Two forms of Wilson disease protein produced by alternative splicing are localized in distinct cellular compartments

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Copper is an essential trace element in prokaryotes and eukaryotes and is strictly regulated by biological mechanisms. Menkes and Wilson diseases are human disorders that arise from disruption of the normal process of copper export from the cytosol to the extracellular environment. Recently a gene for Wilson disease (WD) (also named the *ATP7B* gene) was cloned. This gene encodes a copper transporter of the P-type ATPase. We prepared monoclonal and polyclonal anti-(WD protein) antibodies and characterized the full-length WD protein as well as a shorter form that is produced by alternative splicing in the human brain. We found that the WD protein is localized mainly in the Golgi apparatus, whereas the shorter form is present in the cytosol. These results suggest that the alternative WD proteins act as key regulators of copper metabolism, perhaps by performing distinct roles in the intracellular transport and export of copper.

INTRODUCTION

Copper is an essential trace element in prokaryotes and eukaryotes. In eukaryotic cells, several dozen proteins, including enzymes for electron transport, utilize the unique oxidation–reduction potential of copper for their biological functions. The well-known human diseases of copper imbalance are Wilson and Menkes diseases [1]. In Wilson disease (WD), copper cannot be excreted into the bile and cannot be incorporated into caeruloplasmin in the liver. Instead, it is accumulated in the liver, brain and cornea, which results in cell toxicity in these organs. Patients with WD show a range of clinical symptoms including chronic hepatitis and cirrhosis resulting from toxic accumulation of copper in the liver and neurological damage particularly affecting speech and movement, and leading to psychiatric disturbance in 15% of cases, because of build up of copper in the brain.

The copper transporter has been studied through investigation of copper incorporation into caeruloplasmin in hepatoma cell lines [2] and in the hepatocytes of the Long–Evans Cinnamon (LEC) rat, the bona fide animal of Wilson disease [3,4]. ATP-dependent copper transport has also been studied in isolated rat liver plasma membranes [5] and endoplasmic reticulum [6]. There has been some controversy about the intracellular localization of the copper transporter.

The WD gene has been cloned by a combination of positional cloning strategies and sequence homology to the Menkes disease gene [7–9]; this gene encodes a putative copper transporter of the P-type ATPase [7,9]. The WD protein is composed of six putative copper-binding domains, eight membrane-spanning domains, a phosphatase domain (TGEA), a conserved intramembranous Cys-Pro-X sequence, an aspartyl kinase domain (DKTGT) and an ATP-binding domain (GDGVND). This protein is a member of the CPx-ATPase family conserved from bacteria to humans [10,11].

The other copper transporter which is expressed in all tissues except liver, Menkes protein, has recently been characterized [12,13]. It is localized mainly in the Golgi apparatus [12,13]. One group has also reported that, in conditions of increased copper, there is a rapid redistribution of the Menkes protein from the Golgi to the plasma membrane in copper-resistant cell lines [12]. The other group has reported that, in medium containing basal copper, the Menkes protein is not detected on the plasma membrane in regular cell lines [13]. This and other evidence indicate that the Menkes protein acts as a copper-exporting transporter.

We are interested in copper metabolism in liver. The WD gene is expressed in the liver and brain. Interestingly, although the predominant WD mRNA transcript produced in the human liver encodes the full-length protein, several alternative-splicing forms are detected in other tissues, particularly the brain [14]. One of these, the mRNA lacking exons 6, 7, 8 and 12, was cloned from a brain cDNA library [9]. Reverse transcriptase-PCR analysis of polyadenylated RNA showed that this abbreviated transcript is predominant in the brain, but occurs rarely, if ever, in the liver [14]. Since these deletions occur in-frame, this mRNA could produce a shorter WD protein. We are interested in the physiological roles of the full-length WD protein and this short WD protein. In this study, we characterized these WD proteins using monoclonal and polyclonal anti-(WD protein) antibodies and found that the WD protein is mainly localized in the Golgi apparatus whereas the short WD protein resides primarily in the cytosolic fraction.

