Hepatocyte-specific localization and copper-dependent trafficking of the Wilson’s disease protein in the liver

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Hepatocyte-specific localization and copper-dependent trafficking of the Wilson’s disease protein in the liver. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G639–G646, 1999.—Wilson’s disease is an inherited disorder of copper metabolism characterized by hepatic cirrhosis and neuronal degeneration. In this current study, a polyclonal antiserum specific for the Wilson’s disease ATPase was used to examine the hepatic expression of this protein. Immunoblot analysis of lysates from human and rat liver detected a single 165-kDa protein, which by immunofluorescence was present only in hepatocytes and localized predominantly to the trans-Golgi network and exclusively in this compartment under low hepatic copper concentrations. Although hepatic copper concentration had no effect on the steady-state levels of the Wilson’s disease protein, copper administration in vivo resulted in redistribution of this protein to a cytoplasmic vesicular compartment localized toward the hepatocyte canalicular membrane. The relative abundance of the Wilson’s disease protein in the liver was found to be greatest in the fetus before the onset of biliary copper excretion. Taken together, these studies reveal a novel posttranslational mechanism of copper homeostasis in vivo consistent with the proposed function of the Wilson’s disease protein in holoceruloplasmin biosynthesis and biliary copper excretion. Taken together, these studies reveal a novel posttranslational mechanism of copper homeostasis in vivo consistent with the proposed function of the Wilson’s disease protein in holoceruloplasmin biosynthesis and biliary copper excretion and of relevance to the broad clinical heterogeneity observed in this disease.

Wilson’s disease (WD) is an autosomal recessive disorder of copper metabolism that results in liver disease and neurological symptoms (6, 7). Affected individuals exhibit excessive copper accumulation in the liver, deficient ceruloplasmin biosynthesis, and a marked impairment in biliary copper excretion (28). These findings suggest that the pathogenesis of this disorder results from a defect in the transfer of hepatocyte copper into a compartment essential for the incorporation of this metal into ceruloplasmin as well as for excretion into the bile. Consistent with this concept, the liver has been shown to be the primary site of copper storage and excretion in humans (27), and liver transplantation in affected patients with WD restores copper balance and reverses clinical symptoms (29). Furthermore, the WD gene has been cloned and shown to be expressed in human liver by RNA blot analysis (4, 33, 39).

The deduced amino acid sequence of the WD gene identifies a protein with significant homology to a recently recognized family of polytopic transmembrane cation-transporting P-type ATPases (15, 32). Furthermore, expression of the WD protein in the yeast mutant strain ccc2Δ lacking the homologous ATPase, and in the Long-Evans Cinnamon (LEC) rat, an animal model of WD (38, 40), restores cuproprotein biosynthesis, indicating a direct role for this protein in copper transport (11, 34). In Hep G2 cells, the WD protein is synthesized as a single-chain 165-kDa protein localized to the trans-Golgi network under steady-state conditions (11, 42). An increase in the concentration of copper in these cells results in the rapid movement of this protein out of the trans-Golgi and into a cytoplasmic vesicular compartment (11), similar to what has been observed for the homologous Menkes disease ATPase (23).

Although these data have provided some insights into the potential function of the WD protein, there is currently no information regarding the expression of this protein in vivo. Therefore, the current study was undertaken to define the expression and localization of the WD protein in the liver and to elucidate the effects of hepatic copper concentration on this process. Because dramatic changes in hepatic copper concentration are observed during the perinatal period in the face of very different mechanisms of copper homeostasis (16), the developmental expression of the WD protein under these circumstances was similarly examined.

METHODS

Materials. Chemicals were purchased from Sigma Chemical (St. Louis, MO). Nitrocellulose and nylon membranes as well as enhanced chemiluminescence detection reagents were from Amersham (Arlington Heights, IL). Tissue-Tek and TBS tissue-freezing medium were obtained from Satura Finetek (Torrance, CA) and Triangle Biomedical Sciences (Durham, NC), respectively. Murine monoclonal antibodies directed against the multidrug-resistant P-glycoproteins (clone C219), the rat trans-Golgi network protein TGN38 (clone 2F7.1), and the Golgi-associated rat protein 58K (clone 58K-9) were purchased from Dako (Carpenteria, CA), Affinity Bioreagents (Golden, CO), and Sigma Chemical, respectively. Secondary antibodies conjugated with FITC or lissamine rhodamine isothiocyanate (LRSC) were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). Williams E media, DMEM, and fetal bovine serum were obtained from GIBCO BRL (Grand Island, NY). Normal human liver tissue was obtained at autopsy under approval of the Washington University School of Medicine institutional review board for human studies.

