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# Dominant Mutants of Ceruloplasmin Impair the Copper Loading Machinery in Aceruloplasminemia*[S]

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**The multicopper oxidase ceruloplasmin plays a key role in iron homeostasis, and its ferroxidase activity is required to stabilize cell surface ferroportin, the only known mammalian iron exporter. Missense mutations causing the rare autosomal neurodegenerative disease aceruloplasminemia were investigated by testing their ability to prevent ferroportin degradation in rat glioma C6 cells silenced for endogenous ceruloplasmin. Most of the mutants did not complement (*i.e.* did not stabilize ferroportin) because of the irreversible loss of copper binding ability. Mutant R701W, which was found in a heterozygous very young patient with severe neurological problems, was unable to complement *per se* but did so in the presence of copper-glutathione or when the yeast copper ATPase Ccc2p was co-expressed, indicating that the protein was structurally able to bind copper but that metal loading involving the mammalian copper ATPase ATP7B was impaired. Notably, R701W exerted a dominant negative effect on wild type, and it induced the subcellular relocalization of ATP7B. Our results constitute the first evidence of "functional silencing" of ATP7B as a novel molecular defect in aceruloplasminemia. The possibility to reverse the deleterious effects of some aceruloplasminemia mutations may disclose new possible therapeutic strategies.**

Aceruloplasminemia is a rare autosomal recessive iron overload disease caused by mutations in the gene of the ferroxidase ceruloplasmin (Cp)[3] (1, 2). Cp is a multicopper oxidase mainly secreted by hepatocytes, where the P-type ATPase ATP7B incorporates copper into apo-Cp (3). An alternatively spliced GPI-anchored isoform of Cp has been identified in brain astrocytes (4, 5) and in other cell types (6, 7).

Individuals heterozygous for the disease mutations have a partial Cp deficiency and usually display normal iron metabolism and no clinical symptoms, although a few cases of affected heterozygous patients have been reported. Homozygotes, however, show symptoms of iron overload disease; 37 published mutations (including frameshift, nonsense, and missense mutations) in 45 pedigrees have been described, as reported on line. Magnetic resonance imaging of the brain shows iron deposits in the basal ganglia, striatum, thalamus, and dentate nucleus; iron overload occurs also in the liver, pancreas, and retina despite low serum iron and elevated serum ferritin (8). The onset of clinical manifestations is usually in adulthood, and patients develop retinal degeneration, diabetes mellitus, and neurological symptoms, which include ataxia, involuntary movements, and dementia. The actual pathogenesis of aceruloplasminemia is still unclear, but iron-mediated oxidative stress is thought to contribute to tissue injury and neuronal cell death.

Recent data have demonstrated that the ferroxidase activity of Cp is required for the stability of cell surface ferroportin (Fpn), the only known mammalian iron exporter, and that Fpn is rapidly internalized and degraded in the absence of Cp (9). This finding provides a straightforward explanation for brain iron overload in patients with aceruloplasminemia, where the lack of a functional Cp-GPI would lead to defective export of iron from cells because of degradation of Fpn. Indeed, it has been shown that $Cp^{-/-}$ mice lack Fpn on the astrocyte cell surface (9). However, although it is clear that mutations leading to a truncated protein produce a nonfunctional Cp, the effect of missense mutations on the structure and function of the protein can be much less obvious.

Among the aceruloplasminemia missense mutants described up to now, only a few have been characterized (10–13). Fundamental contributions to the understanding of the molecular basis of this pathology revealed that the mutants were retained totally (P177R) or partially (I9F) in the early secretory pathway of cells, or they were secreted as an inactive apoprotein lacking copper (G631R and G969S). It can be inferred from the x-ray structure of Cp that also other substitutions are expected to perturb copper incorporation because the mutated residues are either copper ligands (His-978) or they are close to copper-binding sites (Ala-331 and Gln-692). The effect of the other mutations on the structure and activity of Cp appears to be less predictable. In particular, mutation R701W is very intriguing because it has been found in an atypical very young heterozygous patient with extremely severe neurologic symptoms

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[3] The abbreviations used are: Cp, ceruloplasmin; Cu(I)-GSH, complex between reduced copper and reduced glutathione; Fpn, ferroportin-1; GFP, green fluorescent protein; GPI, glycosylphosphatidylinositol; hCp, human ceruloplasmin; RT, reverse transcription; MOPS, 4-morpholinepropanesulfonic acid; WT, wild type; siRNA, small interfering RNA; endoH, endo-β-*N*-acetylglucosaminidase H; ER, endoplasmic reticulum.

despite the presence of the wild type allele (14). Arg-701 is located in a long surface exposed loop on the flat basal region of Cp, making it difficult to correlate the disease phenotype to a folding or ferroxidase activity defect of the protein.

Here we show that some mutants of Cp, including one present in an aceruloplasminemic heterozygous patient, have a potentially functional structure yet are not loaded with copper *in vivo*, likely because of defective interaction with the copper-transporting ATPase ATP7B.