MATERIALS AND METHODS

Plasmid construction

The plasmid pCUN-c1 contains nt 223–2031 of the WD cDNA [14] at the *Xmn*I and *Hin*dIII sites of pMAL-c2 (New England Biolabs) to produce maltose-binding protein (MBP)–WD [amino acids (aa) 21–623] fusion protein. The pWD02 plasmid contains

Abbreviations used: aa, amino acids; ER, endoplasmic reticulum; MBP, maltose-binding protein; WD, Wilson disease; WGA, wheat germ agglutinin; GST, glutathione S-transferase; TRITC, tetramethylrhodamine isothiocyanate.

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the *Eco*RI fragment (nt 1489–5581), but it lacks exons 6, 7, 8 and 12 [9]. To convert this deletion form into the wild-type, human liver polyadenylated RNA was reverse-transcribed using the oligonucleotide TGTTCTATTTCTGACACTAC (nt 3237–3218) as a primer. PCR was then performed using this transcript as a template and the oligonucleotides TCAAGTATGACCCAGAGGTC (nt 1751–1770) and TGTTCTATTTCTGACACTAC (nt 3237–3218) as primers. After digestion of the PCR product with *Xho*I and *Bcl*I, the *Xho*I–*Bcl*I fragment (nt 1781–3060) was replaced with the corresponding fragment of pWD02 to produce pWD02comp. PCR was performed with pCUN-c1 as a template using the oligonucleotides GATGTCGAC CATGCCTGAGCAGGAGAGACAGATCACAGCCAGAG AAGGGGCCAGTCGGAAAATCTTATCTAAGCTTTCTT TGCCTACCCGTand CAAGGGCAACGGAGGCATAA (nt 1960–1941) to obtain the N-terminal product. After ligation of the *Sal*I–*Eco*RI fragment of the N-terminal product and the *Eco*RI fragment of pWD02comp, the ligated DNA was joined with the *Sal*I linker and then inserted into the pCX-N2 vector [15] and cut with *Xho*I to obtain the CX-WD plasmid. The *Eco*RI fragment of pWD02 was used instead of pWD02comp to construct the CX-ΔWD plasmid. The oligonucleotides GATGGATCCCTGACGGCATCCCAGGTCAGT and CTA GAATTCCTCACCTTCTACAGTCCAGCC were used to perform PCR with pWD02 as a template to obtain the C-terminal part. After digestion of *Bam*HI and *Eco*RI, the *Bam*HI–*Eco*RI fragment of the PCR product was ligated into the pGEX-2T vector digested with *Bam*HI and *Eco*RI to produce the GST-WD plasmid.

Production of anti-(WD protein) antibodies

The pCUN-c1 and GST-WD plasmids were transformed into *Escherichia coli* LE392. After induction with 0.5–1.0 mM isopropyl β-D-thiogalactopyranoside, the bacterial lysates were prepared and applied to amylose resin (New England Biolab) and glutathione–Sepharose 4B (Pharmacia) respectively. Washing and elution of the MBP–WD protein (which contains aa 21–623) and glutathione S-transferase (GST)–WD protein (which contains aa 1395–1465) proteins were performed according to the supplier's directions.

Purified MBP–WD protein was used to immunize Balb/c mice. After 3 days of booster, isolated spleen cells were fused with P3U1 myeloma cells using poly(ethylene glycol) 4000 (Nakarai, Kyoto, Japan), as described previously [16]. Hybridoma cells secreting anti-(WD protein) antibody were isolated and cloned by immunostaining the CX-WD gene-transfected HTB9 cells with culture supernatants. Cloned hybridoma cell lines were injected into the abdomen of pristane-treated Balb/c mice to obtain ascites. Monoclonal anti-(WD protein) antibodies were purified from ascites using Protein A–Sepharose 4B (Pharmacia). Purified GST–WD protein was used to immunize rabbits to obtain polyclonal anti-(WD protein) serum.

Cell culture and transfection of plasmids

Human bladder carcinoma HTB9 cells were cultured in RPMI1640 containing 10% fetal calf serum. Human hepatoma HepG2 and Hep3B cells, rat hepatoma H4IIEC3 cells and mouse fibroblast L cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. A 20 μg portion of the CX-WD and CX-ΔWD plasmids was transfected into the cultured cells as described previously [17].

