Restoration of Holoceruloplasmin Synthesis in LEC Rat after Infusion of Recombinant Adenovirus Bearing WND cDNA*

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Wilson’s disease, an autosomal recessive disorder, is characterized by the excessive accumulation of copper in the liver. WND (ATP7B) gene, which encodes a putative copper transporting P-type ATPase, is defective in the patients. To investigate the in vivo function of WND protein as well as its intracellular localization, WND cDNA was introduced to the Long-Evans Cinnamon rat, known as a rodent model for Wilson’s disease, by recombinant adenosine-mediated gene delivery. An immunofluorescent study and a subcellular fractionation study revealed the transgene expression in liver and its localization to the Golgi apparatus. Moreover, since the synthesis of holoceruloplasmin is disturbed in the Long-Evans Cinnamon rat, the plasma level of holoceruloplasmin, oxidase-active and copper-bound form, was examined to evaluate the function of WND protein with respect to the copper transport. Consequently, the appearance of holoceruloplasmin in plasma was confirmed by Western blot analysis and plasma measurements for the oxidase activity and the copper content. These findings indicate that introduced WND protein may function in the copper transport coupled with the synthesis of ceruloplasmin and that the Golgi apparatus is the likely site for WND protein to manifest its function.

Wilson’s disease, an autosomal recessive disorder, is characterized by the excessive accumulation of copper in the liver. This phenomenon is thought to be due to reduced biliary excretion of copper and disturbed incorporation of copper into ceruloplasmin (CPN). Hepatic copper at toxic levels causes liver cirrhosis, and extrahepatic copper toxicity occurs especially in the brain due to the released copper from the damaged liver. WND (officially designated ATP7B), identified as the gene responsible for this disease, encodes a putative copper transporting P-type ATPase (2–5). The observations of single base changes or small deletions within WND of Wilson’s disease patients have already been reported (3, 6).

The Long-Evans Cinnamon (LEC) rat, known as an animal model for Wilson’s disease, shows some of the clinical features similar to Wilson’s disease, including hepatic copper accumulation, reduced biliary copper excretion, reduced copper in plasma, and a remarkable decrease of serum CPN activity (7, 8). Atp7b, the rat gene homologous to WND, has been cloned, and a partial deletion at the 3’ end in this gene is reported in the LEC rat (9). It is also known that the expression of Atp7b mRNA is absent in the LEC rat (10).

CPN, a blue copper oxidase in plasma, contains 90–95% of plasma copper. This protein is synthesized mainly in hepatocytes and secreted into plasma with 6 atoms of copper per molecule as the oxidase active holoprotein (11–13). The reduced levels of oxidase activity of CPN in the circulation of Wilson’s disease patients and LEC rats is due to the secretion of apoceruloplasmin, copper-free and oxidase-inactive form, resulting from the disturbed incorporation of copper atoms into the protein (1, 14), while the intracellular synthesis of CPN peptide is normal in the LEC rat (15). Since the incorporation of copper into CPN occurs through the secretory compartments in hepatocytes, the precise site for the incorporation is still controversial. Sato and Gitlin (16) suggested that the rough endoplasmic reticulum (rER) was the likely site for the incorporation, using the human hepatoma cell line HepG2. However, our previous report demonstrated that CPN-bound copper was present in the Golgi apparatus and not the rER isolated from rat liver and that this process was disturbed in the LEC rat (15).

P-type ATPases, defined as those forming a covalent phosphorylated intermediate in their reaction cycle, transport a variety of cations across membranes (17). The general features of those include the TGEA/S motif (phosphatase domain), the DKTGT/S motif (phosphorylation domain), the TGDN motif (ATP-binding domain), and the sequence MxMxX motif (2–5). The ATPases contain several unique features including 6–8 transmembrane segments, 1–6 metal binding motifs, GMTC motif at the N terminus of the molecule, and the CPX motif in an intramembranous region. ATP7B contains 6 metal binding motifs at the N terminus (5), whereas the rat homologue contains only 5 (9). Recent studies have reported that MNK protein, which is also classified as a heavy metal transporting P-type ATPase, and is defective in Menkes disease, localizes to the Golgi apparatus, and may function in transport of intracellular metal ions.
copper (20, 21). Recombinant adenoviruses have been used to deliver genes to several animal models of inherited disorders (22-26) as well as to human individuals (27, 28). Recently, an efficient method for constructing recombinant adenoviral vectors has been established (29). The improved version of adenoviral vector bears the foreign gene under the transcriptional control of a CAG promoter (30) exhibited the efficient expression of the introduced gene (31).