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Animals. Timed pregnant Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA) and fed a nutritionally complete control diet containing 6.5 parts per million (ppm) copper (10) and deionized water until 2 days after parturition. Pups in the treatment group were nursed by dams fed a copper-deficient diet (0.4 ppm) until weaning (postnatal day 21) and then fed this low-copper diet until 42 days of age. Dams and pups in the control group were fed the copper-adequate diet (6.5 ppm) throughout the lactation and postweaning periods. At specific times indicated, animals were killed and the livers were excised and frozen in liquid nitrogen for subsequent analysis. For the copper-loading studies, age-matched male Sprague-Dawley rats (380 g) received daily intraperitoneal injections of 3 mg Cu as CuSO4/kg body wt in 0.9% NaCl over 6 consecutive days (18). Two hours after the last CuSO4 injection, the livers were excised. Copper concentrations in liver and diet samples were determined by atomic absorption spectroscopy as previously described (10). Liver tissue from adult LEC rats was a kind gift from H. Bartsch (DKFZ, Heidelberg, Germany). All animal studies were performed under approval of the animal studies committees of the respective institutions.

RNA analysis. RNA was isolated by CsCl density gradient centrifugation after dissolution in guanidinium isothiocyanate (5). RNA samples were electrophoresed in 0.8% agarose-2.2 M formaldehyde gels, transferred to nylon membranes, and hybridized at 58.5°C, using a 420-bp 32P-labeled complementary RNA probe encoding amino acids 486–621 of the human Wilson ATPase as previously described (39).

Primary cell culture. Hep G2 and HeLa cells were obtained and maintained as previously described (11). Rat hepatocytes were isolated from male Sprague-Dawley rats (300 g) using a modified two-step collagenase perfusion method (31). The isolated cells were resuspended in Williams E medium supplemented with 10% fetal bovine serum, 10−7 M insulin, 10−9 M dexamethasone, and 10 mM nicotinamide, plated on collagen coated coverslips at a cell density of 0.25 × 106 cells/cm2, and cultured in a humidified atmosphere of 5% CO2-95% air at 37°C.

Immunoblotting. Tissue fractions and cell lysates. Total homogenates, postnuclear supernatants, and a Golgi-enriched fraction of human and rat livers were obtained as described previously (19, 34). Hep G2 and HeLa cells and primary rat hepatocytes were lysed in 0.25% Nonidet P-40, 100 mM Tris·HCl, pH 8.0, at 4°C for 20 min and centrifuged at 16,000 g for 20 min as described previously (11). Equal amounts of protein of the liver fractions and postnuclear supernatants of cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes (11), and visualized using enhanced chemiluminescence. A rabbit polyclonal antibody directed against the metal-binding domains of the human WD protein was utilized as described previously (11). A Molecular Dynamics (Sunnyvale, CA) densitometer was used for analysis of immunoblots.

Immunofluorescence. Liver tissue was placed in 1:1 vol/vol Tissue-Tek and TBS tissue-freezing medium, frozen in 2-methylbutane on dry ice, and cut by a standard cryotome into 5- to 10-μm sections. Frozen sections were fixed with acetone at −20°C for 10 min before the application of the primary and secondary antibodies. Primary rat hepatocytes plated on glass coverslips were fixed in 4% paraformaldehyde in PBS for 20 min, quenched with 0.1 M ethanediamine, and permeabilized in 0.2% Triton X-100 in PBS for 10 min. Nonspecific binding was blocked by incubation with 3% bovine serum albumin and 5% donkey serum in PBS for 30 min, followed by incubation with the primary and secondary antibodies as indicated. Murine monoclonal antibodies to the P-glycoproteins were utilized as described to identify the canalicular membrane (35). After staining, slides were mounted on Mowiol 4–88 and analyzed using an Olympus BX 60 microscope (filter sets: Chroma 41001 high-Q for FITC and Chroma 41002 high-Q for LRSC) and a Molecular Dynamics laser scanning confocal microscope model 2001 equipped with an argon/krypton laser (excitation 488 and 568 nm, filter sets 530-DF30 for FITC and 600-DF40 for LRSC). A single 1-μm tissue section and a series of 10 consecutive 1-μm tissue sections were obtained for laser scanning confocal microscopy (LSCM).