Immunostaining and Western-blot analysis

For immunofluorescence staining, the transfected cells were seeded on to coverslips. On the next day, the cells were fixed with 3.5% formalin in PBS and permeabilized with 0.2% Triton-X-100 in PBS. They were then incubated with culture supernatants or diluted antibodies and next with FITC-conjugated anti-mouse IgG antibody (Dako). In the case of double staining, FITC-conjugated wheat germ agglutinin (WGA) (Sigma) (2 μg/ml) was used to stain the Golgi apparatus, and tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG antibody (Organon Teknika Co) was used as the secondary antibody to staining the WD protein. Fluorescence was observed with the confocal laser scanning microscope Axiovert 135 (Zeiss). Monoclonal anti-[γ-adaptin (AP-1)] (A4200; Sigma) and monoclonal anti-(Golgi 58K protein) (G2404; Sigma) antibodies were purchased commercially.

In Western-blot analysis, the transfected cells were collected with a rubber 'policeman' and centrifuged. They were solubilized in 5% SDS and centrifuged to remove cellular debris. Cell lysates were subjected to SDS/PAGE (7% gel) and transferred to PVDF membranes (Millipore) as described previously [3]. Monoclonal anti-(WD protein) antibodies no. 17 and 23 and polyclonal anti-(WD protein) antibody were used as the primary antibodies.

Cell fractionation

HTB9 cells transfected with the CX-WD and CX-ΔWD plasmids were collected and homogenized with a Teflon homogenizer in 3 vol. of 1.54 M (53%) sucrose in 0.1 M Na_2HPO_4, pH 7.0. After removal of the cellular debris by centrifugation at 1000 ***g*** for 10 min at 4 °C, the supernatants (1.28 M) were placed at the bottom of the centrifuge tubes. Then 1.13 M (38.7%), 1.05 M (36%), 0.96 M (33%) and 0.81 M (29%) sucrose in water (5 ml each) were layered sequentially on to the supernatants. After centrifugation at 100000 ***g*** for 60 min at 4 °C, fractions containing the Golgi apparatus, the endoplasmic reticulum (ER) and the cytosol were recovered [3] and subjected to Western-blot analysis. α-Mannosidase II, alkaline phosphodiesterase I, lactate dehydrogenase and glucose-6-phosphatase activities in each fraction were measured [18,19].

Isolation of plasma-membrane lawns

Plasma-membrane lawns were isolated as described previously [20]. Coverslips were coated in poly-L-lysine (0.01%, w/v) by immersion and dried before being seeded with cells. Cultured HepG2 cells on coverslips were washed in PBS and then disrupted by placement under an ultrasonic probe for 2 s (Branson Sonifiec Cell Disrupter 200). Sonicated cells were immediately transferred to 4% paraformaldehyde in PBS and fixed for 15 min before processing for immunofluorescence. Rabbit anti-(porcine Na^+/K^+-ATPase) antibody was a gift from Dr. O. Urayama [21].

RESULTS

The WD gene contains 21 exons and the WD protein is 1465 aa long. The longest WD mRNA is present in the human liver and brain. However, a shorter WD mRNA transcript is also present in the brain, but not in the liver [14]. In accordance with this, we isolated the WD cDNA lacking exons 6, 7, 8 and 12 from a brain cDNA library [9]. Exons 6, 7 and 8 encode 162 aa, and exon

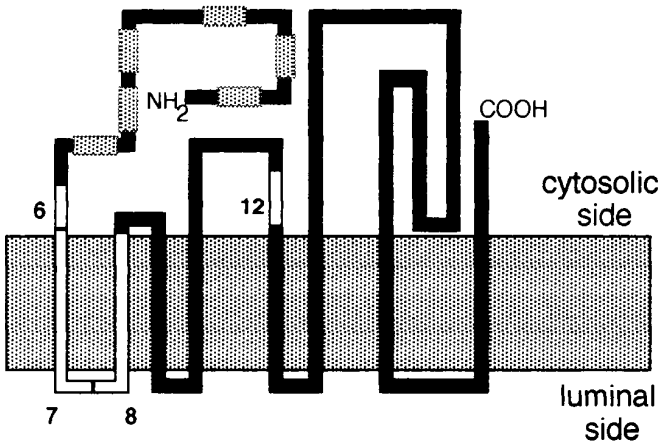

Figure 1 Schematic model of the WD protein outlining its essential structural features: eight transmembrane-spanning domains and six copper-binding domains (light stippled boxes at the N-terminal region)

The regions corresponding to exons 6, 7, 8 and 12 are indicated by open boxes numbered respectively.

