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Copper Transporting P-Type ATPases and Human Disease

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Copper transporting P-type ATPases, designated ATP7A and ATP7B, play an essential role in mammalian copper balance. Impaired intestinal transport of copper, resulting from mutations in the *ATP7A* gene, lead to Menkes disease in humans. Defects in a similar gene, the copper transporting ATPase *ATP7B*, result in Wilson disease. This ATP7B transporter has two functions: transport of copper into the plasma protein ceruloplasmin, and elimination of copper through the bile. Variants of ATP7B can be functionally assayed to identify defects in each of these functions. Tissue expression studies of the copper ATPases and their copper chaperone ATOX1 indicate that there is not complete overlap in expression. Other chaperones may be important for the transport of copper into ATP7A and ATP7B.

KEY WORDS: Copper transport; Menkes disease; Wilson disease; copper trafficking; ATOX1; tissue distribution.

INTRODUCTION

Copper is one of the many metal ions which are required for essential functions, yet are toxic in excess. Approximately half of the daily copper intake of 1–2 mg of copper is absorbed (reviewed in Linder *et al*., 1998. This copper is essential in a number of proteins, including cytochrome oxidase (required for respiration), superoxide dismutase (protects against cellular free radical damage), lysyl oxidase (required for collagen and elastase cross-linking), and dopa beta amino oxygenase (converts dopamine to norepinephrin for neurotransmission). In excess, copper generates free radicals and acts as a potent cellular toxin via the Haber–Weiss reaction (Bremner, 1998). The result of such hydroxyl radical production includes lipid pro-oxidation, mitochondrial damage leading to reduced cytochrome oxidase activity, DNA strand breakage, and protein damage. Mechanisms for homeostasis of copper are therefore critical to survival of the organism, and are highly conserved throughout living species. Two ATPases, ATP7A and ATP7B, are typical P-type ATPases that play a major role in maintaining copper balance. Both share a high homology with various P-type ATPases including those of bacteria (Solioz *et al*., 1994) and yeast cerevisiae (Fu *et al*., 1995).

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COPPER TRANSPORT DISORDERS

The discovery of the important role of these two copper transporting ATPases has had a major impact on our knowledge of metal transport. The transport of copper, as for other metal transport systems yet to be elucidated, has demonstrated the high degree of conservation of metal transport systems from bacteria through to humans. Most of the details of other trace metal transport systems are still to be revealed. Secondly, the discovery of these copper transporters has made possible the understanding and the improved diagnosis for two disorders of copper transport, Menkes disease and Wilson disease.

Menkes Disease

Patients with Menkes disease, an X-linked disorder therefore affecting mainly males, are unable to transport dietary copper from the intestine, resulting in a generalized copper deficiency in most tissues. Dietary copper uptake primarily occurs in the duodenum. The copper is then transported across the intestinal cell membrane, where small amounts enter the body. In Menkes disease,

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mutations occur in the *ATP7A* gene, that is highly expressed in the duodenum and is responsible for transport of copper from that tissue. While small amounts of copper are transported in the body, defective functioning of ATP7A, leads to copper accumulation in selected tissues including kidney and placenta. Patients with Menkes disease have neurodegeneration, developmental delay, connective tissue abnormalities, seizure, kinky twists of the hair, hypothermia, and usually death within the first few years of life.

The biochemical features of Menkes disease include reduction of liver copper and low plasma ceruloplasmin (Tumer and Horn, 1998). A diagnostic feature of Menkes disease is that cultured fibroblasts from patients accumulate high levels of copper due to defective copper efflux from their cells in comparison with those of normal individuals (Horn, 1981). Treatment with copper histidine helps alleviate the clinical manifestations of the disease, except for the connective tissue abnormalities, that begin in utero (Sarkar *et al*., 1993).

The gene for Menkes disease (designated *ATP7A*) was the first human membrane copper transporter identified, by positional cloning using DNA from a female patient with a translocation breakpoint at the Menkes locus (Chelly *et al*., 1993; Mercer *et al*., 1993; Vulpe *et al*., 1993). At that time, sequencing of the cDNA revealed a predicted protein similar to a P-type ATPase previously found to transport copper in bacteria (Odermatt *et al*., 1993). Numerous ATP7A mutations have been identified in Menkes disease patients (Tumer *et al*., 1999). Deletions, some many Kb in length, have been identified in 10–20% of patients with classical Menkes disease (Chelly *et al*., 1993; Mercer *et al*., 1993). Splice site mutations, and a few missense mutations, are typically found in patients with occipital horn syndrome, which is a milder form of the same disease (Kaler *et al*., 1994).