RESULTS

As can be seen in Fig. 1, immunoblotting with an antibody specific to the WD protein identified a single 165-kDa protein in lysates from Hep G2 cells, previously shown to express the WD protein, but not in HeLa cells, which express the homologous Menkes copper-transporting ATPase (Fig. 1, lanes 1 and 2). With the use of these same methods, a similar-sized band was also observed in lysates from human liver, rat liver, and primary rat hepatocytes (Fig. 1, lanes 3 and 5–7). However, preabsorption of the antibody with the glutathione S-transferase (GST)-WD fusion protein used to raise the antibodies prevented the detection of the WD protein in lysates from human liver (Fig. 1, lane 4). The WD protein was not detected in liver lysates from LEC rats (Fig. 1, lane 8), which contain a deletion of the homologous rat WD gene (38, 40). These data indicate that the WD protein is abundantly expressed as a single-chain protein in normal liver tissue and that the

![Fig. 1. Immunoblot analysis of Wilson's disease (WD) protein. Postnuclear supernatants from Hep G2 cells, HeLa cells, rat liver and primary rat hepatocytes, Golgi-enriched fraction from human liver, and total homogenates from normal rat liver and Long-Evans cinnamon (LEC) rat liver were prepared, and 50 μg of protein from each sample were separated by SDS-PAGE, transferred to nitrocellulose, incubated with Wilson antibody (lanes 1–3 and 5–8) or the Wilson antibody preabsorbed with glutathione S-transferase (GST)-WD fusion protein used to raise the antibody (lane 4), and analyzed by chemiluminescence. Molecular mass (Mr, × 10^3) markers are shown at left.](Image)
antibody to the human WD protein readily detects the homologous transporter in the rat.

Immunoblotting revealed comparable levels of the WD protein in rat liver and primary rat hepatocytes (Fig. 1, lanes 5 and 6), suggesting cell-specific expression. To delineate the cell types expressing the WD protein in the liver, immunofluorescence analysis was performed on human and rat liver sections. In this analysis, the WD protein was consistently detected in hepatocytes in both human and rat liver, with no differential gradient of expression throughout the liver parenchyma (Fig. 2, A and D). No expression was observed in vascular endothelium, biliary epithelium, or other liver cell types (data not shown). At higher magnification the WD protein appeared to be distributed in a punctate pattern, oriented toward the canalicular pole directly across from the hepatic sinusoids (Fig. 2, C and F). No specific signal was detected when these sections were incubated with preimmune serum or secondary antibody alone (data not shown). Furthermore, no specific signal was observed in liver sections obtained from LEC rats lacking expression of the WD protein (Fig. 2E).

The significant degree of autofluorescence observed in human liver sections precluded further characterization of the subcellular distribution of the WD protein in human liver. Nevertheless, because the expression pattern of the WD protein appeared identical in human and rat liver, this question was directly addressed in rat liver sections. To characterize the location of the WD protein in more detail, double-label immunofluorescence LSCM studies were performed using an antibody to the trans-Golgi network protein TGN38 (24). As can be seen in Fig. 3, the WD protein was detected in a punctate pattern that showed considerable overlap with the location of the TGN38 protein (Fig. 3, A–C). In contrast, very limited overlap was observed when antibodies to the bile canalicular plasma membrane P-glycoproteins (35) were used in similar experiments (Fig. 3, D–F). As can be seen outlined by the arrowheads (Fig. 3, E and F), the WD protein within the hepatocytes was concentrated at the canalicular pole as revealed by the localization near the bile canalicular marker.
100 µM CuCl₂, the WD protein was detected in a diffuse contrast, 1 h after incubation in medium containing work protein TGN38 (Fig. 4, showed considerable overlap with the trans-Golgi network marker (Fig. 3C, arrows). A similar observation was recently made in Hep G2 cells and found to directly relate to the intracellular copper concentration (17). Because the variability of copper content in individual liver sections precluded a careful analysis of this question, the copper-dependent localization of the WD protein was examined in primary rat hepatocytes. In these studies, freshly isolated hepatocytes were incubated with media containing 100 µM CuCl₂ and then subjected to double-label immunofluorescence microscopy. As was observed in whole liver sections, the WD protein in primary rat hepatocytes under basal conditions showed considerable overlap with the trans-Golgi network protein TGN38 (Fig. 4A, and B, arrowheads). In contrast, 1 h after incubation in medium containing 100 µM CuCl₂, the WD protein was detected in a diffuse vesicular pattern throughout the cytoplasm in the primary rat hepatocytes (Fig. 4C). This effect of copper was specific for the WD protein because the localization of the trans-Golgi network marker TGN38 was not affected in these same cells (Fig. 4D). Further analysis of this process revealed movement of the WD protein from the trans-Golgi network within 5 min of incubation with excess copper that was completed within 15 min (Fig. 5, A–C). This process was dose dependent, being initiated by as little as 10 µM copper but requiring copper concentrations above 40 µM to result in complete disappearance of the protein from the trans-Golgi network (data not shown). The effect of copper was reversible, as chelation of excess copper by the addition of 100 µM bathocuproinedisulfonate for 2 h resulted in a redistribution of the WD protein to a concentrated perinuclear location (Fig. 5D) identical to that observed in control cells (Fig. 5A). This process of copper-dependent trafficking was unaffected by new protein synthesis because similar observations were made in hepatocytes incubated with 10 µg/ml cycloheximide for 2 h before and during the course of the experiments (data not shown).