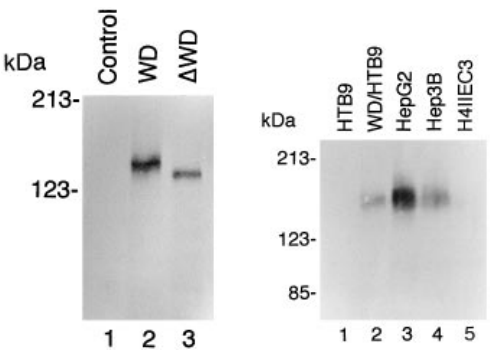

Figure 2 Western-blot analysis of WD protein in cultured cells

Left, HTB9 cells were transfected with the CX-WD or CX-ΔWD plasmid. After 2 days, the cells were solubilized in 5% SDS. Lysates (10 μg) from untransfected cells (lane 1) and cells transfected with the CX-WD gene (lane 2) and the CX-ΔWD gene (lane 3) were analysed by SDS/PAGE (7% gel) and transferred to a PVDF membrane. WD protein was detected by using monoclonal anti-(WD protein) antibody (no. 17) and ECL Western-blotting detection reagents (Amersham). Right, cells were solubilized in 5% SDS. Lysates (100 μg) from HTB9 cells (lane 1), HepG2 cells (lane 3), Hep3B cells (lane 4) and H4IIEC3 cells (lane 5) and 5 μg of the lysate from HTB9 cells transiently transfected with the CX-WD gene (lane 2) were analysed as described above.

12 encodes 45 aa. Since deletions of exons 6, 7 and 8 and exon 12 occur in-frame, the shorter mRNA should be translated into a WD protein of 1258 aa (Figure 1). We constructed a CX-ΔWD plasmid to produce this shorter WD protein (and called it the ΔWD protein) as well as a CX-WD plasmid to produce the full-length WD protein.

In order to obtain anti-(WD protein) antibodies, we produced the recombinant WD proteins in *Escherichia coli* and purified them. The MBP–WD protein contains the N-terminal putative copper-binding domains (aa 21–623), and the GST–WD protein contains 71 aa in the C-terminal region (aa 1395–1465). On SDS/PAGE, the sizes of these proteins were found to be 100 and 34 kDa respectively (results not shown). We immunized rabbits with the purified GST–WD protein to obtain a polyclonal anti-(WD protein) antibody. Next, we immunized Balb/c mice with the purified MBP–WD protein and then fused spleen cells from these immunized mice with myeloma P3U1 cells, as described in the Materials and methods section. We obtained two hybridoma cell lines that produce monoclonal anit-(WD protein) antibodies nos. 17 and 23. These two monoclonal antibodies were of the IgG class (results not shown). We used the three antibodies for experiments and obtained the same results for each one, so we present here the data for monoclonal antibody no. 17 only.

First, we transfected the CX-WD and CX-ΔWD plasmids into human bladder carcinoma HTB9 cells. Two days after transfection, the cells were harvested and lysed in RIPA (1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 50 mM Tris/HCl, pH 7.6). The ΔWD protein was partially solubilized, but the WD protein was hardly solubilized at all in this buffer. Therefore we solubilized the cells in 5% SDS and the cell lysates were processed for Western-blot analysis. When the CX-WD plasmid was transfected, a 160 kDa band was detected with monoclonal antibody 17 (Figure 2, left, lane 2). When the CX-ΔWD plasmid was used, a 140 kDa band was identified (lane 3). The same results were obtained when the plasmids were transfected into mouse fibroblast L cells (results not shown). Next, we analysed the cell lysates from human hepatoma HepG2 and Hep3B cells and rat hepatoma H4IIEC3 cells by the Western-blot method. As shown in the right gel of Figure 2, the WD protein in cell lysates from HepG2 (lane 3) and Hep3B (lane 4) cells was detected migrating at the same mobility as that for the HTB9 cells transfected with the CX-WD plasmid, but not in cell lysates from H4IIEC3 cells (lane 5). These results indicated that our antibodies specifically recognize the human WD protein.

