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**Biological effects of mutant ceruloplasmin on hepcidin-mediated
internalization of ferroportin**

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Abstract

Ceruloplasmin plays an essential role in cellular iron efflux by oxidizing ferrous iron exported from ferroportin. Ferroportin is posttranslationally regulated through internalization triggered by hepcidin binding. Aceruloplasminemia is an autosomal recessive disorder of iron homeostasis resulting from mutations in the ceruloplasmin gene. The present study investigated the biological effects of glycosylphosphatidylinositol (GPI)-linked ceruloplasmin on the hepcidin-mediated internalization of ferroportin. The prevention of hepcidin-mediated ferroportin internalization was observed in the glioma cells lines expressing endogenous ceruloplasmin as well as in the cells transfected with GPI-linked ceruloplasmin under low levels of hepcidin. A decrease in the extracellular ferrous iron by an iron chelator and incubation with purified ceruloplasmin in the culture medium prevented hepcidin-mediated ferroportin internalization, while the reconstitution of apo-ceruloplasmin was not able to prevent ferroportin internalization. The effect of ceruloplasmin on the ferroportin stability was impaired due to three distinct properties of the mutant ceruloplasmin: namely, a decreased ferroxidase activity, the mislocalization in the endoplasmic reticulum, and the failure of copper incorporation into apo-

ceruloplasmin. Patients with aceruloplasminemia exhibited low serum hepcidin levels and a decreased ferroportin protein expression in the liver. The *in vivo* findings supported the notion that under low levels of hepcidin, mutant ceruloplasmin cannot stabilize ferroportin because of a loss-of-function in the ferroxidase activity, which has been reported to play an important role in the stability of ferroportin. The properties of mutant ceruloplasmin regarding the regulation of ferroportin may therefore provide a therapeutic strategy for aceruloplasminemia patients.

Key words: aceruloplasminemia, ceruloplasmin, ferroportin, haemochromatosis, hepcidin,

1. Introduction

In the majority of physiological conditions, the extracellular transport of iron by ferroportin (Fpn) is regulated by the hepatic peptide hepcidin in response to inflammation and hepatic iron overload . Hepcidin binds to Fpn, which is present on cell surfaces, and thereby induces the phosphorylation of two tyrosine residues on an intracellular loop of Fpn. These phosphorylation events result in the internalization of the Fpn-hepcidin complex, ubiquitination of Fpn, and lysosomal degradation of both proteins . Fpn transports intracellular ferrous iron (Fe^{2+}) to transferrin via the oxidization of ferrous iron to ferric iron (Fe^{3+}) by the multicopper oxidase ceruloplasmin (Cp) . Serum Cp is primarily synthesized in hepatocytes as the secreted form of Cp (Cp-S). Bioactive Cp is a holo form of Cp (holo-Cp) and six atoms of copper are incorporated during biosynthesis. The failure to incorporate copper into Cp results in the secretion of an unstable apo form of Cp (apo-Cp), which is devoid of any oxidase activity and rapidly degrades in the serum. Cp is located on the surface of astrocytes in the brain, and the glycosylphosphatidylinositol (GPI)-linked Cp (Cp-GPI) plays a major role in the mobilization of iron in the central nervous system .

Aceruloplasminemia is a hereditary iron overload disease caused by loss-of-function mutations in the *Cp* gene, resulting in impaired iron efflux from the cells. Affected

patients present with hepatic iron overload, iron-refractory anemia, retinal degeneration, diabetes mellitus, and neurological symptoms due to parenchymal iron accumulation . Our previous studies have identified two biological properties of mutant Cp that resulted in misfolding and retention in the endoplasmic reticulum (ER) and interfered with copper incorporation in the late secretory pathway . The present study identified a third property of mutant Cp associated with decreased ferroxidase activity resulting in loss of Fpn stability under low levels of hepcidin, although the mutant Cp was synthesized and secreted with normal kinetics. The *in vitro* findings support the *in vivo* findings observed in patients with aceruloplasminemia: namely, a decreased Fpn protein expression in the liver under low serum hepcidin levels.

2. Methods and materials

2.1 Expression plasmids

Human GPI-linked Cp cDNA was cloned into the expression vector pcDNA3 (Invitrogen, Carlsbad, CA, USA). A plasmid encoding the full-length human *SLC40A1* Fpn gene (IMAGE CLONE ID 5213437) was purchased from Thermo Scientific Open Biosystems (Huntsville, AL, USA). The cDNA was then subcloned into PcDNA6.2/GW/D-TOPO (Invitrogen) to generate the V5-epitope fusion protein at the

C-terminus, and site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Agilent Technologies, La Jolla, CA, USA) according to the manufacturer's protocol to generate the Cp-GPI mutants and an Fpn mutant.

2.2 Cell Culture and Transfection

Hela cells, C6 cells and U251MG cells were provided by RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. The cells were maintained in basal media containing DMEM with 10% fetal bovine serum and were transiently transfected with LipofectAMINE2000 (Invitrogen) according to the manufacturer's instructions. Thereafter, the immunofluorescent and immunoblotting analyses were performed as described . In the cells incubated with and without human hepcidin-25 (Bachem, Bubendorf, Switzerland), the cells were treated for 4 h in serum-free medium, OPTI-MEM (Invitrogen) with and without apo-transferrin or holo-transferrin (AppliChem, Darmstadt, Germany) or were pretreated for 24 h in 250 μ M bathocuproinedisulfonic acid (BCS; Sigma-Aldrich, St.Louis, MO, USA), 100 μ M tetraethylenepentamine (TEPA; Sigma-Aldrich), 600 μ M bathophenanthroline disulfate (BPS), or purified human ceruloplasmin (Sigma-Aldrich).