The mottled mouse serves as an excellent model for Menkes disease, with alterations in the orthologous gene, *Atp7a* (Levinson *et al*., 1994; Mercer *et al*., 1994). There are at least 20 mutations identified in *Atp7a* that lead to the mottled (Mo) phenotype, with a wide range of disease severity, from prenatal lethality to only connective tissue abnormalities (Levinson *et al*., 1994; Masson *et al*., 1997; Reed and Boyd, 1997).

The Copper Transporter ATP7B, Defective in Wilson Disease

The second copper transporting P-type ATPase gene to be cloned was that for *ATP7B* (Bull *et al*., 1993; Tanzi *et al*., 1993), located on human chromosome 13. Wilson disease (hepatolenticular degeneration) is an autosomal recessive disorder of hepatic transport copper transport and storage, first described in 1912. Wilson disease affects approximately 1 in 30,000 individuals, with a frequency three times as high in certain populations. Previous to identification of the basic defect, the typical biochemical and clinical changes of the disease could not be entirely explained. Two of the major problems in this disorder are reduced excretion of copper into the bile, resulting in a toxic accumulation of copper in the liver, and an increase of copper in the urine. The second defect is reduced incorporation of copper into the serum copper protein ceruloplasmin.

The gene defective in Wilson disease, *ATP7B*, is highly expressed in the liver, where *ATP7A* is not expressed. Because of the defect in transport of copper from the liver due to mutations in the gene, copper accumulates in the liver, first inducing the production of metallothionein, which can apparently maintain copper in a relatively harmless state. This accumulation of copper causes damage to mitochondria initially, and eventually leads to destruction of the hepatocyte. Copper is also deposited in renal tubules, and kidney damage occurs to varying degrees. A combination of copper accumulation in the blood as well as impaired expression of *ATP7B* in the brain, leads to neurological disease, by mechanisms not entirely explained. The clinical presentation of Wilson disease is highly variable, with an age of onset from less than 5 years to greater than 50 years, and clinical manifestations presenting as neurologic or hepatic disease (Cox and Roberts, 1998; Danks, 1995).

The Long Evans Cinnamon (LEC) rat has biochemical abnormalities and clinical features similar to those seen in Wilson disease, including autosomal recessive inheritance, and liver damage due to a secretion defect in the hepatocyte. We cloned *Atp7b*, the orthologue of the human copper transporting gene, and have shown that 25% of the gene at the 3′ end is deleted in the mutant LEC rat (Wu *et al*., 1994). The LEC rat is being used effectively to study the functional characteristics of Atp7b. Two types of toxic milk mouse (tx, tx_J) also phenotypically similar to Wilson disease, have two different mutations in the mouse orthologue, *Atp7b* (Coronado *et al*., 2001; Theophilos *et al*., 1996).

Yeast as a Model for the Copper Transport Function

ATP7B is likely to play a dual functional role in the cell. One role is biosynthetic, delivering copper to ceruloplasmin within the Golgi apparatus (Murata *et al*., 1995). The other role is to transport excess copper out of the hepatocyte. The transport function is reflected in a yeast assay. The components of the mammalian transport system are

the same as those in the yeast *Saccharomyces cerevisiae*. Yeast has a copper transporting P-type ATPase (Ccc2p) similar to ATP7B, that transports copper within the cell and is critical for iron transport. Human *ATP7B* can replace the yeast gene and provide full transport function. The plasma membrane protein Ctr1p transports copper with high-affinity into the cytoplasm (Dancis *et al*., 1994), to the soluble copper chaperone Atx1p, (Lin *et al*., 1997) that delivers copper to Ccc2p. Copper is supplied by the protein, Ccc2p, to the plasma membrane oxidase Fet3p, similar to ceruloplasmin in humans, that functions, with the high-affinity iron permease Ftr1p, to import iron into the yeast cell (Stearman *et al*., 1996; Yuan *et al*., 1995). When yeast cells lack Ccc2p, copper is not incorporated into Fet3p, and subsequently the cells lack high-affinity iron uptake and will not grow (Yuan *et al*., 1995). ccc2 mutant yeast are unable to grow on iron deficient medium lacking added iron or copper. This connection with iron is also observed in humans. The rare recessive condition aceruloplasminemia leads to cellular iron storage (Harris *et al*., 1995; Yoshida *et al*., 1995).