We next investigated the localization of WD proteins in the cell by the indirect immunofluorescence method. When HTB9 cells transfected with the CX-WD gene were stained, fluorescence was observed in the coarse granular substance around the nucleus (Figure 3B). In contrast, cells transfected with the CX-ΔWD gene showed diffuse staining throughout the whole cytoplasm (Figure 3C). To delimit the localization of WD protein more precisely, we used a double-staining method. The Golgi apparatus was stained with FITC (green)-conjugated WGA, and the WD protein was stained with monoclonal antibody and TRITC (red)-conjugated secondary antibody. As shown in Figures 3D and 3F, compared with Figures 3E and 3G, the two colours had similar locations.

Next, we fractionated the subcellular compartments of cells transfected with the CX-WD and CX-ΔWD genes, as described in the Materials and methods section. The purity and degree of cross-contamination of the subcellular fractions were determined by assaying marker enzymes. As shown in Table 1, the Golgi apparatus, ER and cytosol were almost completely separated from each other. We used the fractions of 0.81 M/0.96 M plus 0.96 M/1.05 M, precipitate and 1.28 M as the Golgi apparatus, ER and cytosolic fractions, respectively. Recoveries of marker enzymes in the Golgi apparatus, ER and cytosolic fractions were 20, 80 and 80% respectively. Fractions containing the Golgi apparatus, the ER and the cytosol were subjected to Western-blot analysis. As shown in Figure 4 (left), the WD protein was present in the Golgi apparatus fraction (lane 2), but not in the ER (lane 3) or cytosolic (lane 4) fractions. In contrast, the ΔWD protein was present in the cytosolic fraction (Figure 4, right, lane 4), but not in the Golgi apparatus or ER fractions (lanes 2 and 3). These results clearly show that the WD protein is localized mainly in the Golgi apparatus, whereas the ΔWD protein is found mainly in the cytosol.

We then stained HepG2 cells by the indirect immunofluorescence method using monoclonal anti-(WD protein) anti-

