Analysis of functional domains of Wilson disease protein (ATP7B) in *Saccharomyces cerevisiae*

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Received 11 March 1998; revised version received 30 April 1998

Abstract Wilson disease is a genetic disorder of copper metabolism characterized by the toxic accumulation of copper in the liver. The *ATP7B* gene, which encodes a copper transporting P-type ATPase, is defective in patients with Wilson disease. To investigate the function of ATP7B, wild type or mutated *ATP7B* cDNA was introduced into a yeast strain lacking the *CCC2* gene (Δ*ccc2*), the yeast homologue of *ATP7B*. Wild type and the H1069Q mutant could rescue Δ*ccc2*, however, the N1270S mutant could not, reflecting phenotypic variability of Wilson disease. In addition, the mutant containing only the sixth copper binding domain could rescue Δ*ccc2*, indicating its functional importance.

Key words: ATP7B; Copper; Wilson disease; Yeast Ccc2 protein

1. Introduction

Copper is an essential trace metal for prokaryotes and eukaryotes, and is a required component for a variety of enzymes, including cytochrome oxidase and other copper containing enzymes [1,2]. However, excess accumulation of copper ion in the body is toxic, because it acts predominantly through the formation of highly reactive hydroxyl radicals, which lead to damage of cell membranes, mitochondria, proteins and DNA [3].

Wilson disease is an autosomal recessive disorder of copper transport and has a worldwide frequency of between one in 35 000 and one in 100 000 live births [4]. The disorder manifests as chronic liver disease and/or neurological impairment, frequently with kidney malfunction, due to toxic accumulation of copper in several tissues, principally the liver, brain and kidney. This disease is believed to be caused by the mutation of the gene which encodes a copper transporting P-type ATPase (ATP7B) [5–7] and various mutations in this gene have been reported in patients with Wilson disease [7–9]. The excessive accumulation of copper in affected liver is thought to be due to reduced biliary excretion of copper and disturbed incorporation of copper into ceruloplasmin (CPN) [10].

P-type ATPases, defined as those forming a covalent phosphorylated intermediate in their reaction cycle, transport a variety of cations across membranes [11]. 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 MXGDGXNDXP that connects the ATP binding domain to the transmembrane segment. ATP7B protein is further classified as a heavy metal transporting P-type ATPase which pumps copper or cadmium [12–14]. The six repeated motifs, GMTCXXC, at the N-terminus of ATP7B are thought to be copper binding domains from their homology with CopA, a copper transporting ATPase in copper resistant strains of *Enterococcus hirae* [15]. A recent study has demonstrated that this motif has specificity for copper and 6 mol of copper bind to 1 mol of N-terminus of ATP7B [16]. ATP7B also contains the SEHPL and CPC motifs, conserved in heavy metal transporting ATPases and involved in heavy metal translocation [12–14].

The yeast *CCC2* gene, homologous to the human Wilson and Menkes genes, has been shown to encode a copper transporting P-type ATPase with two copies of the conserved copper binding motif [17]. Fet3 protein (Fet3p, multi-copper oxidase), known as a membrane bound CPN-like protein in yeast, is required for ferrous transport, indispensable for yeast growth [18,19]. The copper binding to Fet3p is necessary to manifest its oxidase activity and this process is mediated by Ccc2p [17]. Thus, Ccc2p is involved in high affinity iron uptake. On the other hand, Δ*ccc2*, the yeast mutant strain lacking *CCC2*, can survive in an iron rich environment, suggesting the existence of an alternative pathway for iron transport into cells. Our recent report has demonstrated that Ccc2p function is complemented by the introduction of *cua-1* cDNA, encoding a copper transporter of *Caenorhabditis elegans*, into the Δ*ccc2* strain [20].

In this study, we constructed several mutated cDNAs reported in Wilson disease patients as well as deleted cDNAs within the copper binding domains of ATP7B, and introduced these cDNAs into the yeast Δ*ccc2* strain to investigate their functions.