The observation of copper-dependent trafficking of the WD protein in primary hepatocytes suggested that the variation in localization of WD protein observed previously in liver sections might be related to the intracellular hepatic copper concentration. However, the observations in primary hepatocytes are subject to the caveats of in vitro experiments, including the loss of polarization that occurs with cell isolation. Therefore, to more directly address this question, LSCM was performed on liver sections obtained from copper-deficient and copper-loaded rats. The hepatic copper concentrations of these animals revealed significant alterations of copper status in the sections examined (see Fig. 6, legend). As demonstrated by overlay images, the WD protein was concentrated in the trans-Golgi network within hepatocytes in the copper-deficient state (Fig. 6B), and no overlap was detected with the canalicular membrane marker P-glycoprotein (data not shown). In contrast to these findings, analysis of the WD protein in liver sections from copper-loaded rats revealed this protein distributed in multiple vesicular structures with limited trans-Golgi network localization (Fig. 6C) and concentrated near the bile canalicular plasma membrane (data not shown). In contrast to these differences in the intracellular localization, the hepatic copper concentration had no effect on the relative abundance of the WD protein in lysates from these same livers (Fig. 6A).

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**Fig. 4.** Double-label immunofluorescence of primary rat hepatocytes. Primary rat hepatocytes were grown under standard conditions (A and B) or in medium supplemented with 100 µM CuCl₂ for 1 h (C and D). After fixation, coverslips were incubated with antibodies directed against WD protein (WD) detected by FITC-conjugated secondary antibodies and with murine monoclonal antibodies directed against the trans-Golgi network protein TGN38 and detected by LRSC-conjugated secondary antibodies. Arrowheads mark overlap of signals. Single exposures of identical cells (A and B; C and D) were taken in the FITC and LRSC filter set. Original magnification: ×600.

**Fig. 5.** Copper-induced trafficking of WD protein in primary rat hepatocytes. Primary rat hepatocytes preincubated with 50 µM bathocuproinedisulfonate (BCS) (A–D) for 8 h were incubated with medium containing 40 µM CuCl₂ for times indicated and fixed immediately thereafter (A–C) or kept in medium supplemented with 40 µM CuCl₂ for 15 min followed by incubation in medium containing 100 µM BCS for 2 h before fixation (D). Coverslips were incubated with WD protein antibodies detected by LRSC-conjugated secondary antibodies. Arrowheads indicate trans-Golgi network localization of WD protein (A, B, and D). Original magnification: ×600.
The results of the copper studies both in vitro and in vivo suggested that the WD protein is regulated in a posttranslational fashion by the copper concentration of the cell. Because hepatic copper concentrations increase significantly during development before the onset of biliary copper excretion, the developmental expression of the WD gene was analyzed. Total RNA was isolated from the liver tissue of fetal and adult rats, and RNA blot analysis was performed using a human WD cRNA probe. This analysis revealed a transcript of 7.4 kb, readily detected in all samples, which decreased in abundance with increasing age of the animals (Fig. 7A). Reprobing of these samples with a 28S RNA probe revealed loading of equivalent amounts of RNA in each lane (data not shown). Consistent with these RNA data, immunoblot analysis of rat livers during late gestation and the postnatal period demonstrated abundant protein during the earliest fetal stages examined, with decreasing amounts as the animals aged (Fig. 7B). Reprobing the same blot with an antibody to P-glycoproteins revealed a different pattern of developmental expression and confirmed that the decrease in WD protein at later stages of development was not due to changes in the protein content of the samples (Fig. 7B). A quantitative analysis of hepatic WD protein expression during development revealed ∼15% of the protein in the liver of adult animals compared with embryonic day 17 (Fig. 7C), whereas the hepatic copper concentration was highest in the postnatal period (Fig. 7D).

