourne campus, 221 Burwood Highway, Burwood, Victoria 3125, Australia. e-mail: Ujmercer@deakin.edu.au; fax: (61) 3-9251-7328.
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