2. Materials and methods

2.1. Construction of mutated ATP7B cDNAs

Full length *ATP7B* cDNA was constructed as described previously [21]. To obtain deleted cDNAs within copper binding domains, the full length *ATP7B* cDNA was digested with restriction enzymes as indicated in Fig. 1A. As shown in Fig. 1A, Cu0 has the deletion of *ATP7B* cDNA from nucleotides (nt) 320 to 1991. Cu1–2, from nt 811 to 1991. Cu1–3, from nt 1132 to 1991. Cu1–4, from nt 1489 to 1991. Cu1–5, from nt 1781 to 1991. Cu6, from nt 320 to 1804. To introduce point mutations into *ATP7B* cDNA (Fig. 1B), site directed mutagenesis using the polymerase chain reaction (PCR) was performed [22]. A *Hinc*II-*Sac*I fragment (nt 2795–3762) containing the DKTG motif and the SEHPL motif and a *Sac*I-*Pvu*II fragment (nt 3762–4729) containing the GDGVND motif were cloned into vector pUC18 and used as

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0014-5793/98/$19.00
PII S0014-5793(98)00546-8

templates for PCR. Primers used for D1027A were 5′-ACTGTGATGTTTGCCAAGACT-3′, for T1029A, 5′-TTTGACAAAGGCTGGCACCATT-3′, for H1069Q, 5′-AGTGAACAACCCTTGGGCGT-3′, and for N1270S, 5′-CGGGGAGTCACTGACCCCAT-3′. MUT1 primer, 5′-CATGATTACGAGTTCTAGCT-3′ (Takara, Kyoto, Japan), was used as a counterpart for each primer. The presence of the specific mutations or deletions, as well as the fidelity of the entire cDNA sequence, were confirmed by using Abi Prism Dye terminator cycle sequencing ready reaction kit (Perkin-Elmer).

To detect the expression of the above cDNAs, the hemagglutinin (HA) epitope (Boehringer Mannheim) was inserted into each constructed cDNA at the *Eco47*III site, corresponding to nt 4327 in wild type *ATP7B* cDNA. All of the constructed cDNAs were inserted into the mammalian expression vector pRC/CMV (Invitrogen) and the yeast expression vector pSY114 [20].

2.2. Yeast strains, growth conditions, and transformations

The *CCC2* gene was disrupted from chromosomal DNA of *Saccharomyces cerevisiae* YPH499 (*MAT*a, *ura3-52*, *leu2-Δ1*, *trp1-Δ63*, *his3-Δ200, ade2-101, lys2-801*) [20]. Δ*ccc2* yeast cells were transformed with the constructed cDNAs by the modified lithium acetate method [20], and the transformants were selected on solid synthetic dextrose (SD) medium (0.67% yeast nitrogen base without amino acids, 2% glucose, 2% agar) supplemented with adenine, uracil, tryptophan, and lysine

