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FAILURE OF COPPER INCORPORATION INTO CERULOPLASMIN IN THE GOLGI APPARATUS OF LEC RAT HEPATOCYTES

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SUMMARY: Copper incorporation into ceruloplasmin during ceruloplasmin synthesis was studied by comparing LEC and control rats. Major 132 and 136 kDa ceruloplasmins were found in microsomes and the Golgi apparatus, respectively, isolated from liver homogenates of LEC and control rats. Copper analysis showed that no copper was detected in the ceruloplasmin in the microsomes of either rat. Copper was present in ceruloplasmin in the Golgi apparatus and serum of controls, while it was not detected in ceruloplasmin in the Golgi apparatus and serum of the LEC rat. These results indicate that copper is incorporated into ceruloplasmin in the Golgi apparatus of normal hepatocytes. LEC rats fail to incorporate copper into ceruloplasmin in the Golgi apparatus.

Wilson disease is a genetic disorder characterized by hepatic copper accumulation and low ceruloplasmin and copper levels in the serum. Generally, copper is incorporated into ceruloplasmin during its synthesis in normal hepatocytes, and then secreted into the serum as holoceruloplasmin (copper-bound form)(1). In patients with Wilson disease, however, ceruloplasmin is secreted into

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the serum as apoceruloplasmin (copper-unbound form)(2). These facts suggest that copper incorporation into ceruloplasmin is reduced in the liver of Wilson disease. Recently, the pathogenesis of Wilson disease has been suggested to be a deficiency of copper-transporting ATPase (3-5). As this enzyme is known to be a membrane protein, the reduction of copper incorporation in the liver of Wilson disease seems to be caused by a defect of copper transport through the membrane of the organelle in which copper is incorporated into ceruloplasmin. Sato et al. (1) reported that copper is incorporated into newly synthesized ceruloplasmin in the early stage of biosynthesis in normal hepatocytes. However, the stage of copper incorporation has not been elucidated.

The Long Evans Cinnamon (LEC) rat, which has been recently identified as a bonafide animal model of Wilson disease (6), has biochemical abnormalities that are similer to those of patients with Wilson disease (7, 8). In this study, we showed that hepatic copper is combined with ceruloplasmin in the Golgi apparatus in normal rat hepatocytes. We also showed that copper incorporation is impaired in the Golgi apparatus of LEC rat hepatocytes.

MATERIALS AND METHODS

Fractionation of Rat Liver Homogenates: Eight-week-old female LEC rats were used. Before use, the liver function of these rats was confirmed to be normal by liver function tests. Age- and sex-matched Long-Evans Agouti rats were used as controls. Microsomal and Golgi (GF_{1+2}) fractions were isolated from rat liver homogenates prepared from 16 specimens by the method of Howell et al.(9). The purity of each fraction was analyzed by the marker enzymes of cytosol, lysosome and mitochondria such as glucose-6-phosphate dehydrogenase, acid phosphatase, cytochrome c oxidase and ornithine transcarbamylase as reported previously (10). Separation of the Golgi apparatus from microsomes was also examined morphologically by electron microscopy.

Detection of Ceruloplasmin in Each Subcellular Fraction and in the Serum: Hepatic subcellular fractions obtained from LEC and control rats were suspended in a small amount of PBS, mixed with 20% Triton X to final concentration of 1%, and centrifuged. Portions of each supernatant were diluted with an equal volume of sample buffer (0.25 M Tris HCl, 2% SDS, 30% glycerol, 10% mercaptoethanol, 0.01% BPB, pH 6.8), heated at 100℃ for 3 min and

electrophoresed on 7.5% SDS-PAGE. After being transferred to an Immobilon Transfer membrane (Millipore), the blot was incubated with polyclonal rabbit antibody against rat ceruloplasmin which was prepared from the antiserum made by immunization of rabbits with ceruloplasmin highly purified from the serum of Wister rats according to the method of Weiner et al (11). Immune complexes on the blot were detected with an Amplified Alkaline Phosphatase Goat Anti-Rabbit Immu-Blot Assay Kit (BIO-RAD). Ceruloplasmin in the serum of LEC and control rats was analyzed in the same way.

A portion of the supernatant (50 mg protein) from each subcellular fraction was diluted with 20 mM phosphate buffer, pH 6.8, and applied to a HiLoad 26/10 Q-Sepharose Fast Flow in an FPLC system (Pharmacia). Ceruloplasmin adsorbed to the column was eluted at 5 ml/min with a 0-1 M NaCl linear gradient at 4℃ in 900ml of the same buffer. Eight ml-fractions were collected. The serum (3 ml) of both kinds of rats and authentic ceruloplasmin (Sigma) were treated in the same manner. The eluate fractions of the microsomal and Golgi fractions from the column were concentrated to one-tenth volume by centriprep 30 (Amicon). The copper concentration was analyzed with an atomic absorption spectrometer (Hitachi Z-8100) after wet-digestion with HNO_3. Ceruloplasmin in these fractions was also detected by Western blot analysis as described above. All glassware used in the chromatography and copper analysis was washed with 2N HNO_3 before use to avoid metal contamination. The amount of protein was assayed by the method of Lowry et al.(12).