Copper Induced Intracellular Trafficking of ATP7B

The ATP7B protein is localized to the trans Golgi network in HepG2 hepatoblastoma cells (Hung *et al*., 1997). When copper is added to growth medium, ATP7B redistributes from the trans Golgi network (TGN) to cytoplasmic vesicles and returns to the trans Golgi location when copper is removed (Hung *et al*., 1997). This redistribution event is specific for copper: zinc, cadmium, iron, and cobalt have no effect. In polarized HepG2 cells, ATP7B is localized in the trans Golgi network only at a low copper concentration. Redistribution to vesicular structures, and then to apical vacuoles occurs at increased copper levels (Roelosfen *et al*., 2000). This redistribution has also been demonstrated in copper loaded rats, indicating physiological relevance of this process (Schaefer *et al*., 1999a). The same copper dependent trafficking event has been shown for the ATP7A copper transporter, except that the movement upon copper stimulation is to the plasma membrane (Petris *et al*., 1996). Immuno histochemistry on human liver has revealed intracellular punctate staining of hepatocytes, as well as canalicular membrane staining, adjacent to bile canaliculae (Schaefer *et al*., 1999b). The observed copper induced trafficking may represent a posttranslationally inducible switch from a primarily biosynthetic role in the TGN to a primarily excretory role under conditions of copper loading.

The signals which facilitate this intracellular trafficking of the copper transporters are currently under investigation. A C-terminal dileucine motif is generally involved in recycling proteins from the plasma membrane to late endosomes (Calvo *et al*., 1999). Molecular studies on ATP7A indicate that a C-terminal dileucine motif is required for relocalization of the protein back to the trans Golgi network (Francis *et al*., 1998; Petris *et al*., 1998). Mutations in this motif resulted in *ATP7A* proteins which were localized entirely to the plasma membrane and could not recycle back to the trans Golgi network in response to copper depletion. The mutant proteins could still mediate copper efflux, suggesting that the dileucine motif is not involved in transport function, and that the plasma membrane is a site of ATP7A dependent copper efflux from the cell. A similar leucine-containing motif is present in ATP7B, that may provide a similar function. A Golgi localization signal has also been reported in the third transmembrane segment of ATP7A (Francis *et al*., 1998). This may be due to a specific sequence targeting signal, but could be the result of a conformational change. Effective intracellular transport is a prerequisite for normal hepatic excretion of copper.

An interesting new protein in the copper transport pathway has been identified through studies in dogs. A naturally occuring mutation is found in terriers, particularly Bedlington terriers, that results in canine copper toxicosis. This condition shows a recessive mode of inheritance, failure of biliary excretion of copper, liver copper accumulation, and resultant copper induced liver disease (Twedt *et al*., 1979). Although very similar to Wilson disease, *ATP7B* is not involved. Mapping studies in the dog localized the gene to a region of dog chromosome 10, similar to a gene region on human chromosome 2 (van de Sluis *et al*., 2000), and different from that of *ATP7B* (van de Sluis *et al*., 1999). We (Coronado and Cox, unpublished) and others (van de Sluis *et al*., 2002) identified a deletion of a complete exon in a small gene, *MURR1*, for which the function is not yet known. Since the ceruloplasmin concentration is normal in affected dogs, the basic defect is more likely to involve the excretion function only. The corresponding human disease has not yet been reported.

Study of Missense Mutations in ATP7B

A study of the mutations in ATP7B in Wilson disease allows us to learn more about the functional significance of amino acids within the various domains of the ATPase. In addition, there is an important practical application, both in understanding the clinical variability and in providing a definitive diagnosis for Wilson disease. More than 230 mutations have now been described and are listed in a mutation database: (www.medgen.med.ualberta.ca/database.html).

The spectrum of known mutations is different from that of *ATP7A*, particularly in the lack of large deletions.