2.3 Immunoblot and immunofluorescent analysis

The transfected cells were plated on coverslips, fixed with 4% paraformaldehyde, and quenched with 1 M ethanolamine. The cells were permeabilized in 0.1% Triton X-100 and analyzed with a goat anti-ceruloplasmin antibody (1:200, Bethyl Laboratories, Montgomery, TX, USA), a rabbit anti-V5 antibody (1:250, Kamiya biomedical company, Seattle, WA, USA), an anti-rabbit IgG Alexa488 secondary antibody, or an anti-goat IgG Alexa593 secondary antibody (1:1000; Invitrogen). For the Western blot analyses the cell lysates or liver homogenates were immunoblotted as previously described using a goat anti-ceruloplasmin antibody (1:1000, Bethyl Laboratories), a rabbit anti-V5 antibody (1:1000, Kamiya biomedical company), a rabbit anti-ferroportin antibody (1:750, Alpha Diagnostics, San Antonio, TX, USA), and the anti-rabbit IgG HRP and anti-goat IgG HRP antibodies (1:2000; Santa-Cruz Biotechnology, Santa Cruz, CA, USA). A quantification analysis of Fpn localized on the cell surface was performed by counting the cells expressing Fpn on the cell surface among the transfected cells in a 0.8 mm² area of a coverslip using the image J software program (<http://rsbweb.nih.gov/ij/index.html>). The analysis was independently performed three times by two blinded investigators.

2.4 Oxidase assay

The serum-free media from HeLa cells expressing wild-type or Cp-S mutants were concentrated and dialyzed, followed by an analysis of the oxidase activity. A *para*-phenylenediamine (*p*PD) oxidase staining of SDS gels was performed by incubation in 0.1 μ M pH 5.7 sodium acetate at 37 °C as previously described . The ferroxidase assay of the concentrated media was performed as previously described .

2.5 Patients

The clinical features and mutations in the Cp gene of patients with aceruloplasminemia in the present study have been previously reported . Fasting serum samples were obtained from control subjects or patients who had not received iron chelation therapy or a phlebotomy, and were stored in -80 °C until the assays were performed. The serum hepcidin-25 levels were measured using surface-enhanced laser deposition/ionization time of flight mass spectrometry-based Protein Chip System array technology (CIPHERGEN Biosystems, Palo Alto, CA, USA) . All patients gave their written informed consent for this study. The study protocol conforms to ethical guidelines of the 1975 Declaration of Helsinki as reflected by the approval by the Ethical Committee of The Hamamatsu University School of Medicine.

2.6 Real-time PCR analysis

The first-choice human total RNA survey kit (Applied Biosystems, Foster City, CA, USA) was used to investigate the RNA levels in human tissues. Total RNA from the liver biopsy samples of three control subjects and two patients with aceruloplasminemia who had not received iron chelation therapy or phlebotomy was extracted using the MELT Total Nucleic Acid Isolation System (Applied Biosystems). For the real-time RT-PCR analyses, 2 µg of RNA were reverse-transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems). Semiquantitative PCR was used to quantify the RNA levels of the secreted form of Cp, the GPI-linked form of CP RNA, *SLC40A1* encoding Fpn, and the *HAMP* gene encoding hepcidin. RNA expression was calculated using a control mRNA plasmid standard curve, and was normalized to the *GAPDH* or *B2M* housekeeping gene. TaqMan expression assays for the secreted form of Cp (Applied Biosystems; Hs00375596_m1), *HAMP* (Applied Biosystems; Hs00221783_m1), and *SLC40A1* (Hs00205888_m1) were used for the quantification of the transcripts. The primers for the analyses of RNA specific for GPI-kinked Cp with a TaqMan assay were: forward, 5'-AATGGAAACCACTTACACCGTTCTA-3' and reverse, 5'-GATCACATTCCATATTCTCCTGTGA -3'. The results are expressed as the

mean of three independent experiments.

3. Results

3.1 *Cp-GPI stabilized cell surface Fpn*

The cellular property of Cp-GPI associated with stability of Fpn on the cell surface was examined. HeLa cells lacking both endogenous Cp and Fpn synthesis were chosen for the present study and were used for the following biological experiments with Cp-GPI mutants. The cells transfected with Fpn with the V5 immunotag (Fpn-V5) were internalized, thus resulting in the formation of intracellular vesicles following the treatment with 0.15 and 0.5 μM hepcidin for 4 h (**Fig. 1A**). However, the internalization of Fpn-V5 after the treatment with 0.15 μM hepcidin was not observed in the cells transfected with Cp-GPI. Hepcidin binding to Fpn induces the phosphorylation of two Y302 and Y303 tyrosine residues of Fpn, therefore resulting in the internalization of Fpn. The cells transfected with Fpn-V5 bearing the two Y302F and Y303F mutations stably expressed Fpn on the cell surface, even after treatment with hepcidin, thus indicating that the Fpn internalization is caused by the interaction with hepcidin (**Fig. 1A**). Human U251MG glioma cells and rat C6 glioma cells expressed endogenous Cp-GPI. Treatment with 0.15 μM hepcidin in these glioma cells did not result in

internalized Fpn. Although the cells expressing either endogenous or exogenous Cp-GPI stabilized Fpn following treatment with 0.15 μ M hepcidin, the addition of 0.5 μ M hepcidin was sufficient to induce Fpn internalization in these cells (**Fig. 1A**). A quantification analysis revealed that treatment with 0.5 μ M hepcidin was sufficient to display an inverse correlation between the concentration of hepcidin and the number of the cells expressing cell surface Fpn (**Fig. 1B**). Following treatment with 0.15 μ M hepcidin, approximately 35% of the transfected cells exhibited cell surface Fpn, while the Hela cells transfected with Cp-GPI and the C6 and U251MG glioma cells prevented Fpn internalization (**Fig. 1B**). However, hepcidin concentrations of greater than 0.15 μ M induced Fpn internalization in the cells expressing Cp-GPI which occurred in a concentration-dependent manner. A Western blot analysis of Fpn confirmed these results (**Fig. 1C**). These findings showed that hepcidin and Cp-GPI acted as competitors affecting Fpn internalization, and that Cp-GPI had only a limited effect on Fpn stability under low hepcidin concentrations.