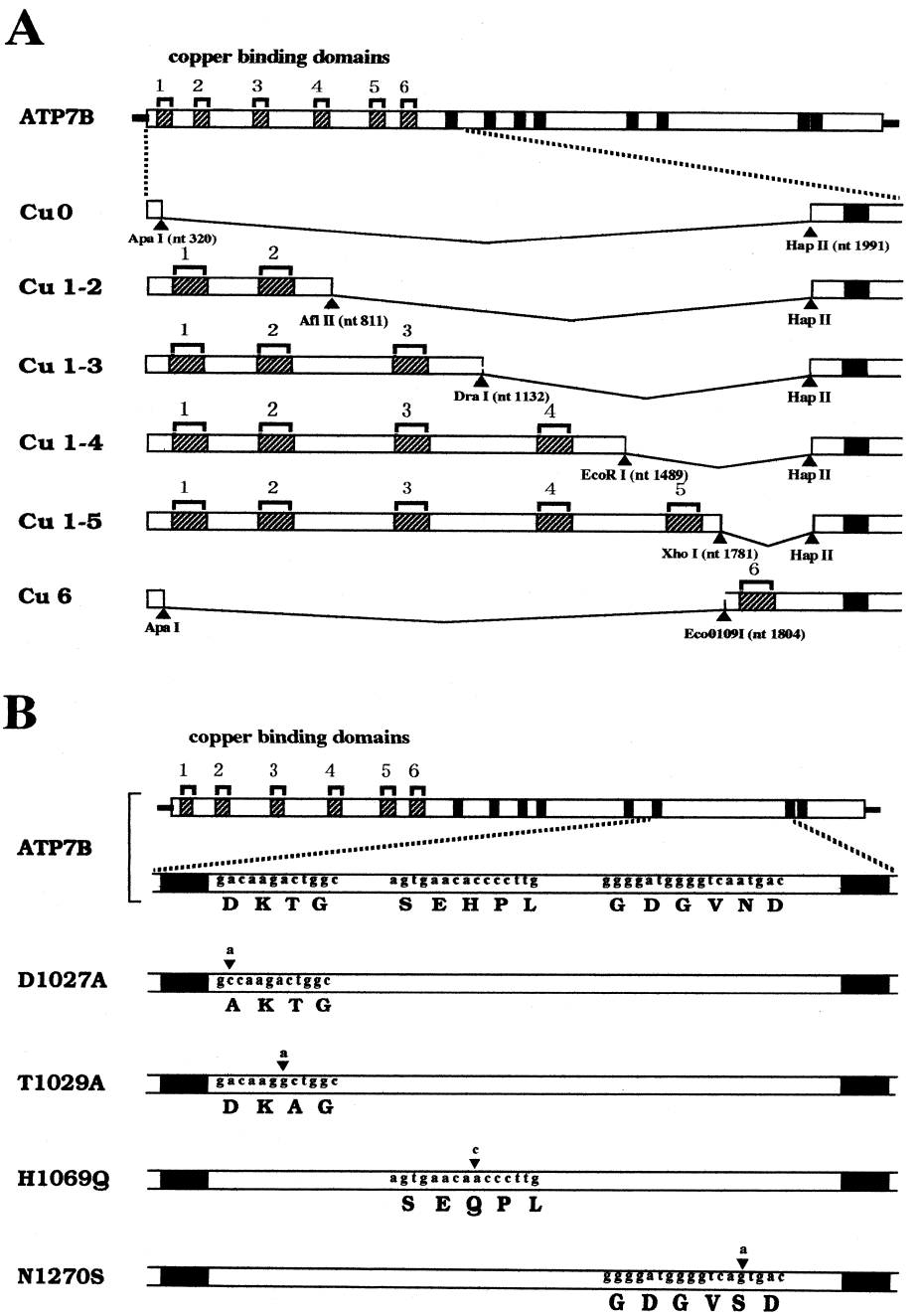

Fig. 1. Construction of mutated *ATP7B* cDNA. A: Each *ATP7B* cDNA with a deletion in copper binding domains was constructed by cleaving the full length *ATP7B* with indicated restriction enzymes. B: Point mutated *ATP7B* cDNAs were produced by site directed mutagenesis with the oligonucleotide primers, described in Section 2. Asp1027Ala (D1027A) and Thr1029Ala (T1029A) are point mutated in the phosphatase domain. His1069Gln (H1069Q) in the SEHPL motif, highly conserved in heavy metal transporting ATPases. Asn1270Ser (N1270S) in the hinge domain. Each constructed cDNA was inserted into the expression vectors for mammalian and yeast.

[20]. For metal dependent tests, histidine (20 mg/ml) and leucine (60 mg/ml) were added to the same medium with 2% agar instead of 0.7% agarose (test medium). The selected transformants were grown at 30°C for 4 days on the test medium containing 500 μM of bathocuproine disulfonate and/or 500 μM ferrozine (3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine), or nothing.