RESULTS AND DISCUSSION

No activity of marker enzymes was detected either in the Golgi or microsomal fractions, showing that contamination of the cytosol, lysosome and mitochondria was negligible. Electron microscopic observation also showed that the Golgi apparatus and microsomes were separated from each other (data not shown). Fig. 1 shows the Western blot analysis of the serum and hepatic subcellular fractions. Four bands (Cp1, 2, 3 and 4) were observed at the molecular mass of Mr 127, 132, 136 and 200 kDa, respectively, in both LEC and control rats. The faint Cp1 band was found only in the microsomal fractions of both kinds of rats. Cp2 was observed clearly in the microsomal fractions, but that observed in the Golgi fractions was faint. On the other hand, the Cp3 band appeared in the Golgi fractions and serum of both kinds of rats. Cp4 appeared clearly in the serum.

These results suggest that the Cp2 (Mr 132 kDa) in hepatic microsomes is a premature ceruloplasmin that could be converted to Cp3 (Mr 136 kDa), a mature

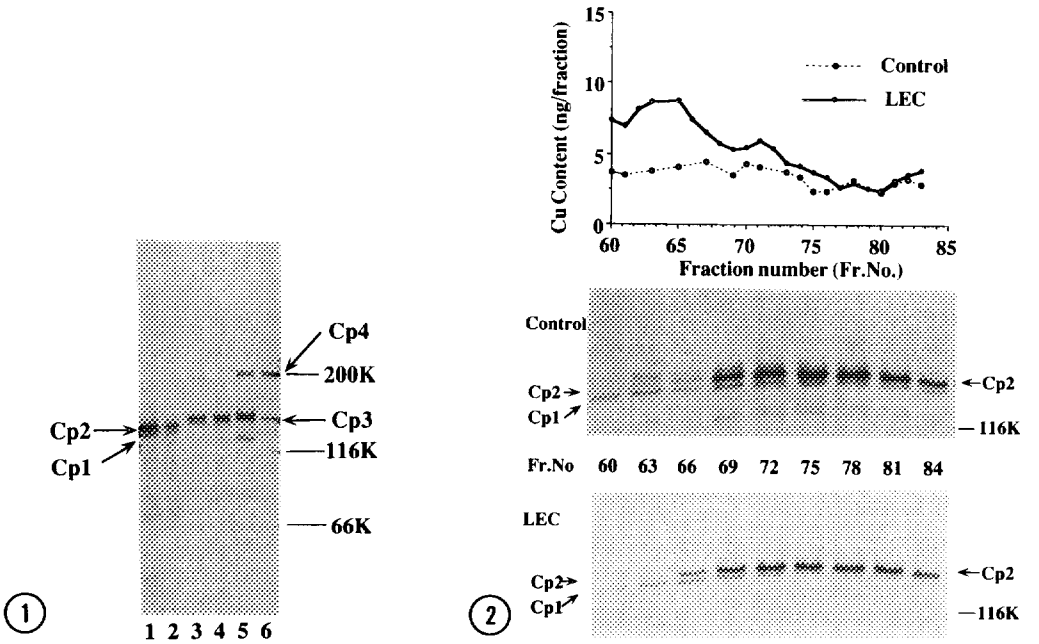

Fig. 1. Immunoblotting of ceruloplasmin in the microsomes (30μg protein, lines 1 and 2) and Golgi fractions (10μg protein, lines 3 and 4) from liver homogenates and in the serum (25nl,lines 5 and 6). Lines 1, 3 and 5, the control rat; lines 2, 4 and 6, the LEC rat.

Fig. 2. Detection of ceruloplasmin in microsomal fractions. Microsomal fractions were chromatographed on a Hiload 26/10 Q-Sepharose Fast Flow column, and 8ml of each fraction was collected. Upper, copper content; middle (control) and lower (LEC rat), immunoblotting of ceruloplasmin.

type, in the Golgi apparatus by some processing such as carbohydrate addition. The intensity of the Cp1, 2 and 3 bands in the microsomal and Golgi fractions from LEC rat hepatocytes was similar to that from control rats, suggesting that ceruloplasmin synthesis in LEC rat hepatocytes is not impaired. However, the Cp3 band in the serum from the LEC rat was faintly stained as compared with that in the control. Ceruloplasmin in the serum of the LEC rat has been reported to be stained similarly to that of control rats on Western blot analysis, although copper was not bound to ceruloplasmin in the LEC rat (8, 13, 14). On the contrary, It has been reported that the amount of apoceruloplasmin in the serum of patients with Wilson disease is nearly the same as that of controls (about 10% of total

ceruloplasmin protein in the control serum), whereas no significant holoceruloplasmin is detected in the serum of these patients, that is, the amount of ceruloplasmin protein in the serum of patients with Wilson disease is much less than that in control (2, 15). Our results coincide well with those of patients with Wilson disease. Cp4 with a larger molecular mass (Mr 200 kDa) was observed in the serum of LEC and normal rats. Sato et al.(16) reported a similar band in the serum of normal humans and suggested that it may be the result of post-translation modification of ceruloplasmin with Mr of 135kDa .