3.2 Ferroxidase activity of Cp is necessary for cell surface Fpn stability

An immunoblot analysis of cell lysates treated with copper chelators of BCS or TEPA revealed the presence of a 130 kDa band, representing the apo form of Cp-GPI, and an

80 kDa band, representing the holo-Cp, therefore suggesting that the copper chelators can reconstitute the apo form of Cp-GPI without ferroxidase activity (**Fig. 2A**) as described previously. Incubation of cells transfected with Fpn-V5 and Cp-GPI with the copper chelators induced Fpn internalization following hepcidin treatment (**Fig. 2A**). The addition of bioactive purified human Cp prevented hepcidin-mediated Fpn internalization (**Fig. 2B**). A quantification analysis revealed that incubation with 600 μM of the impermeable ferrous iron chelator BPS displayed a correlation between the concentration of BPS and the number of the cells expressing the cell surface Fpn under treatment with 0.15 μM hepcidin (**Fig. 2C**). Reducing the extracellular ferrous iron concentration led to the accumulation of Fpn on the cell surface. The addition of exogenous apo-transferrin or holo-transferrin in the cells transfected with Fpn-V5 did not prevent hepcidin-induced Fpn internalization, thus indicating that transferrin could not be substituted for Cp in stabilizing Fpn (**Fig. 2D**).

These results indicated that the ferroxidase activity of Cp promoted low extracellular ferrous iron conditions and stabilized Fpn to maintain efficient iron transport.

3.3 Cp-GPI mutants impaired cell surface Fpn stability

The biosynthesis of either wild-type Cp-GPI or seventeen Cp-GPI mutants, including three novel Y356H G873E and M966V mutants, was performed to examine the effects of these residues. The immunofluorescence analysis of transfected cells with or without permeabilization with Triton X-100 revealed that the mutants are either diffusely localized on the cell surface or reside in the perinuclear compartment (**Fig. 3A**). The mutants that trafficked to the perinuclear region colocalized with the ER resident protein disulfide isomerase (data not shown), indicating that the mutants are retained in the ER as previously described . The cells treated without Triton X-100 expressed Cp on the entire cell surface, therefore suggesting its cell surface localization. This finding showed that the Cp-GPI mutants, including Y356H, G631R, Q692K, R701W, G876A, M966V, and G969S, are localized on the cell surface, while the I9F, D58H, G176R, P177R, F198S, W264S, A331D, G606E, and G873E mutants are retained in the ER. Treatment with 200 μ M CuCl₂ in the culture media increased the expression of the holo form of Cp (holo-Cp) . An immunoblot analysis of cell lysates from M966V, G631R, G969S, and Q692K Cp-GPI cells revealed the mutants to be synthesized as apo-Cp, even though the transfected cells were incubated in the medium containing excess CuCl₂. This finding suggested that the mutants had impaired copper incorporation. Y356H, R701W, and G876A mutants were synthesized as the apo-Cp and holo-Cp (**Fig. 3B**). An examination

of the biosynthesis of the mutants by an immunofluorescence analysis and an immunoblot analysis revealed findings consistent with those observed in other cell culture systems, including Chinese hamster ovary cells or COS7 cells, which have no endogenous Cp synthesis (data not shown).

The M966V, G631R, G969S, and Q692K mutants had an impaired copper incorporation, the P177R and W858X mutants were retained in the ER as previously described, and the Y356H, R701W, and G876A mutants had identical synthesis and trafficking characteristics to wild-type Cp, but each failed to stabilize Fpn on cell surface (**Fig. 3C**). Although the Y356H, R701W, and G876A mutants could form the holo-Cp, the mutants were unable to prevent the Fpn internalization. The oxidase activity of the mutants was investigated because the stability of Fpn was associated with the ferroxidase activity of wild-type Cp. Although the Y356H, R701W, and G876A Cp-S mutants were present in a ratio of apo-to-holo Cp equivalent to that observed for wild-type Cp (**Fig. 3D**), both the *p*PD oxidase gel staining (**Fig. 3D**) and the ferroxidase assay (**Fig. 3E**) revealed decreased catalytic activity of these mutants. These findings suggested that the mutants which constitute a holo form of Cp on the cell surface cannot prevent Fpn internalization because of the inherently impaired oxidase activity.

3.4 Hepatic Fpn levels and serum hepcidin levels decrease in patients with aceruloplasminemia

The human tissue distribution of RNA expression of Cp-S and Cp-GPI was examined by a semiquantitative real-time PCR analysis. The human genome contains a single gene for Cp, but cells can synthesize either Cp-S or Cp-GPI. The Cp-GPI is generated by alternative RNA splicing which occurs downstream of exon 18 and replaces the C-terminal 5 amino acids of the secreted form, and incorporate an alternative 30 amino acids that signal the addition of the GPI anchor. The GPI-linked form of Cp is the major form in the brain, whereas the secreted form of Cp is predominant in the liver. RNA specific for Cp-S was mainly expressed in the liver, while Cp-GPI was expressed in all tissues including the liver, trachea, heart, and brain (**Fig. 4A**). The Fpn expression in patients with aceruloplasminemia was investigated in the liver, where both Cp-GPI and Cp-S were expressed. An immunoblot analysis for Fpn revealed low Fpn levels in the liver samples of two patients (**Fig. 4B**); however, the RNA levels of the *SLC40A1* gene encoding Fpn were increased in the livers of the patients (**Fig. 4C**). Patients exhibited decreased serum hepcidin levels (**Table 1**) and decreased RNA levels of the *HAMP* gene encoding hepcidin (**Fig. 4D**). These findings indicated that the hepatic expression of Fpn was suppressed in patients with

aceruloplasminemia, due to Fpn degradation rather than decreased Fpn synthesis.