In this study, we introduced WND cDNA to LEC rats using this adenoviral vector construct to investigate the in vivo function of the gene with respect to copper transport in the liver. Consequently, we observed transgene expression in the liver and the secretion of holo-CPN in the plasma of the treated LEC rats.

EXPERIMENTAL PROCEDURES

Construction of Full-length WND cDNA—Two partial cDNA clones, pCUN-C1 and pWD02, covering WND cDNA (GenBank accession number U17700) from nucleotide number 223 to 2031 and from 1489 to 5581, respectively, were kindly provided by Dr. T. C. Gilliam, Columbia University School of Medicine. Animals had free access to water and standard rat chow. Recombinant adenoviruses, 1010 plaque forming unit (pfu) in 0.5 ml of saline, were administered to 4- or 5-week-old LEC rats by tail vein injection. All experiments were performed in accordance with the guidelines of Akita University School of Medicine. Animals had free access to water and standard rat chow. Recombinant adenoviruses, 1 x 1010 plaque forming unit (pfu) in 0.5 ml of saline, were administered to 4- or 5-week-old LEC rats by tail vein injection. All experiments were performed in accordance with the guidelines of Akita University School of Medicine. Animals had free access to water and standard rat chow. Recombinant adenoviruses, 1 x 1010 plaque forming unit (pfu) in 0.5 ml of saline, were administered to 4- or 5-week-old LEC rats by tail vein injection. All experiments were performed in accordance with the guidelines of Akita University School of Medicine. Animals had free access to water and standard rat chow. Recombinant adenoviruses, 1 x 1010 plaque forming unit (pfu) in 0.5 ml of saline, were administered to 4- or 5-week-old LEC rats by tail vein injection. All experiments were performed in accordance with the guidelines of Akita University School of Medicine. Animals had free access to water and standard rat chow. Recombinant adenoviruses, 1 x 1010 plaque forming unit (pfu) in 0.5 ml of saline, were administered to 4- or 5-week-old LEC rats by tail vein injection. All experiments were performed in accordance with the guidelines of Akita University School of Medicine.
maximal. Consistent with the above results, a band corresponding to WND was detected in the sample infused with AxCAWD but not with AxCAwt (Fig. 1B, lanes 3 and 4). The lower weight bands below WND protein (Fig. 1B, lane 4) seem to be degradation products since the protein sample was prepared from frozen liver tissue. Protein samples obtained from freshly isolated liver show no degradation products (describe below, Fig. 2). The Coomassie staining shows equivalent amounts of protein samples loaded (Fig. 1B, lanes 1 and 2).

Localization of WND Protein in Liver Cells—The above immunofluorescent study suggests that the WND protein is present in the cytoplasm of liver cells (Fig. 1A, e); however, its precise subcellular localization could not be determined. To this end, Golgi, rER, lysosomal, and cytosolic fractions of liver were freshly prepared from LEC rat 3 days after infusion of AxCAWD for use in immunoblot analyses. To assess the enrichment of each fraction, immunoblotting was performed using antibodies to marker proteins with known subcellular locations as described in "Experimental Procedures." Mannosidase II, PDI, and cathepsin D are detected mainly in the Golgi, the rER, and the lysosomal enriched fractions, respectively, suggesting the efficacy of subcellular fractionation study (Fig. 2, B-D). As shown in Fig. 2A, WND is detected mainly in the Golgi enriched fraction (lane 4), and not in the rER, lysosomal, nor...
Localization and Function of WND Protein

In this study, we demonstrate the appearance of holo-CPN, copper-bound and oxidase-active form, in plasma of the LEC rats after the introduction of human WND cDNA using the recombinant adenovirus. Additionally, we provide evidence that introduced WND protein localizes to the Golgi apparatus.