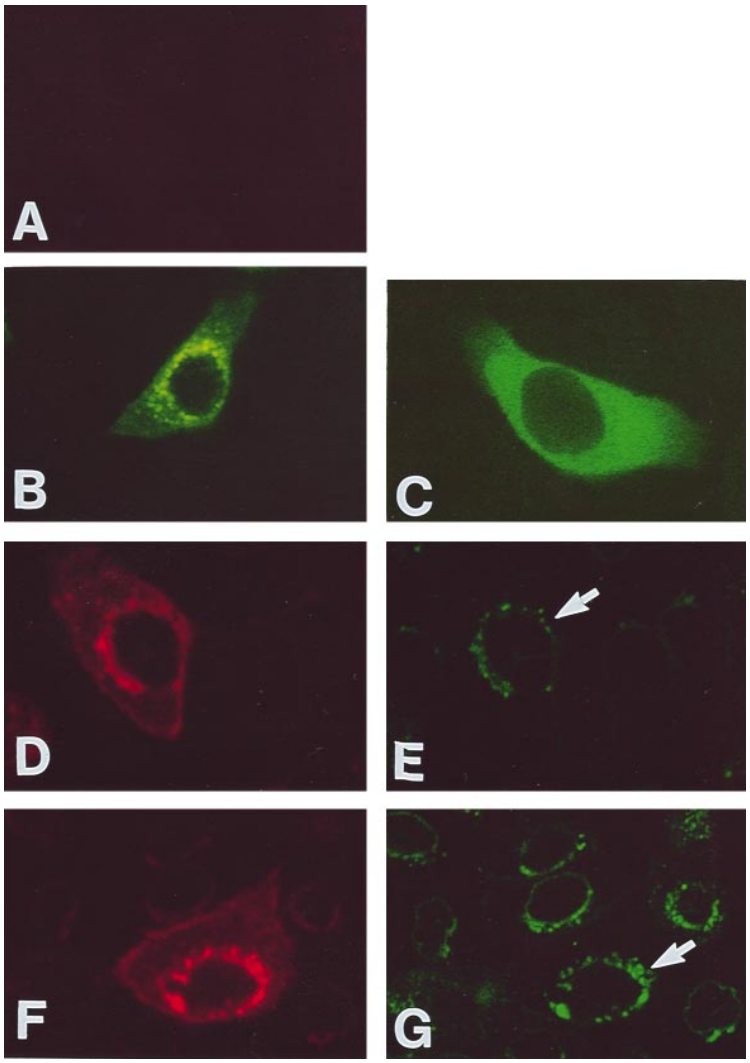

Figure 3 Localization of WD protein in transfected HTB9 cells

(**A**–**C**) Untransfected HTB9 cells (**A**) and HTB9 cells transfected with the CX-WD gene (**B**) and the CX-ΔWD gene (**C**) were fixed and incubated with monoclonal anti-(WD protein) antibody (no. 17). FITC-conjugated anti-mouse IgG antibody was used as the secondary antibody. (**D**)–(**G**) HTB9 cells transfected with the CX-WD gene were doubly stained with monoclonal anti-(WD protein) antibody (no. 17) and TRITC-conjugated anti-mouse IgG antibody (**D** and **F**) and FITC–WGA (**E** and **G**). (**E**) and (**G**) correspond to (**D**) and (**F**) respectively. The CX-WD gene-transfected cells are indicated by arrows (**E** and **G**). Magnification: **A** and **B**, ×522; **C**, ×870; **D**–**G**, ×652.

Table 1 Assay of marker enzymes in the subcellular fractions

Values are means ± S.D. ($n = 3$).

Fraction	α-Mannosidase II (fluorescence units/min per mg)	Glucose-6-phosphatase (absorbance units/min per mg)	Alkaline phosphodiesterase I (absorbance units/h per mg)	Lactate dehydrogenase (absorbance units/min per mg)
0.81 M	10.0 ± 1.2	0.06 ± 0.01	15.00 ± 0.90	11.10 ± 0.98
0.81 M/0.96 M	135.6 ± 12.0	0.06 ± 0.02	7.16 ± 0.30	9.70 ± 0.88
0.96 M/1.05 M	35.2 ± 6.5	0.05 ± 0.01	6.10 ± 0.12	9.55 ± 0.56
1.05 M/1.13 M	18.8 ± 3.1	0.08 ± 0.01	4.90 ± 0.37	7.65 ± 0.66
1.13 M/1.28 M	22.0 ± 3.7	1.63 ± 0.25	1.52 ± 0.15	3.23 ± 0.31
1.28 M	22.4 ± 2.9	1.13 ± 0.48	0.76 ± 0.06	49.90 ± 1.87
Precipitate	8.8 ± 1.0	7.30 ± 0.94	1.37 ± 0.07	5.52 ± 0.63

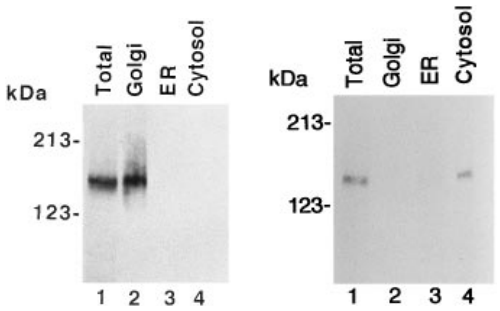

Figure 4 Western-blot analysis of fractions from HTB9 cells transfected with the CX-WD and CX-ΔWD genes

The CX-WD gene (left) and the CX-ΔWD gene (right) were transfected into HTB9 cells. After 2 days, the cells were homogenized and fractionated as described in the Materials and methods section. Left, 180 μg of the total homogenate (lane 1), 10 μg of the Golgi apparatus fraction (lane 2) and cytosolic fraction (lane 4) and 30 μg of the ER fraction (lane 3) were processed as in Figure 2 (left). Right, 10 μg of the total homogenate (lane 1) and the cytosolic fraction (lane 4) and 20 μg of the Golgi apparatus fraction (lane 2) and the ER fraction (lane 3) were processed as in Figure 2 (left).