The abundant expression of the WD protein before biliary copper excretion and holoceruloplasmin biosynthesis was somewhat surprising. Because the liver at this stage is a prominent site of hematopoiesis, immunofluorescence microscopy was performed to examine the cell type-specific expression of the WD protein during embryonic development. As can be seen in Fig. 8, at gestational day 17 the WD protein was exclusively expressed in hepatocytes (Fig. 8, A and B, arrowheads), with no signal detected in hematopoietic cells (Fig. 8, A and B, asterisks). During these early developmental stages, when hepatic copper concentrations were 4- to 30-fold greater than in the normal adult liver (Fig. 7), the WD protein showed considerable overlap with the Golgi-associated rat protein 58K (2) (Fig. 8, C and D), as well as with the trans-Golgi network resident protein TGN38 (data not shown), indicating a distribution

![Image of Fig. 6. Immunoblot analysis and double-label LSCM of WD protein in rat liver. A: lysates were prepared from copper-loaded (lane 2), copper-deficient (lanes 4 and 6), and control (lanes 1, 3, and 5) rat livers, and equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose, incubated with Wilson antibody, and analyzed by chemiluminescence to detect WD protein (WD). Copper concentration (in µg/g wet liver wt): lane 1, 4.4; lane 2, 3.84; lane 3, 2.71; lane 4, 1.0; lane 5, 3.12; lane 6, 0.44. Frozen liver sections from copper-deficient (B) and copper-loaded (C) rats were acetone fixed and incubated with WD protein antibody detected by LRSC-labeled secondary anti-rabbit IgG antibody and monoclonal antibody detecting trans-Golgi network protein TGN38 utilizing secondary FITC-conjugated anti-mouse-IgG antibody. LSCM overlay images of single 1-µm planes were obtained. Arrowheads indicate WD protein colocalization with TGN38 under low copper (lane 2, C) and high copper (lane 5, C). Original magnification: ×600.

![Image of Fig. 7. Developmental expression of WD protein. A: equal amounts of RNA from rat liver tissue at gestational day 17 (E17), at E20, and from an adult rat were separated by electrophoresis, transferred to nitrocellulose, and hybridized with a 32P-labeled cRNA probe specific for human Wilson gene mRNA (WD mRNA). B: postnuclear supernatants of rat livers from E17 and E21 and postnatal days 2, 12, 21, and 42 (P2, P12, P21, and P42) were prepared, and 25 µg of protein from each sample were separated by SDS-PAGE, transferred to nitrocellulose, and incubated with Wilson protein antibody (WD) or P-glycoprotein antibody (pgp). C: densitometric analysis of 3 sets of Wilson protein expression during development was performed to determine expression levels in relation to E17 (3 data sets, ±SD). D: copper concentrations of rat liver tissue as determined by atomic absorption spectroscopy (3 animals/data point; ±SD).]
in such circumstances, the excretion of copper by the liver remains negligible until the level of hepatic copper reaches a specific threshold (21). The observations in this study suggest a mechanistic explanation for these data, where the copper concentration-dependent movement of the WD protein from the trans-Golgi network represents the rate-limiting event required for biliary copper excretion. The hypothesis that biliary copper excretion requires this intermediate step of post-Golgi copper sequestration into a vesicular compartment is entirely consistent with the immunofluorescence studies in copper-deficient and copper-loaded hepatocytes and rats (Figs. 4–6). Such a concept is also supported by recent studies on the homologous copper-transporting P-type ATPase in Saccharomyces cerevisiae, which demonstrate that localization of this protein to a post-Golgi vacuolar compartment is essential for copper homeostasis in this organism (43).

On the basis of the methods employed here, it does appear that a small amount of the WD protein may localize to the canalicular membrane (Fig. 3C). Further analysis of this question will require thin-section immunoelectron microscopy and the development of domain-specific antibodies. However, the predominant pericanalicular localization of the Wilson protein observed in vivo suggests that once the cytoplasmic concentration of the copper is decreased by copper sequestration into vesicles the protein is recycled back to the trans-Golgi network as the vesicles traffic to the canalicular membrane for copper excretion. This model is consistent with the failure to observe either the Wilson's or ApoCeruloplasmin in these conditions.