Figs. 2-4 show ceruloplasmin detected by immunoblot assay and the copper content in each subcellular fraction and the serum after partial purification by Sepharose chromatography. The experiments were repeated three times using different animals, and the results were similar in all three. In the microsomes of both the LEC and control rats, The Cp2 band was observed in fractions 69-84. However, no significant copper was detected in either strain of rat (Fig. 2). On the other hand, in the Golgi fraction of the control, the Cp3 band was detected in fractions 63-84. In this case, copper was detected in fractions 63-75 (Fig. 3). The Cp3 band of the Golgi apparatus was most intensively stained in fraction 69, in which the copper level was maximum. This was clearer in the case of control serum (Fig. 4). These results indicate that the ceruloplasmin in fractions 63-75 is holoceruloplasmin.

In contrast, the Cp3 band in the Golgi fraction and serum of the LEC rat was more intensely stained in fractions 72-84 (most intensively stained in fraction 78), that is, the elution time of Cp3 in these samples was later than that of Cp3 in the control (Figs. 3 and 4). No significant copper was detected in these fractions of samples. These results show that the ceruloplasmin in fractions around 78 is apoceruloplasmin which is not bound with copper. From these results, we conclude that copper is incorporated into ceruloplasmin in the Golgi apparatus in normal rat hepatocytes and that this copper incorporation is impaired in LEC rat hepatocytes.

The pathogenesis of the LEC rat as well as Wilson disease is probably a dysfunction of copper-transporting ATPase, an integral membrane protein (6).

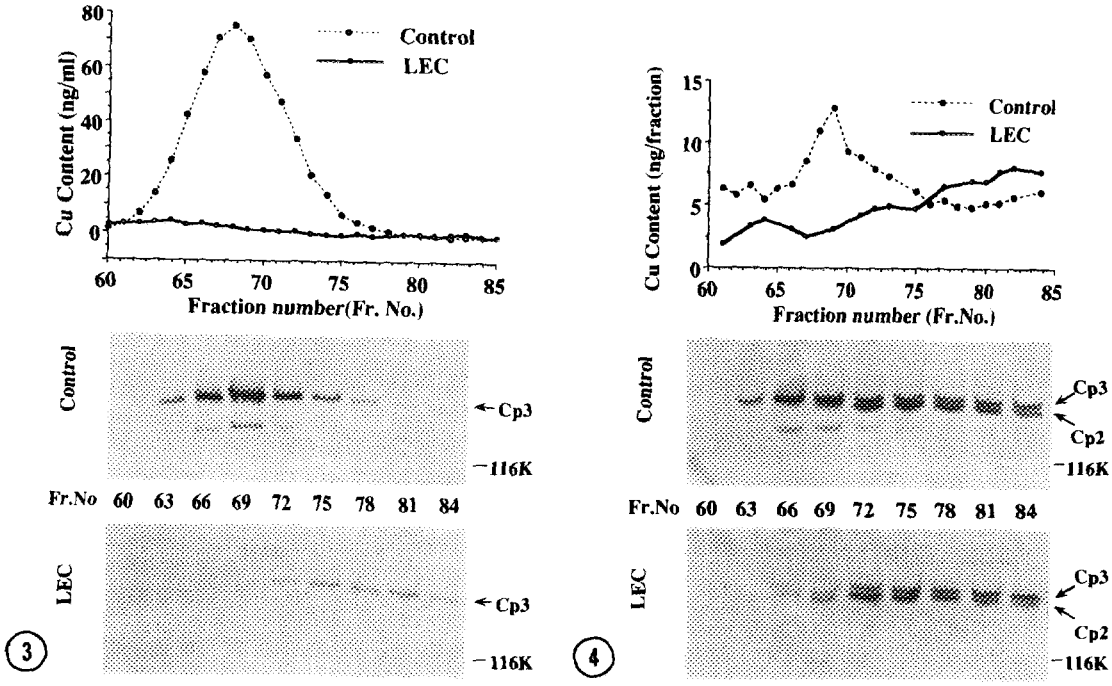

Fig. 3. Detection of ceruloplasmin in Golgi fractions. Golgi fractions were chromatographed in the same manner as microsomal fractions. Upper, copper content; middle and lower, immunoblotting of ceruloplasmin.

Fig. 4. Detection of ceruloplasmin in the serum. Sera (3ml) were chromatographed in the same manner as microsommal fractions. Upper, copper content; middle and lower, immunoblotting of ceruloplasmin.

Copper transport from the cytosol to the noncytosolic fractions is reported to be reduced in LEC rat hepatocytes (7, 17, 18). Our results suggest that P-type ATPase is in the membrane of the Golgi apparatus and transports copper from the cytosol into the inside of the Golgi apparatus. Copper in the Golgi apparatus is considered to be incorporated into ceruloplasmin in normal hepatocytes. The defect of copper transport to the Golgi apparatus in LEC rat hepatocytes could explain the reduced efficiency of copper incorporation into ceruloplasmin in the Golgi apparatus of LEC rat hepatocytes.

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