2.3. Protein extraction from the yeast and immunoblotting

2×10^7 yeast cells were suspended in 1 ml of 1 M sorbitol containing 0.2 N NaOH and 8% 2-mercaptoethanol, and incubated on ice for 10 min. Subsequently, 100 μl of 30% trichloroacetic acid was added and protein was precipitated on ice for 10 min. Protein was collected by centrifugation at 12 000 rpm for 10 min, washed with ice cold acetone twice, and then dissolved in 50 μl of SDS sample buffer (62.5 mM Tris-HCl, 0.01% phenol red, 10% glycerol, 40 mM dithiothreitol, 5% sodium dodecyl sulfate). 25 μl of protein samples was resolved by polyacrylamide gel electrophoresis (PAGE), and immunodetection was performed as described [21].

3. Results

To obtain deleted cDNAs within copper binding domains, the full length *ATP7B* cDNA was digested with restriction enzymes as indicated in Fig. 1A. These mutants lack the indicated copper binding domains. Site directed mutagenesis was applied to produce the mutants D1027A, T1029A, H1069Q, and N1270S, as described above (Fig. 1B). The mutations of D1027A and T1029A, located in the DKTG motif, lead to the disruption of phosphorylation of the motif. H1069Q in the SEHPL motif and N1270S in the hinge domain may affect heavy metal translocation and hinge region, respectively.

Since our previous reports conducted by in vitro and in vivo studies [21,23] have revealed that the wild type ATP7B protein localizes to the Golgi apparatus, we examined the localization of the mutated ATP7B proteins by the introduction of mutated cDNAs into COS1 cells. The immunofluorescence study showed that the products of wild type and mutated cDNAs except Cu1–5 are detected in the perinuclear region in transfected COS1 cells, indicating the mutated ATP7B proteins mainly reside in the Golgi apparatus (data not shown). However, the product of the Cu1–5 mutant mainly showed a diffused staining in the whole cytoplasm (data not shown), implying that the deletion surrounding the sixth copper binding domain of ATP7B protein may lead to missorting of the protein.

To confirm the expression of mutated ATP7B proteins in the Δ*ccc2* yeast strain, we performed Western blot analysis using cell homogenates prepared from the Δ*ccc2* strains transfected with mutated *ATP7B* cDNAs. The molecular masses of mutants with deletion in copper binding domains, such as Cu0, Cu1–2, Cu1–3, Cu1–4, Cu1–5, and Cu6, were 120 kDa, 135 kDa, 155 kDa, 160 kDa, 170 kDa, and 125 kDa, respectively (Fig. 2A, lanes 3–8), consistent with the molecular masses deduced from the amino acid sequences. However, the level of expression of Cu1–5 (Fig. 2A, lane 7) was significantly low compared with those of other deleted mutants. This is probably due to the degradation of translated protein resulted from missorting of the protein as suggested above, since Southern blot and Northern blot analyses revealed that the amount of transfected DNAs and the resulting mRNA expression were equivalent (data not shown). In addition, the molecular masses of mutated ATP7B proteins with a single amino acid substitution, such as D1027A, T1029A, H1069Q, and N1270S, were identical with that of the wild type ATP7B protein and their expression was equivalent with the wild type (Fig. 2B, lanes 10–14).