4. Discussion

Cellular iron export requires the concentrated actions of a ferroxidase known to be the copper-containing protein Cp and a ferrous iron transporter Fpn, which is regulated by hepcidin. Previous studies showed that Cp-GPI was essential for the stability of cell surface Fpn in C6 rat glioma cells with endogenous Cp-GPI, and that cell surface Fpn is rapidly internalized and ubiquitinated for degradation by silencing of the endogenous Cp gene . However, transfected Fpn is localized on the cell surface in a variety of cultured cells without endogenous Cp, as observed in the current study . De Domenico et al. speculated that Cp-GPI expressing cells may have cellular properties recognizing the ferrous iron-bound form of cell surface Fpn by an E3 ligase specific to these cells . The current cell culture model using Hela cells without endogenous Cp-GPI and Fpn revealed that either Cp ferroxidase activity or extracellular chelation of ferrous iron is required for cell surface Fpn stability, as observed in a previous study using C6 cells . The stabilization of Fpn mediated by Cp was observed in the human glioma U251MG cells expressing endogenous Cp as well as in C6 cells, but this observation was restricted by the treatment under low hepcidin concentrations, namely as low as 0.15

μM . Incubation with hepcidin concentrations over $0.15 \mu\text{M}$ caused internalization of Fpn in HeLa cells transfected with Cp-GPI, U251MG cells and C6 cells. Therefore, the present study demonstrated that hepcidin and Cp-GPI acted as competitors affecting Fpn internalization. A dose-responsive counteracting effect of hepcidin on Fpn stability mediated by Cp revealed hepcidin to be a primary negative regulator of Fpn expression, while Cp was a limited positive regulator effecting Fpn stability under low hepcidin conditions.

The biological effects of Cp mutants on Fpn stability were investigated using the culture model. More than thirty aceruloplasminemia-causing mutations have been identified. This biosynthesis study of sixteen missense mutants revealed three distinct pathological mechanisms. As previously seen with the I9F, G176R, and P177R mutants, the D58H, F198S, W264S, A331D, G606E, and G873E mutants were retained in the ER. This mechanism presumably results from the misfolding that is common to many inherited diseases. We previously reported that the analysis of the amino acid sequence of Cp revealed a novel G(FLI)(LI)GP repeat motif, which is believed to affect folding during the early secretory pathway. The G873E, G176R, and P177R mutants affect the conserved repeated G(FLI)(LI)GP motif, which is consistent with this hypothesis, whereas the G876A mutant was not retained in the ER. The mutants located beside this

motif are speculated to have other molecular mechanisms in cellular trafficking of Cp. A second form of the M966V and Q692K mutants identified in this study was synthesized and was secreted with normal kinetics, but failed to incorporate copper during the late secretory pathway, resulting in the constitution of apo-Cp as has been previously reported for the G631R and G969S mutants . The G631R and G969S mutations are located in the nearby type I copper-binding His637 and His975 sites, respectively. Q692K and M966V mutations are also located near the type I copper-binding sites of M690 and His975, respectively. A site-directed mutagenesis analysis of the type I copper binding site indicated that these mutants failed to incorporate copper into the apo-Cp . These biochemical studies demonstrated that the type I copper binding site did not affect either protein folding for intracellular trafficking from the ER to the Golgi body or the subsequent protein secretion from the cell. The copper binding site may play an essential role in the protein structure for copper incorporation into the apo-Cp. The Y356H, R701W, and G876A mutants reconstituted both apo and holo proteins and were localized to the cell surface. However, these mutants failed to stabilize cell surface Fpn because the mutations impaired the ferroxidase activity which is required for Fpn stability. These findings therefore revealed the third biological property of the Cp mutant, which is distinct from the impairment of cellular trafficking in the ER and

copper incorporation in the late secretory pathway. These mutants may alter the iron binding site or the tri-nuclear copper cluster, which are essential for oxidase activity. It will be necessary to analyze the crystal structure of mutant Cp proteins, which will provide insight into the mechanism of ferroxidase activity.

The current biological analyses *in vitro* suggested that the Cp mutants impaired Fpn stability. However, Fpn and hepcidin expression *in vivo* has not yet been investigated in patients with aceruloplasminemia. The present study in patients with aceruloplasminemia revealed that serum hepcidin levels and *HAMP* RNA levels in the liver are lower than in control subjects. There may be an inverse correlation between serum hepcidin levels and serum ferritin levels, suggesting that an inappropriately low transcriptional level of hepcidin exists for the degree of systemic iron stores. The low hepcidin levels may contribute to the pathological mechanism of aceruloplasminemia, because low serum hepcidin levels induce increased iron absorption in the intestine where the Cp homolog hephaestin retains ferroxidase activity that is involved in basolateral intestinal iron transport. Chronic iron overload *in vivo* promotes the hemojuvelin (HJV)-dependent bone morphogenetic proteins (BMPs)/SMAD4 signaling, resulting in increased hepcidin gene transcription. The iron-dependent regulation of hepcidin in hepatocytes is regulated by competitive interactions with cell surface multi-

protein complexes including HJV, HFE, transferrin receptor 1 (TFR1), transferrin receptor 2 (TFR2), and diferric transferrin. In hereditary haemochromatosis, the levels of plasma and urinary hepcidin and hepatic *HAMP* expression are inappropriately low for the degree of systemic iron stores due to mutations in the genes encoding *HFE*, *HAMP*, *HJV*, and *TFR2*. Transferrin saturation in patients with aceruloplasminemia has been observed to decrease because Cp mutants fail to oxidize ferrous iron into ferric iron, therefore resulting in less incorporation of ferric iron into transferrin. Preliminary data regarding hepcidin levels in patients with aceruloplasminemia suggested that a decrease in diferric transferrin may impair interactions of the multi-protein complexes, resulting in decreased hepcidin synthesis. Future investigation of hepatic expression of the BMPs, SMAD4, HJV, HFE, TFR1, and TFR2 in aceruloplasminemia will elucidate the iron-dependent regulation of hepcidin expression.