## EXPERIMENTAL PROCEDURES

*Constructs*—The cDNA for human Cp-GPI was cloned by RT-PCR on total RNA from U373MG human glioma cells with the cMaster RT-PCR System (Eppendorf). The secreted form of Cp was generated by PCR by substituting the region coding for the last 30 residues of the GPI-anchored isoform with the five amino acids of secreted Cp. For expression in mammalian cells, the Cp-GPI or secreted Cp cDNA was cloned SacI-XhoI in the pCMVTag4b vector (Stratagene). All mutants were produced either with the QuikChange II XL mutagenesis kit (Stratagene) or by megaprimer PCR. The FLAG or Myc tag was introduced by PCR in the coding sequence of Cp to replace Arg-481. *Saccharomyces cerevisiae* Ccc2p coding sequence was obtained by PCR on genomic DNA and cloned EcoRI-XhoI in pCMVTag4b to generate a C-terminally FLAG-tagged protein. All constructs were verified by automated DNA sequencing at Biogen-ENEA (Italy). The pFpn-EGFP expression plasmid was a generous gift from J. Kaplan.

*Cells and Media*—Rat C6 glioma cells were purchased from the ATCC and maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Cambrex) and 40 $\mu$g/ml gentamicin (Sigma). Confluent monolayers were subcultured by conventional trypsinization, and cells were seeded in 35-mm tissue culture dishes for transfections.

*Small Interfering RNA (siRNA) and Transient Transfection*—siRNA oligonucleotide pool matching selected regions of rat Cp-GPI was obtained from Dharmacon (ON-TARGETplus SMART pool L-089853) and was specific for the rat protein, allowing expression of transfected human Cp isoforms (both GPI and secreted). Sense sequences of oligonucleotide pools were as follows: gaaaugguccagaucguauuu; gaaugaaguugacgugcauuu; gaaugaaucuugguacuuauu; and ggagaaaggaccuacuauauu.

C6 cells were transfected with siRNAs at a final concentration of 100 nM by using Oligofectamine (Invitrogen). Eighteen to 24 h after silencing, cells were transfected with pFpn-EGFP and pCMVhCp constructs using Lipofectamine enhanced by the Plus reagent (Invitrogen). Cells were grown for 18–24 h and then processed for immunofluorescence microscopy, Western blot analysis, or iron export measurement. Ferritin measurements were carried out as reported previously (9). Briefly, cells were incubated with 20 $\mu$M ferric ammonium citrate for 24 h. After a further 24 h in the absence of ferric ammonium citrate, cellular proteins were extracted with 150 mM NaCl, 10 mM EDTA, 10 mM Tris (pH 7.4), 1% Triton X-100, and a protease inhibitor mixture (Sigma), and 10–20 $\mu$g was used for ferritin measurement. Ferritin levels were determined by an enzyme-linked immunosorbent assay (Biotech Diagnostic, Laguna Niguel, CA) according to the manufacturer's instructions and were normalized for the total protein concentration in each sample.

*Immunofluorescence Microscopy*—Cell staining for immunofluorescence microscopy was performed as described previously (9), using the following primary antibodies: rabbit anti-Cp (1:100, Dako), mouse anti-FLAG M2 (1:100, Sigma), mouse anti-Myc 9E10 (1:100, Santa Cruz Biotechnology), mouse anti-TGN-38 (1:50, BD Biosciences), mouse anti-GM130 (1:50, BD Biosciences), rabbit anti-ATP7A/B (1:50, Santa Cruz Biotechnology), rabbit anti-FLAG (1:100, Sigma), and rabbit anti-Myc (1:100, Sigma), followed by treatment with either Alexafluor 594- or fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:750, Invitrogen), or Alexafluor 594-conjugated goat anti-mouse IgG (1:750, Invitrogen) as secondary antibodies. NBD-C6-ceramide complexed to bovine serum albumin (Molecular Probes) was used, following the manufacturer's instructions, to produce selective staining of the Golgi complex for visualization by fluorescence microscopy.

Cells were visualized using an inverted DMI 6000 confocal scanner microscope TCS SP5 (Leica Microsystems CMS GmbH) with a 63× oil immersion objective. Images were acquired using Leica application suite advanced fluorescence software. Each fluorochrome was scanned individually, and each image included 3–8 cells. The number of cells showing a certain pattern was counted in every image and expressed as a percentage of all cells in the image. Then the median percentage from different images was calculated.