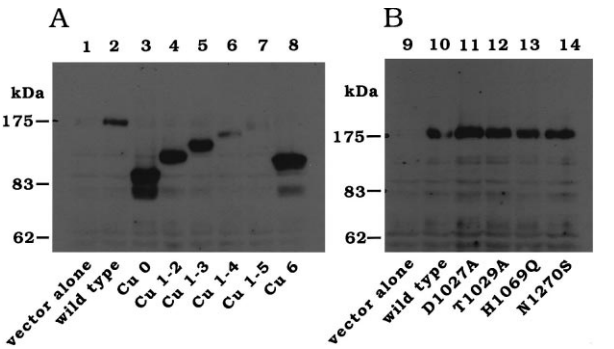

Fig. 2. Detection of wild type and mutant ATP7B proteins in the Δ*ccc2* strain of yeast. Western blot showing the chemiluminescent detection of ATP7B proteins lacking the copper binding domains (A) and point mutated ATP7B proteins (B) in the transformed Δ*ccc2*. Total protein was extracted from 2×10^7 cells of each transformant, applied to 7% SDS-PAGE, and transferred to a PVDF membrane. The blots were probed with mouse anti-HA monoclonal antibody (1:50) followed by horseradish peroxidase conjugated anti-mouse IgG (1:4000). Bound antibody was detected by enhanced chemiluminescence. A: Lane 1, vector alone; lane 2, wild type ATP7B; lane 3, Cu0; lane 4, Cu1–2; lane 5, Cu1–3; lane 6, Cu1–4; lane 7, Cu1–5; lane 8, Cu6. B: Lane 9, vector alone; lane 10, wild type ATP7B; lane 11, D1027A; lane 12, T1029A; lane 13, H1069Q; lane 14, N1270S.

We then transfected mutated *ATP7B* cDNAs into the Δ*ccc2* strain to investigate their function with respect to the growth of Δ*ccc2* under various metal concentrations. As shown in Fig. 3A, all of the transfected Δ*ccc2* strains were able to grow on an SD medium which contained sufficient iron and copper. Additionally, no growth impairment was observed in each Δ*ccc2* strain on an SD medium supplemented with a copper chelator, bathocuproine disulfonate (Fig. 3B), indicating iron was taken up from the environment through the low affinity uptake pathway. In contrast, the Δ*ccc2* strains which could grow in a medium containing the iron chelator ferrozine were only those transfected with wild type *ATP7B*, Cu6, or H1069Q cDNAs (Fig. 3C). However, the growth of Δ*ccc2* with H1069Q (Fig. 3C, area 13) was slower than that of Δ*ccc2* rescued by wild type *ATP7B* and Cu6 (Fig. 3C, areas 4 and 10). In addition, Δ*ccc2* with H1069Q could not grow in a medium containing less copper (data not shown). None of the transfected Δ*ccc2* strains could grow on medium supplemented with both chelators (Fig. 3D). These results suggest that products of wild type *ATP7B*, Cu6, or H1069Q cDNA recover iron uptake in a copper dependent manner and complement the function of Ccc2p.

4. Discussion

In this study we demonstrated that human ATP7B protein was able to rescue the yeast Δ*ccc2* mutant. In addition, the Cu6 mutant bearing the sixth copper binding domain and the H1069Q mutant, the most common mutation reported in Wilson disease patients [7–9], were also found to restore the function of yeast Ccc2p.

A previous report demonstrated that the yeast *CCC2* gene encoded copper transporting P-type ATPase and was a yeast counterpart for human *ATP7A* or *ATP7B*, defective in Menkes disease or Wilson disease, respectively [17]. This re-