The liver is the predominant source of serum Cp, and extrahepatic Cp expression has been shown in several tissues, including the retina and brain. The present study revealed that two forms of Cp-S and Cp-GPI were expressed in the liver. The hepatic expression of Fpn proteins and its transcription was analyzed to evaluate the stability of Fpn *in vivo*. In patients with aceruloplasminemia, the hepatic Fpn protein levels were decreased despite the presence of high *SLC40A1* RNA levels. Decreased Fpn protein

levels in the liver may be due to degradation due to the absence of Cp rather than decreased synthesis of Fpn at the transcriptional level. In patients with aceruloplasminemia, hepatic Fpn protein was expressed at low levels, even though the serum hepcidin levels were low. These *in vivo* findings were consistent with our finding in a cell culture study, which showed that under low hepcidin levels mutant Cp-GPIs that impaired the ferroxidase activity could not stabilize Fpn on cell surface, while wild-type Cp-GPI inhibited Fpn internalization by competing with hepcidin. Therefore, the degradation of Fpn observed in aceruloplasminemia patients may result from a loss of function in Fpn stability caused by mutations in Cp.

5. Conclusions

The results of the present study suggested that Cp-GPI mutants fail to stabilize the cell surface localization of Fpn *in vitro* and *in vivo*. The biosynthetic analysis of Cp mutants revealed a novel biological property of impaired ferroxidase activity, resulting in a loss of Fpn stability. This observation suggests that iron chelation therapy for aceruloplasminemia patients harboring these mutations may decrease the levels of extracellular ferrous iron, thereby preventing the degradation of cell surface Fpn.

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Figure legends

Figure 1

GPI-linked ceruloplasmin stabilizes cell surface ferroportin. (A) HeLa cells were transiently transfected with plasmids containing Fpn-V5, Fpn-V5 with the Y302F and Y303F mutations, and Cp-GPI and U251MG and C6 cells were also transfected with Fpn-V5. Sixteen hours post-transfection, the cells were incubated with 0.15 or 0.5 μ M human hepcidin-25 and were examined for the cellular localization of Fpn and Cp-GPI by an immunofluorescence analysis. (B) The cells transfected with Fpn-V5 were incubated with 0.15, 0.3 or 0.5 μ M human hepcidin-25 in serum-free media for 4 h, and the cells expressing Fpn localized on the cell surface were quantified by counting the transfected cells. The results represent the mean \pm the standard error from three independent experiments. The statistical data analyses were performed using the Mann-Whitney U-test. *P < 0.01 compared to the cell counts untreated with hepcidin. (C) The transfected cells were analyzed by a Western blot analysis using an anti-V5 antibody and an anti-actin antibody.

Figure 2

Ferroxidase activity of ceruloplasmin is necessary for cell surface ferroportin stability. (A) Cells were transfected with Fpn-V5 and pretreated in the presence or absence of copper chelators BCS (250 μM) or TEPA (100 μM) for 24 h. The cells expressing Fpn on the cell surface among the transfected cells were analyzed as described in Fig. 1B and were examined by a Western blot analysis using an anti-ceruloplasmin antibody. * $P < 0.01$ versus the cell counts untreated with hepcidin. (B) Human ceruloplasmin (1 μM) purified from serum was pretreated with cell culture medium for 24 h, and the cells were transfected with Fpn-V5, quantified and examined by an immunofluorescence analyses in the presence or absence of 0.15 μM hepcidin. The statistical data analyses were performed using the Kruskal-Wallis U test followed by the Mann-Whitney U-test with Bonferroni's correction for the post hoc test. * $P < 0.01$ versus the cell counts untreated with hepcidin or treated with ceruloplasmin. (C) The cells transfected with Fpn-V5 were incubated with 300 or 600 μM BPS in the presence or absence of 0.15 μM hepcidin. (D) The cells transfected with Fpn-V5 were incubated with 5 or 50 μM apo-transferrin and holo-transferrin in the presence or absence of 0.15 μM hepcidin. A quantification analysis was performed as described in Fig. 1B and cells were examined by an immunofluorescence analysis. * $P < 0.01$ versus

the cell counts untreated with hepcidin. Results represent the mean \pm the standard errors from three independent experiments.

Figure 3

Biosynthetic analysis of ceruloplasmin mutants. (A) The cells transfected with sixteen Cp-GPI mutants were examined by an immunofluorescence analysis using an anti-ceruloplasmin antibody with or without 0.1% Triton X-100 permeabilization. (B) The cells transfected with the Cp-GPI mutants incubated with 200 μ M CuCl₂ in the culture medium were examined by an immunoblot analysis. (C) The cells expressing Fpn localized on the cell surface were analyzed as described in Fig. 1B. *P < 0.01, **P < 0.05 versus the cell counts untreated with hepcidin. (D) Concentrated medium from the cells transfected with wild-type and mutant Cp-S constructs were analyzed by a Western blot analysis or were incubated with *para*-phenylenediamine (pPD) for a pPD oxidase assay. (E) The concentrated medium from the cells transfected with Cp-S wild type or mutants was analyzed with a ferroxidase assay. *P < 0.01 versus the wild-type medium. Results represent the mean \pm the standard errors from three independent experiments.