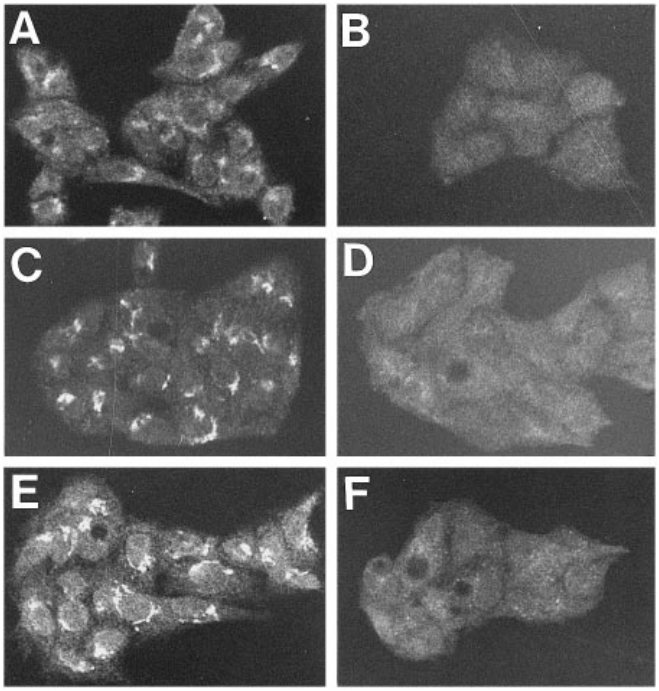

Figure 5 Effect of brefeldin A on localization of endogenous WD protein in HepG2 cells

HepG2 cells were incubated in medium without (**A**, **C** and **E**) or with brefeldin A (Nakarai; 10 μg/ml) for 1.5 h (**B**, **D** and **F**), fixed, incubated with monoclonal anti-(WD protein) antibody (**A** and **B**), monoclonal anti-AP-1 antibody (**C** and **D**) and monclonal anti-(Golgi 58K protein) antibody (**E** and **F**), and then with FITC-conjugated anti-mouse anti-IgG antibody as the secondary antibody. Magnification, $\times$ 522.

bodies. As shown in Figure 5(A), the fluorescence signal was detected in the coarse granular substance in the perinuclear areas. In order to define further the location of the WD protein, we incubated HepG2 cells for 1.5 h in medium containing brefeldin A, a fungal metabolite that disrupts the morphology of the Golgi apparatus. γ-Adaptin (AP-1) and Golgi 58K proteins,

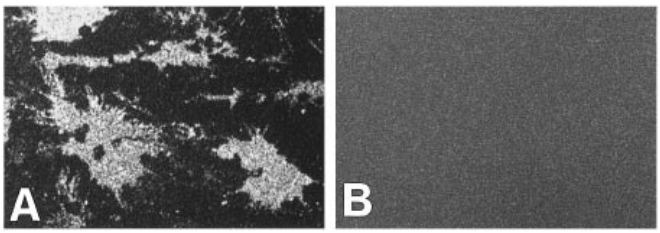

Figure 6 Investigation of WD protein association with plasma-membrane lawns of HepG2 cells grown in the presence of elevated copper levels

Plasma-membrane lawns were isolated and fixed from HepG2 cells cultured for 24 h in medium containing 200 μM Cu–histidine complex before processing for immunofluorescence analysis using rabbit anti-(porcine Na^+/K^+-ATPase) (**A**) or monoclonal anti-(WD protein) antibody (**B**). Magnification, $\times$ 522.

which are markers of the Golgi apparatus, were localized in perinuclear regions (Figures 5C and 5E), but the perinuclear localization was not detected after treatment with brefeldin A (Figures 5D and 5F). Similarly the perinuclear distribution of WD protein disappeared after treatment with brefeldin A (Figure 5B). These results indicate that the endogenous WD protein is localized mainly in the Golgi apparatus in HepG2 cells.

Next we investigated whether the WD protein was redistributed under conditions of increased copper. We cultured HepG2 cells in medium containing basal copper or 200 μM Cu–histidine complex for 1 or 24 h and stained the cells by the indirect immunofluorescence method. Even after culture in the presence of high concentrations of copper for 1 and 24 h, the WD protein was localized in the perinuclear regions, as in medium containing basal copper (results not shown).

Finally we determined whether the WD protein was present on the plasma membrane. Because it was not solubilized in conditions suitable for immunoprecipitation, we used plasma-membrane lawns instead of cell surface labelling. Procedures for obtaining these plasma-membrane lawns involve sonication of cells grown on coverslips to yield plasma-membrane fragments with their cytosolic surfaces exposed and which are free from contamination by intracellular membranes [20]. Immunofluorescence labelling of plasma-membrane lawns by confocal laser-scanning microscopy was used to investigate whether the WD protein was associated with the plasma membrane. The plasma-membrane lawns from cultured cells in medium containing 200 μM copper and basal copper showed strong fluorescence with antibodies to a marker for the plasma membrane, Na^+/K^+-ATPase (Figure 6A and results not shown). In contrast, no fluorescence signal was detected when the lawns were processed for immunostaining with anti-(WD protein) antibody (Figure 6B and results not shown). These results indicate that the WD protein is not redistributed in response to elevated copper levels, and not associated with the plasma membranes.