The data in this study are also relevant to earlier observations on copper metabolism in the rat liver under high hepatic copper concentrations (Fig. 6C).

**DISCUSSION**

Biliary copper excretion is the primary mechanism for maintaining copper balance in humans. Patients with WD have a marked impairment in biliary copper excretion, suggesting that the protein encoded by the WD gene is a key factor in this process. The data in this paper are consistent with this concept, demonstrating that the hepatocyte is the site of expression of this protein in vivo. The localization of the WD protein to the trans-Golgi network suggests that the function of this protein is to transport copper into the secretory pathway of hepatocytes, consistent with observations in the hepatoblastoma-derived Hep G2 cell line (11, 42) as well as studies on the homologous Menkes copper transporter in human and rodent cell lines (8, 23, 41). Individuals with WD are also unable to synthesize holoceruloplasmin (6, 7, 28). Because copper is incorporated into this protein in the secretory pathway (20, 26), the hepatocyte-specific trans-Golgi localization of the WD protein shown here provides an explanation for this biochemical phenotype observed in affected patients.

The data in this study are also relevant to earlier observations on copper metabolism in the rat liver during copper deficiency. On reinstating dietary copper...
Menkes disease protein on the plasma membrane in cell lines under endogenous circumstances (11, 41). Furthermore, the model is also consistent with previous studies on the biochemistry of copper metabolism in the LEC rat (30) and the detection of an intact copper excretory mechanism in hepatocellular canalicul membranes from these same animals (1). Taken together, these findings suggest a posttranslational mechanism for hepatocyte copper transport and excretion that is regulated by the copper concentration during recycling of the WD protein between the trans-Golgi network and a post-Golgi vesicular compartment (Fig. 9). Such a process is analogous to the signal-mediated recycling of the mannose 6-phosphate receptor in the targeted delivery of lysosomal enzymes (13). This implies that the final step of copper excretion may involve previously defined processes of vesicle delivery and insertion into the bile canalicul plasma membrane (3). Further characterization of the processes responsible for docking, trafficking, and protein retrieval of the vesicles containing the WD protein as well as the mechanism involved in the final steps of copper excretion into bile will therefore be crucial for the understanding of the regulation of copper homeostasis. Elucidation of such pathways may also be relevant to our understanding of a wide range of biliary excreted substances.

WD is a clinically heterogeneous disorder, and the molecular genetic analysis of patients has revealed a series of distinct mutations (36). The data in this paper suggest a mechanism for this clinical heterogeneity whereby specific mutations may affect individual processes of copper sensing, copper-mediated trafficking, and copper transport, resulting in phenotypic variations analogous to what has been observed with different cystic fibrosis transmembrane conductance regulator mutations in patients with cystic fibrosis (37). Furthermore, patients with WD have marked clinical variation even within affected families carrying the same mutation (24). This may also be related to the mechanisms outlined in this paper, whereby genetic variations in cofactors necessary for the sensing or trafficking functions of the WD protein or environmental factors affecting hepatocyte copper concentrations may result in individual differences in the function of the WD protein. Recent studies identifying the chaperone essential for delivery of copper to the WD protein (12, 14) and elucidating the role of the GEF1-encoded chloride channel in the function of the homologous yeast ATPase suggest potential factors that might be examined for contribution to such heterogeneity (9).

In contrast to the observations in the adult, in the fetal liver the WD protein is predominantly localized to the trans-Golgi network despite a marked elevation of hepatic copper. This difference in localization correlates directly with the lack of biliary copper excretion in the fetal period. The failure of copper to induce movement of the WD protein at this stage of development may be due to the lack of biliary flow; to factors involved in embryonic copper metabolism, such as the known increase in metallothionein concentration (25); or to more general features of hepatocyte development. Consistent with the findings obtained for adult animals (Fig. 6A), there was no direct correlation between the hepatic copper concentration and the relative abundance of the WD protein during the perinatal period. Presumably, the concentration of the WD protein is high during the embryonic period in anticipation of the postnatal excretion of this markedly increased copper load (Fig. 7, C and D). The mechanisms regulating this developmental increase in WD protein expression are unknown but may merit further investigation given recent studies that demonstrate that the WD protein can functionally substitute for the Menkes protein (22). Understanding the factors regulating WD protein expression might therefore permit a novel genetic approach to the treatment of Menkes disease by induction of expression of the WD protein in affected cells of Menkes disease patients during a critical period of development.

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