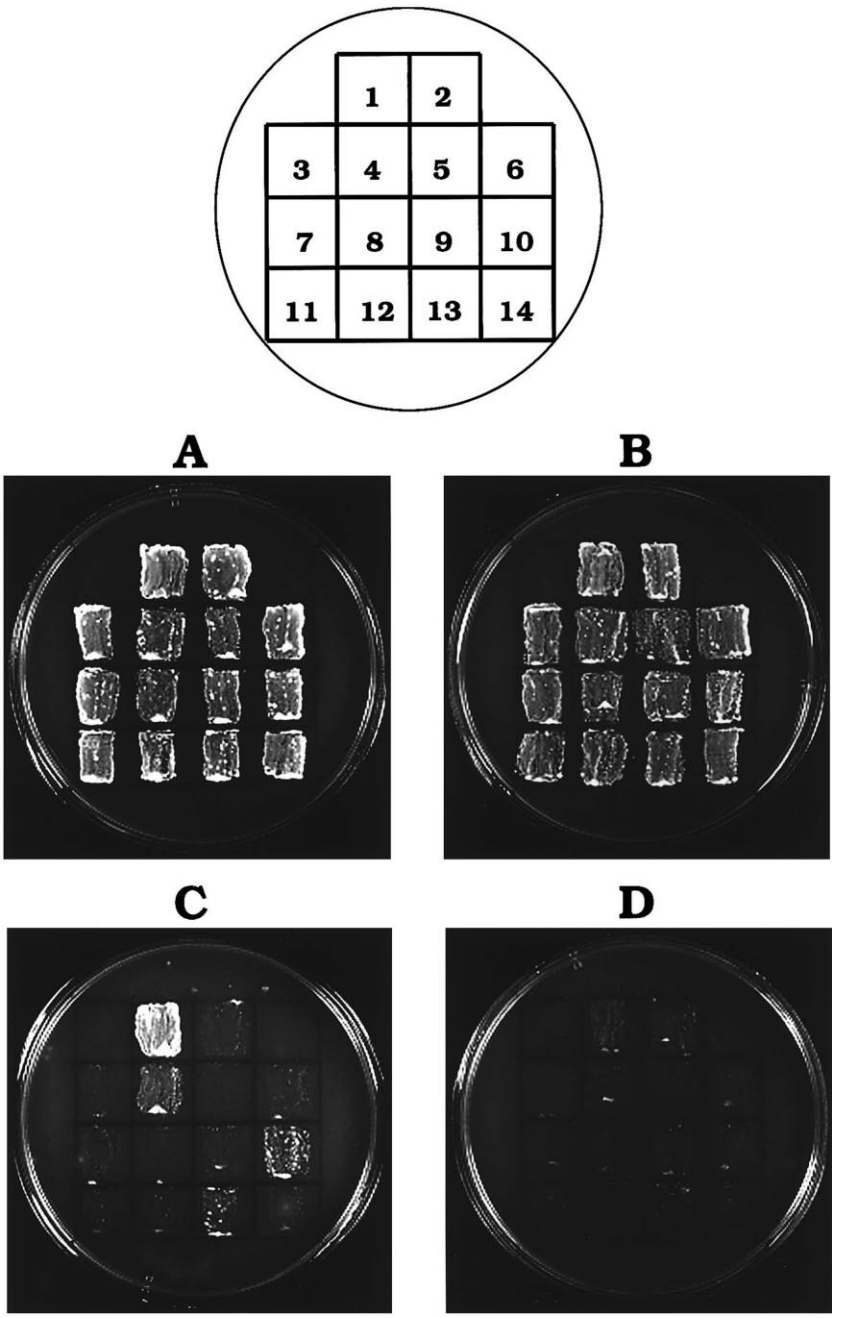

Fig. 3. Metal dependent test for the growth of the yeast Δ*ccc2* strain transformed with wild type and mutated *ATP7B* cDNAs. The Δ*ccc2* yeast cells were transformed with the mutated *ATP7B* cDNAs and the transformants were selected on solid synthetic dextrose (SD) medium as described in Section 2. The transformed cells were grown at 30°C for 4 days on SD medium containing metal ion chelators. Plate A, no chelators; plate B, 500 μM bathocuproine disulfonate for chelating copper; plate C, 500 μM ferrozine for chelating iron; plate D, 500 μM bathocuproine disulfonate and 500 μM ferrozine. Strains grown on each plate are YPH499 (wild type strain, area 1), Δ*ccc2* (area 2), Δ*ccc2* transformed with vector alone (area 3), Δ*ccc2* with wild type *ATP7B* (area 4), Δ*ccc2* with Cu0 (area 5), Δ*ccc2* with Cu1–2 (area 7), Δ*ccc2* with Cu1–4 (area 8), Δ*ccc2* with Cu1–5 (area 9), Δ*ccc2* with Cu6 (area 10), Δ*ccc2* with D1027A (area 11), Δ*ccc2* with T1029A (area 12), Δ*ccc2* with H1069Q (area 13), and Δ*ccc2* with N1270S (area 14).