Figure 4

Hepatic expression of Fpn proteins and its transcription in the patients with aceruloplasminemia. (A) The RNA levels of the GPI-linked form and secreted form of ceruloplasmin in different human tissue specimens. A semiquantitative real-time PCR analysis was performed using human total RNA from several tissue specimens. Ceruloplasmin expression was calculated using a control mRNA plasmid standard curve, then was normalized to *GAPDH* expression. (B) An immunoblot analysis using ceruloplasmin (Cp), ferroportin (Fpn), and beta-2-microglobulin (B2M) antibodies was performed using liver biopsy samples obtained from three control subjects and two patients with aceruloplasminemia (patients 5 and 7 in **Table 1**). The RNA levels of (C) *SLC40A1* encoding ferroportin and (D) *HAMP* encoding hepcidin in the liver were examined by a semiquantitative real-time PCR analysis. *SLC40A1* and *HAMP* expression levels were calculated using a control mRNA plasmid standard curve, and were then normalized to *B2M* expression. The results represent the mean \pm the standard errors from three independent measurements.

Table 1.

Clinical data and the serum hepcidin levels in patients with aceruloplasminemia

Patient	Sex	Age	Genotype	TS	Fe	Ferritin	Hepcidin-25
1	M	60	W858X	8.0	22	1,936	6.9
2	F	58	W858X	9.8	25	1,610	8.8
3	F	55	c.2482delG	13.3	33	1,180	10.3
4	M	58	c.2602delG	10.7	27	1,775	7.4
5	M	60	c.607insA	6.5	18	2,465	4.8
6	F	70	G969S	13.2	33	980	15.0
7	F	66	c.1286insTACAC	10.6	29	1,875	4.5

Normal values for adults are as follows: hepcidin-25 (AU); 14.8-27.0, ferritin ($\mu\text{g/L}$); 39.4-320 for males, 3.6-114 for females, iron ($\mu\text{g/dL}$); 54-200 for males, 48-154 for females. TS indicates transferrin saturation (%); 25-47 for males, 18-40 for females.

Figure 1

A

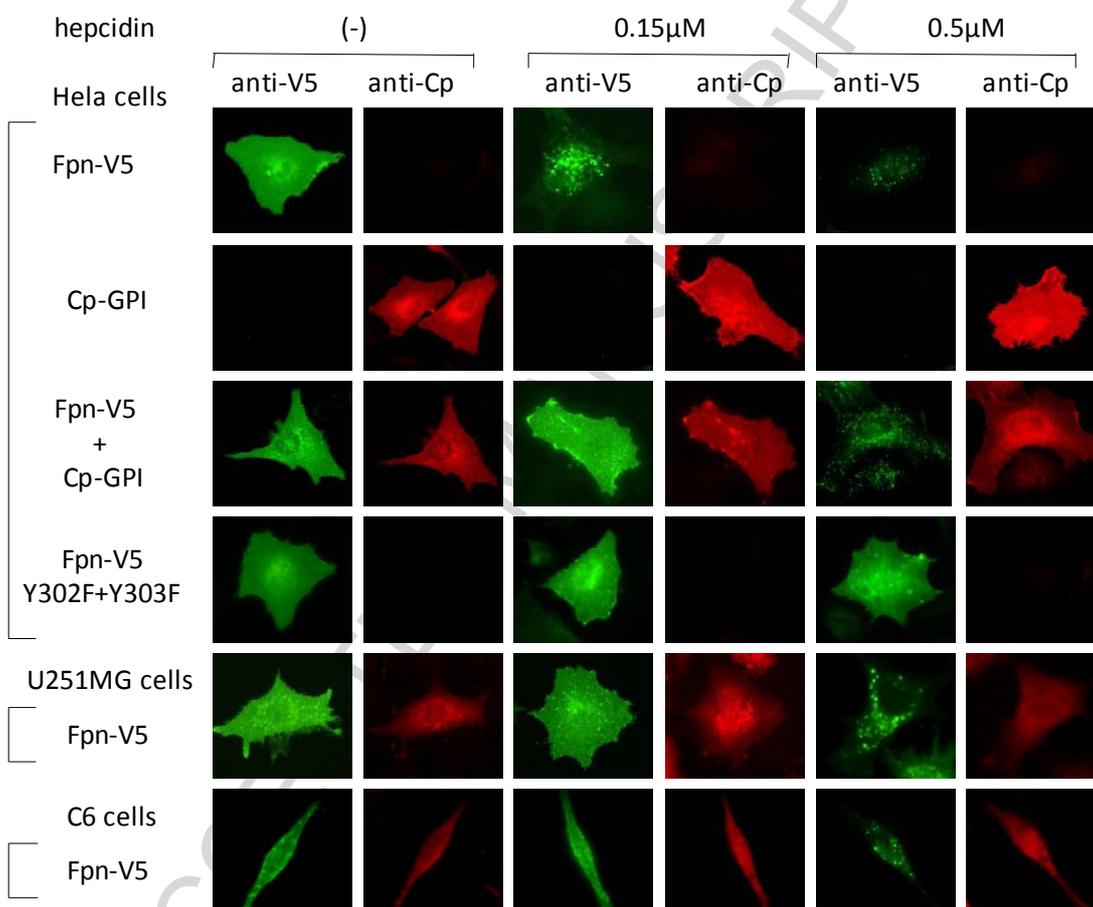
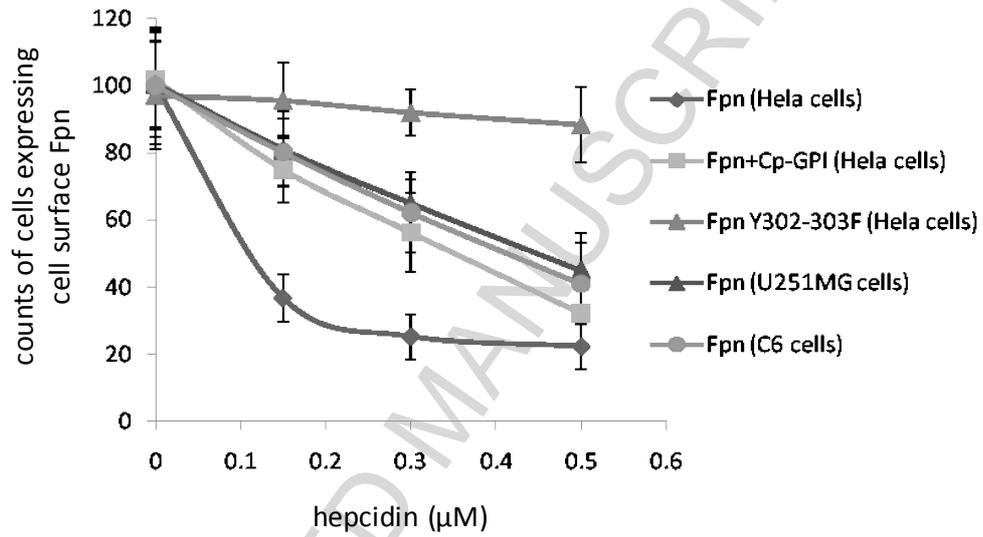