DISCUSSION

The WD protein is a putative copper transporter of the P-type ATPase [7,9]. Alternative splicing has been detected in the human liver and brain [14]. We were interested in an alternative-splicing form lacking exons 6, 7, 8 and 12, which is present in the brain but not in the liver [14]. Therefore we made two constructs to overexpress the WD protein and the shorter ΔWD protein. By using monoclonal antibodies and a polyclonal antibody, we found that the WD protein is mainly localized in the Golgi apparatus and the ΔWD protein is present in the cytosol. Since

exons 7 and 8 encode putative membrane-spanning domains and exon 12 does not (Figure 1), the deletion of exons 6, 7 and 8 could be responsible for the failure of the ΔWD protein to become inserted into ER membranes. Even though the ΔWD protein contains six other putative membrane-spanning domains [7,9], these domains do not operate as stop-transfer signals. That is, the first membrane-spanning region of the WD protein appears to be important for insertion into the ER membrane.

Clearly, our results indicate that the WD protein is localized mainly in the Golgi apparatus. This indicates that copper is transported from the cytosol into the spaces inside intracellular membranes mainly by means of the Golgi membranes. Although it cannot be excluded that copper is transported to the ER from the Golgi apparatus via membrane trafficking, it is reasonable to assume that incorporation of copper into caeruloplasmin occurs in the Golgi apparatus. Our result is consistent with defective copper incorporation into caeruloplasmin during its synthesis in the Golgi apparatus in hepatocytes of LEC rats reported previously [3,4]. It is also known that bile contains copper, some of which is protein-bound [22–26]. Incorporation of copper into unknown bile proteins might also occur in the Golgi apparatus. We tried to detect the WD protein on the plasma membranes in HepG2 cells, but we could not detect it even in culture in conditions of increased copper.

The Menkes gene is expressed in all tissues but liver, and the WD gene is expressed in the liver and brain. The Menkes and WD proteins are structurally similar and both are proposed to act as copper-exporting transporters. It has been recently reported that the Menkes protein is mainly located in the Golgi apparatus [12,13], as we have found for the WD protein in this study. However, there appear to be some differences between the Menkes and WD proteins. The Menkes protein is solublized in 0.25% Nonidet P40 [13], whereas the WD protein is not solubilized in 1% Nonidet P40/0.5% deoxycholate/0.1% SDS. When cultured in medium containing high copper, the Menkes protein is redistributed from the Golgi apparatus to the plasma membranes [12], whereas the WD protein remains in the Golgi apparatus.

The physiological significance of the WD protein in the brain is not known. The mammalian brain is one of the richest copper-containing tissues, and the copper distribution corresponds to the distribution of various copper enzymes, including superoxide dismutase, tryptophan 2,3-dioxygenase, lysine oxidase, cytochrome oxidase, monoamine oxidases, dopamine β-hydroxylase and tyrosinase [27–29]. The shorter splicing form of the WD mRNA is also detected in the human brain [14]. Although we have no direct evidence, this mRNA should be translated into the ΔWD protein, which is localized in the cytosol in the brain as it is in the transfected cells. Metallothioneins play a central role in homoeostasis and detoxification of heavy metals, including copper in the liver and kidneys [30]. Although a metallothionein isoform (MT-III) has been reported to exist in brain tissue [31–33], it is expressed at a low level in the normal brain. It is possible that the short WD protein could bind and sequester cytosolic copper as a copper reservoir in brain tissue.

We found the endogenous WD protein localized mainly in the Golgi apparatus in HepG2 cells by Western-blot and indirect immunofluorescence analyses. This result is consistent with the previous finding that copper is incorporated into caeruloplasmin to make holocaeruloplasmin in HepG2 cells [2]. A biochemical study of the transporter proteins purified from the overexpressed materials in eukaryotic cells should clarify the structure and function of the WD protein in membranes.

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