The large deletions, which occur in 15–20% of ATP in patients with Menkes disease have not been reported in Wilson disease. Of the *ATP7B* mutations, 31% are small deletions and insertions of one or a few base pairs, 8% are nonsense mutations, and 7% are mutations affecting splice sites. The majority of know mutations in *ATP7B*, 54%, are single base pair missense mutations. These single base pair substitutions occur throughout the gene, but are particularly prevalent in transmembrane motifs two to six (of the total of eight), and in the extra cellular loop on which the ATP binding motif occurs. Not all of the base pair substitutions lead to disease-causing mutations: some are rare normal variants. While a study of normal chromosomes helps to differentiate between these options, a method for functional assessment of these mutations is useful.

The two functions of ATP7B, copper incorporation into ceruloplasmin, and excretion of copper through the bile, can be assayed in separate systems. A yeast assay detects the ability of mutant ATP7B proteins to transport copper, reflecting the ability to incorporate copper into ceruloplasmin (Forbes and Cox, 1998). Using this assay, in combination with direct observance of copper trafficking in CHO cells, several types of mutant ATP7B proteins have been identified (Forbes and Cox, 2000). Some variants are unable to transport copper in the yeast system and appear to aggregate in the endoplasmic reticulum. One particularly interesting mutant, glycine 943 serine, in the fifth transmembrane motif showed an almost normal transport activity in yeast, but was completely unable to traffick from the trans Golgi network in response to copper (Forbes and Cox, 2000). This type of defect has been reported for the most common mutation in WD patients, histidine1069glutamine (Payne *et al*., 1998). This type of analysis of other mutants in missense mutations occuring in patients with Menkes disease or Wilson disease should help shed light on the specific amino acid residues required for proper transport and trafficking functions.

Tissue Expression of the Copper ATPases

The expression patterns of the ATP7A and ATP7B transporters have been shown by northern blot analysis, in situ hybridization, and immunohistochemistry. For ATP7B, northern blot analysis revealed that expression is highest in adult liver, kidney, and slight in brain, placenta, heart, and lungs (Bull *et al*., 1993; Muramatsu *et al*., 1998; Tanzi *et al*., 1993). On the other hand, *ATP7A* expression is high in all tissues except the liver.

In the adult liver, ATP7B acts to export copper via the bile, balancing the copper levels in the body. Bile is excreted from hepatocytes and collects in the bile canaliculus. However, tissue localization in rat liver indicates that ATP7B shows an intracellular punctate pattern oriented toward the canalicular pole (Schaefer *et al*., 1999a). In an additional study involving human liver, ATP7B was localized to two regions (Schaefer *et al*., 1999b). In the first region, immunostaining is in an intracellular punctate pattern within specific hepatocytes. The second region is the pericanalicular area, only found in the presence of elevated copper, associated with copper induced trafficking.

Normally, very little copper is excreted from the kidney. However, Wilson disease patients have renal copper accumulation and elevated urinary copper levels, and approximately 45% of patients have renal tubular dysfunction. In contrast, Menkes disease patients do not have elevated urinary copper levels, nor do they have renal dysfunction, but they do have renal copper accumulation (Kodama *et al*., 1992). Northern blot analysis, indicates that both *ATP7A* and *ATP7B* are expressed in the kidney (Bull *et al*., 1993; Vulpe *et al*., 1993). Detailed localization studies of and ATP7A and ATP7B in mouse kidney, idenitified structures important for copper transport. ATP7A has been localized to the proximal and distal renal tubules (Grimes *et al*., 1997; Murata *et al*., 1997), and recently to the glomeruli (Moore *et al*., 2002). ATP7B has been shown to be localized to the glomeruli, and the metulla most likely in the loops of Henle (Moore and Cox, 2002). The loops of Henle have previously been suggested to be involved in the reabsorption of copper (Wareing *et al*., 2000). These expression data support the loops of Henle as important in renal copper homeostasis, and implicate the glomeruli in copper regulation.