Figure 1

B



C

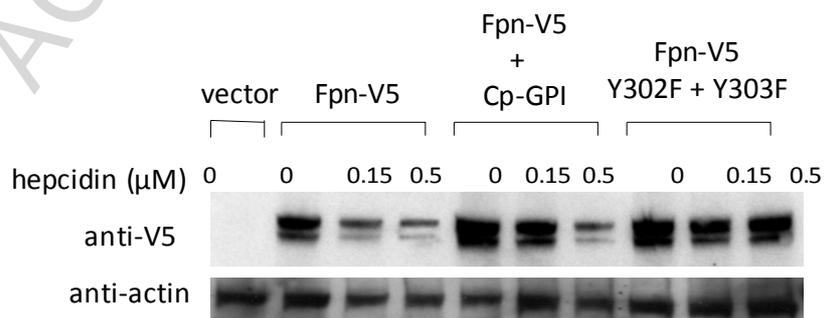
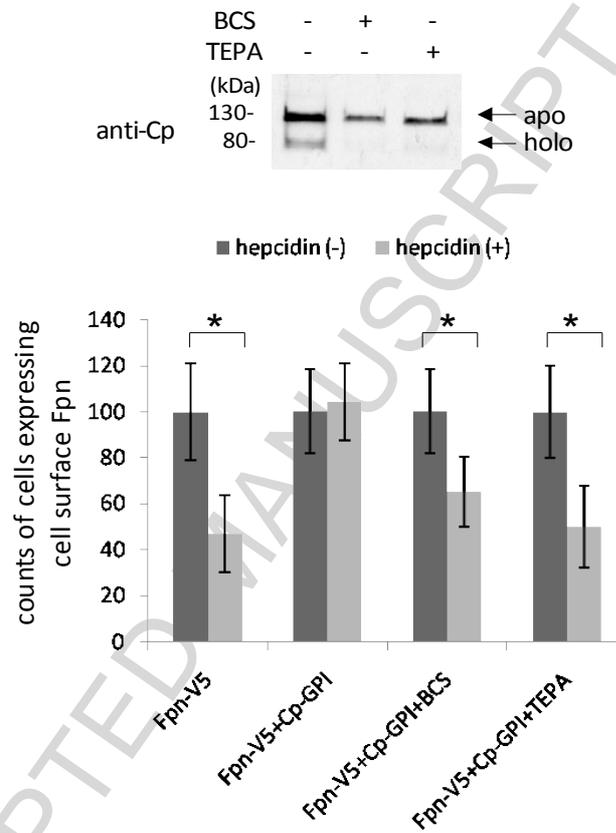


Figure 2

A



B

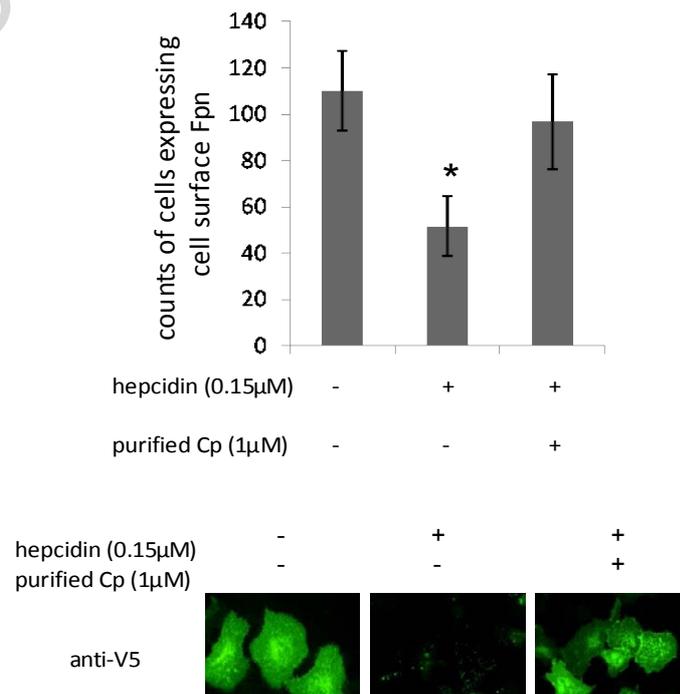
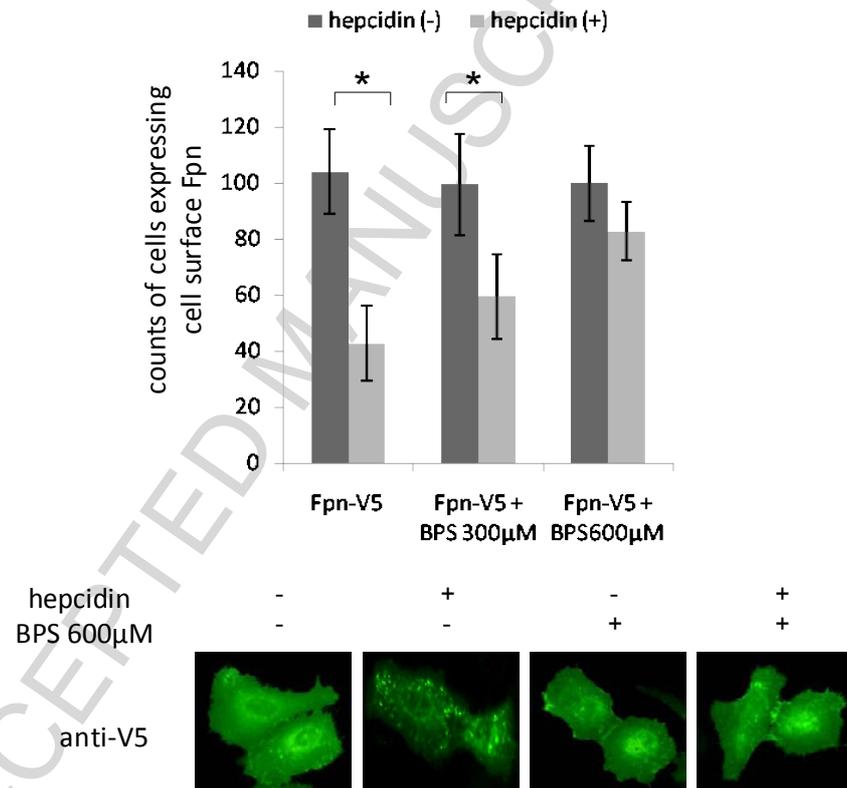