The gene transfer mediated by recombinant adenoviruses has been applied to a variety of metabolic diseases in liver (22–28, 39). To introduce human WND cDNA into the LEC rat liver, we constructed the recombinant adenovirus by using the COS-TPC method (29) by which the desired recombinant adenovirus is efficiently obtained. Additionally, the high-level expression of the transgene can be achieved by using the CAG promoter. The major problem in the use of adenoviral vectors, so far, are host immune responses that result in transient expression of the transgene and the inability to readminister the same virus. Previous reports suggested that transient expression of the transgene was due to destructive immune responses directed against the non-self transgene product are the major determinants of the stability of the transgene expression (42). To minimize these problems, we used the recombinant adenovirus lacking regions of E1A, E1B, and E3 within the viral genome and employed the CAG promoter to achieve the efficient expression of the transgene to reduce the number of viral particles to be infused. Despite these attempts, the expression of WND protein lasted only 10–14 days in the present study. We postulate that this transient expression is due to the induction of host immune responses against transduced cells expressing the exogenic transgene product efficiently, as previously suggested (24, 42, 43).

The export pathways for the hepatic copper consist of the secretion into plasma and excretion into bile. In the LEC rats, as well as in patients with Wilson’s disease, both pathways are thought to be impaired, leading to the accumulation of copper in the liver (1, 7, 8, 14, 15, 37, 44). Normally, CPN is secreted as a copper-bound form; however, evidence from studies of the LEC rats suggest that the process of copper incorporation into CPN is disturbed in the secretory compartments of affected livers (14–16). On the other hand, the reduced biliary excretion of copper is considered to be due to a defect in the entry of copper into lysosomes (37). These phenomena may be attributed to a defect in translocation of copper across the membrane of subcellular compartments in the secretory and excretory pathways. The WND protein is likely to play an important role in these processes, since the protein is believed to be a member of copper-transporting P-type ATPases from its deduced amino acid sequence and thus capable of transport copper into subcellular compartments. A recent report has indicated that MNK protein, which also encodes a putative copper-transporting P-type ATPase and shows 55%
identity in amino acids to WND protein, localizes to the trans-Golgi network and may function in delivering copper to cuproproteins through the secretory pathway (20, 21). Similarly, CCC2 protein, the copper transporting P-type ATPase in yeast, was found to deliver cytosolic copper into an extracytosolic compartment and give copper to a CPN-like oxidase, FET3 protein (45).

In this study, we introduced WND cDNA into LEC rat liver using the recombinant adenovirus, AxCAWD, to examine the function of WND protein with respect to the synthesis of holo-CPN as well as the intracellular localization of the protein. The results obtained by PAGE under non-denaturing conditions and the plasma measurement for the oxidase activity and the copper concentration reveal that the LEC rats infused with AxCAWD secrete holo-CPN into plasma. This indicates that the introduced WND protein functions in the copper transport and the incorporation of copper into CPN. Moreover, this last process was found to occur in the Golgi apparatus, suggesting that this is the likely site for the WND protein to manifest its function. Subcellular fractionation studies performed in this report and the recent reports of others (35, 46) revealing the localization of WND protein to the Golgi apparatus support this notion. However, levels of circulating holo-CPN in the treated rats could not be restored to the normal level in the present study. This may be due to the insufficient transduction resulting from a reduced number of infused viral particles as described above. It is also possible that the product of transgene derived from human is not able to manifest its complete function in the rodent model due to the differences in amino acid sequence (24). While the homology between human WND and rat Atp7b are 82% in amino acids and each functional domain is well conserved, there is the difference in the number of metal binding motifs, which is six in the human protein and five in the rat protein (5, 9). These data suggest that the WND protein participates in the copper transport coupled with CPN synthesis; however, the association of WND protein with the biliary excretion pathway of copper still remains to be determined.

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