Interestingly, ATP7B is not expressed in regions that accumulate copper in Wilson disease patients. In the LEC rat, an animal model for Wilson disease, the low molecular weight copper binding proteins, metallothioneins (MT), are proposed to be involved in copper accumulation in the proximal tubules (Nomiyama *et al*., 1999). However, this could be secondary to, and not causative for, copper accumulation. In both Menkes and Wilson disease patients, copper metallothioneins accumulate in the intestine and liver respectively (Shiraishi *et al*., 1991). Studies with cadmium demonstrate that orally ingested cadmium induces intestinal metallothionein and is at least partially responsible for downstream renal accumulation in the proximal tubules (Elsenhans *et al*., 1992). Although *ATP7B* is not expressed in the proximal tubules of the kidney, the site of copper accumulation in Wilson disease patients, a similar mechanism may be involved in renal copper accumulation.

Interaction of ATOX1 With Copper ATPases

Yeast two-hybrid interaction studies have shown that the yeast copper chaperone Atx1p interacts with the

copper transporter Ccc2p, the orthologue of the P-type ATPases ATP7A and ATP7B (Pufahl *et al*., 1997). The human orthologue of Atx1p, ATOX1, has also been shown to interact with ATP7A and ATP7B, by yeast two-hybrid studies, mammalian two-hybrid studies (Larin *et al*., 1999) and coimmunoprecipitation (Hamza *et al*., 1999). In addition, crystal structures suggest a molecular mechanism for protein recognition and metal ion exchange between the chaperone and the copper transporters (reviewed in Rosenzweig, 2001). ATOX1 has also been shown to transfer copper to the N-terminal domain of ATP7B that ultimately regulates its catalytic activity (Walker *et al*., 2002). These mammalian studies suggest that ATOX1 acts as a copper chaperone for the copper transporters ATP7A and ATP7B. Therefore expression patterns for these ATPases and the chaperone would be expected overlap.

In the liver, ATOX1 expression is limited to hepatocytes that surround central and hepatic veins (Moore *et al*., in press). These hepatocytes surround the veins that carry blood that has already passed through the liver and is being redistributed into the body. Therefore hepatic expression of *ATOX1* apparently does not overlap with that of ATP7B. The possibility remains that ATOX1 is not the sole chaperone for the transport of copper to ATP7A and ATP7B.

The kidney is particularly interesting because ATP7A, ATP7B, and ATOX1 are all expressed there. As noted above, *ATP7A* is expressed in the proximal and distal convoluted tubules (Grimes *et al*., 1997; Murata *et al*., 1997) as well as the glomeruli (Moore and Cox, in press) of the mouse kidney. ATP7B renal expression has been shown to be limited to the glomeruli and the loops of Henle (Moore and Cox, in press). Renal ATOX1 expression was localized to the glomeruli and the renal medulla (containing the loops of Henle), the same expression pattern as that of ATP7B, but not of ATP7A. These differences in localization raise the possibility that there may be another chaperone that functions in association with ATP7A in the distal and proximal convoluted tubules. An alternative chaperone for ATP7A may also provide a possible explanation for expression results by northern blot analysis, in that skeletal muscle expresses *ATP7A*, but not *ATOX1* (Moore and Cox, unpublished data).

ATOX1 Copper Chaperone and Disease

The usual cause of Wilson disease is the toxic accumulation of copper in the liver and brain, due to a mutation in *ATP7B*. However, if *ATOX1* is an essential protein in the pathway to excrete copper via ATP7B from the liver, mutations in ATOX1 may lead to an accumulation of hepatic copper as seen in Wilson disease patients. Alternatively, the phenotype resulting from a complete disruption of both functional alleles of ATOX1 could result in a copper deficiency, as seen in Menkes disease patients and the *Atox*$1^{-/-}$ knockout mouse (Hamza *et al*., 2001). The production of an Atx1 knockout mouse has shown that ATOX1 is essential for normal embryonic development. However, a less severe defect might be predicted to allow normal development, but result in impaired transport of copper into ATP7B producing a Menkes like phenotype, or into ATP7B producing a Wilson disease like phenotype. Although selected patients with Wilson-like and Menkes-like phenotypes have been screened for mutations in *ATOX1*, none have been found to date (Moore *et al*., in press).

CONCLUSION

Studies in yeast, rodents, dogs, and humans have all contributed to our understanding of the proteins involved in copper transport. However, we do not yet have a full understanding of this pathway. Discovery of the basic defect in human disorders can lead to our understanding of copper pathways. For several of the genes in this pathway, no human or naturally-occurring mouse mutations have yet been identified.

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