Figure 2

C



D

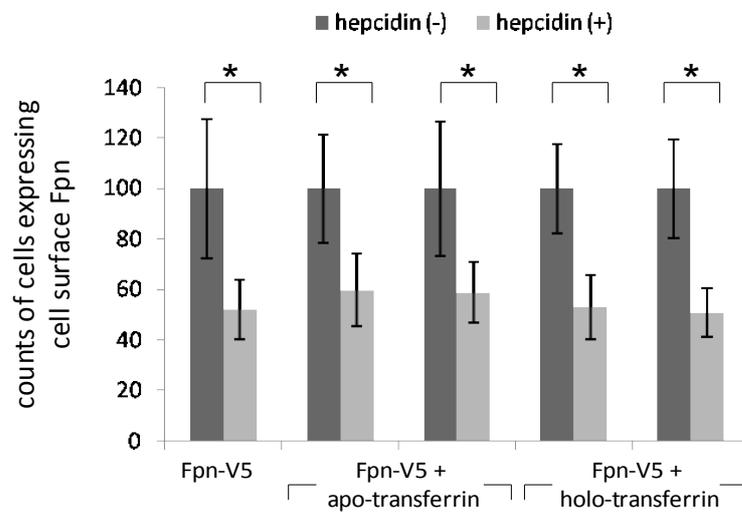
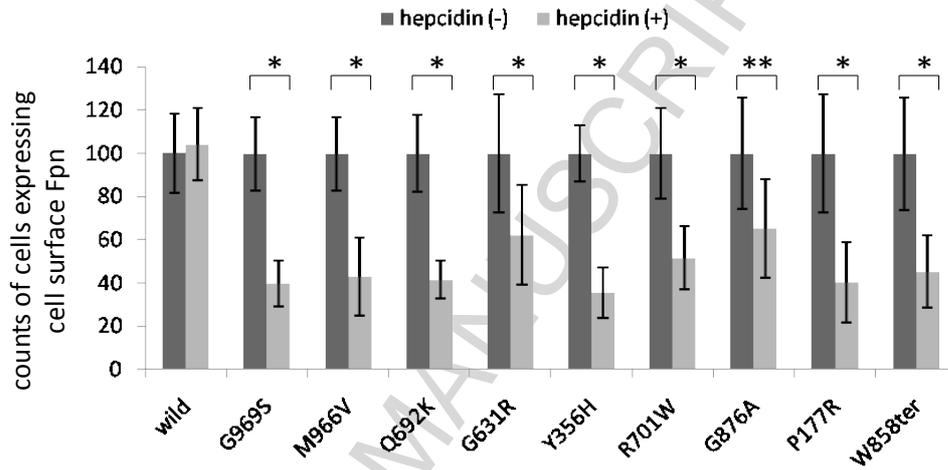
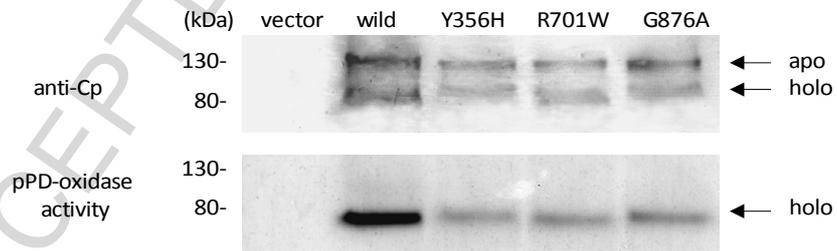


Figure 3

C



D



E

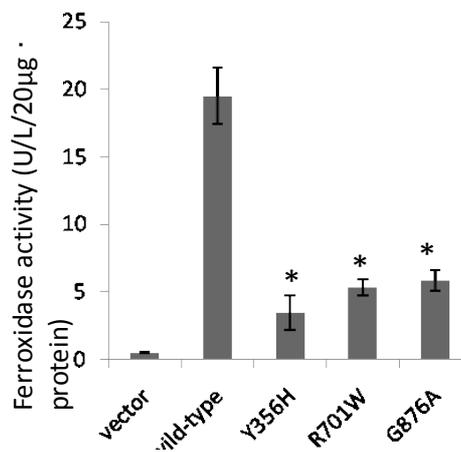


Figure 4