port also revealed that Ccc2p was required for the oxidase activity of Fet3p and the high affinity iron uptake [17]. The yeast Δ*ccc2* mutant lacking the *CCC2* gene is not able to grow in iron-deficient medium due to the reduced uptake of iron resulting from the disturbed incorporation of copper into Fet3p. However, the Δ*ccc2* mutant was rescued by expression of human ATP7B protein in a copper dependent manner (Fig. 3), indicating that ATP7B protein complements Ccc2p function. This notion is supported by recent reports that introduction of *C. elegans cua-1* cDNA, homologous to *ATP7A*/*ATP7B*, rescued the Δ*ccc2* mutant [20], and that expression of ATP7B protein in the Δ*ccc2* mutant restored copper incorporation into Fet3p [24].

To examine how the products of mutated *ATP7B* genes, reported in Wilson disease patients, manifest their function, the Δ*ccc2* strain was transformed with the H1069Q mutant, the N1270S mutant, and the Cu1–5 mutant lacking the sixth copper binding domain. H1069Q is the most common muta-

tion in the patients, representing approximately 38% of samples studied by Shah et al. [9]. This mutation, located in the SEHPL motif, may impair heavy metal translocation across membranes [13,14]. The partial restoration of Ccc2p function by mutant H1069Q in the Δ*ccc2* strain (Fig. 3) may in part explain the variable clinical manifestation in patients with this mutation [9]. By contrast, the N1270S mutation, located in the hinge domain and found predominantly in patients from Costa Rica [9], was not able to rescue the Δ*ccc2* mutant (Fig. 3). This result may correlate with a high incidence of the fulminant type of the disease in those samples [9]. Cu1–5 mutant lacking the sixth copper binding domain of ATP7B protein, mimicking the mutation with skipping exon 5 [8], also failed to restore the Ccc2p function (Fig. 3). However, this is probably because of inadequate expression of this mutant. Taken together, the variety of phenotypic manifestation of the disease mutation studied in this report may imply that epigenetic or environmental factors, other than genetic factor, influence the phenotypic outcome among Wilson disease patients. In addition, we introduced single amino acid substitutions in the DKTG motif, such as D1027A and T1029A, to produce missense mutations, since an aspartic acid residue in this motif is thought to be phosphorylated. Both mutants could not rescue the Δ*ccc2* mutant, suggesting the DKTG motif is essential for the manifestation of ATP7B function.

Copper transporting P-type ATPases identified in various species show differences in the number of metal binding motifs, which are six in ATP7B, five in Atp7b (the rat homologue) [25], three in Cua-1 of *C. elegans* [20], two in Ccc2p [17], and one in CopA of *E. hirae* [15]. Thus, to evaluate the necessity of six domains for copper binding, various deleted mutants within these domains were constructed and transfected into the Δ*ccc2* mutant. Consequently, while none of the mutants lacking the sixth copper binding domain could not rescue the Δ*ccc2*, the Cu6 mutant bearing only the sixth copper binding domain complemented Ccc2p function (Fig. 3). This result indicates that the sixth copper binding domain is necessary to manifest the function of ATP7B protein and that the copper binding domains are not functionally equivalent. A recent report has shown that the third copper binding domain in ATP7A is critical for copper transport [26]. This discordance with our current data is probably due to the differences in construction of mutated DNA. In this study we deleted the copper binding domains of ATP7B, while amino acid substitutions were introduced into the copper binding domains of ATP7A in the other report. This may lead to a conformational alteration of the proteins responsible for their function. The importance of the sixth copper domain in ATP7B is probably supported by the evidence that CopA has only one copper binding domain. However, questions on how the five other domains associate with the function of ATP7B with respect to copper transport still remain.

Acknowledgements: We thank Dr. George Iida for critical reading of the manuscript. This work was supported by a grant-in-aid for Scientific Research on Priority of 'Channel-Transporter Correlation' (to T.S.) and for Scientific Research (C, to K.T.) from the Ministry of Education, Science, Sports and Culture of Japan, by the Senri Life Science Foundation (to K.T.), and by CREST (to M.F.).

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