*Western Blot, Immunoprecipitation, and RT-PCR*—The Cu(I)-glutathione complex was prepared as described previously (15). For Western blot analysis of secreted Cp, culture supernatants of transfected cells grown in serum-free medium were supplemented with protease inhibitors (phenylmethylsulfonyl fluoride 1 mM, leupeptin 2 $\mu$g/ml, pepstatin 2 $\mu$g/ml), concentrated with 30 units of Microcon (Millipore), and fractionated by SDS-PAGE under denaturing (samples heated in the presence of reducing agents) or nondenaturing (samples loaded as such) conditions. This technique was used to discriminate apo- and holo-Cp, as reported (16). For analysis of Cp-GPI, cells were solubilized in 25 mM MOPS buffer (pH 7.4), 150 mM NaCl, 1% Triton X-100, supplemented with protease inhibitors, by incubation on ice for 45 min. Protein content of cell extracts was determined by either the microBCA method (Pierce) or Bradford assay (Bio-Rad). Equal amounts of lysates were fractionated by SDS-PAGE, transferred to nitrocellulose, and probed with rabbit anti-Cp antibody (1:5000, Dako), mouse anti-FLAG M2 (1:2000, Sigma), mouse anti-Myc 9E10 (1:1000, Santa Cruz Biotechnology), and rabbit anti-ATP7A/B (1:500, Santa Cruz Biotechnology) antibodies. The appropriate peroxidase-conjugated secondary antibody (Sigma) was used at a 1:10,000 dilution. The blot was visualized with ECL Plus (GE Healthcare) or Blue POD (Roche Applied Science). For immunoprecipitation, cellular proteins were extracted with 20 mM Tris (pH 7.5), 100 mM NaCl, 0.5% Nonidet P-40, 0.5 mM EDTA, supplemented with a protease inhibitor mixture (Sigma). Cp was immunoprecipitated using rabbit anti-Cp antibody (1:250, Dako) and protein A/G-agarose (Santa Cruz Biotechnology) at 4 °C overnight; either WT or mutant Cp (FLAG) was immuno-

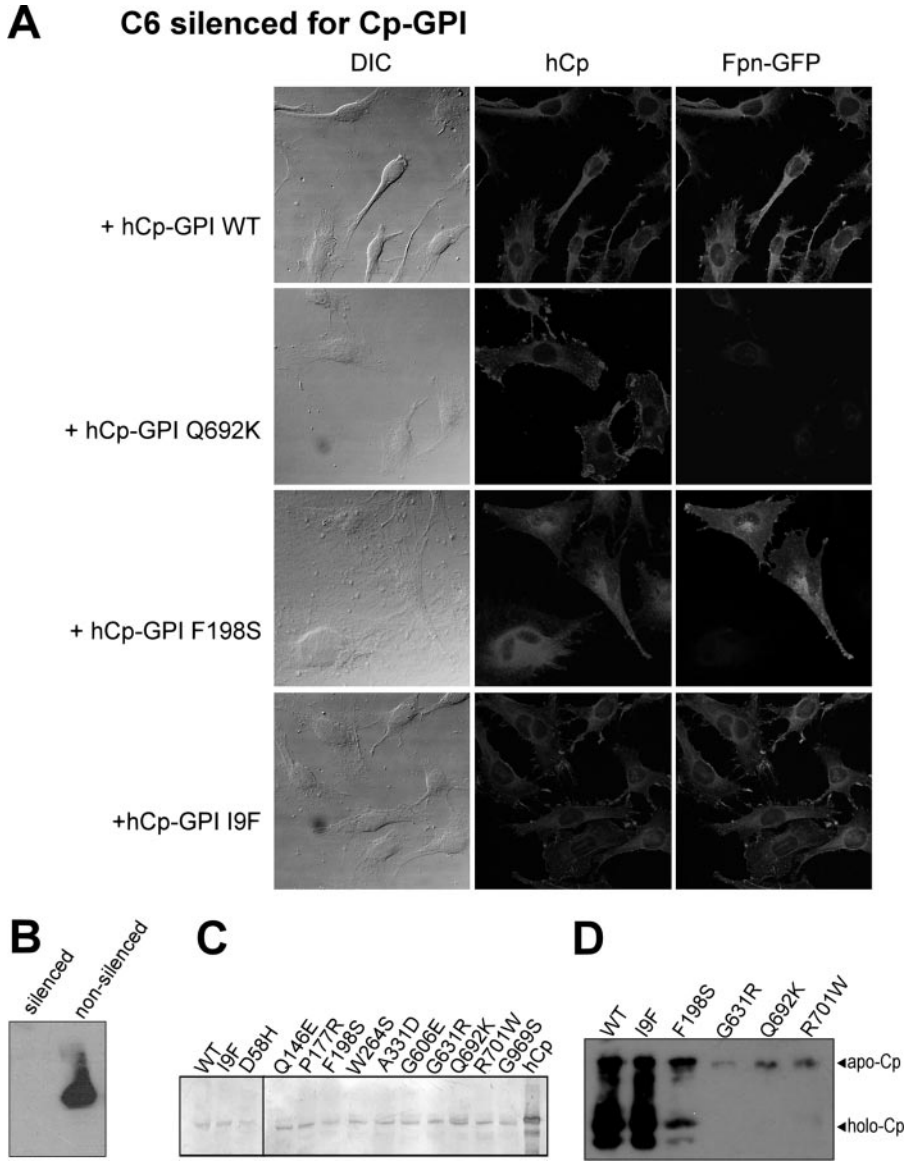


FIGURE 1. **Functional complementation by hCp-GPI missense mutants.** *A,* rat glioma C6 cells were silenced for Cp-GPI. After 24 h they were transfected with Fpn-GFP and human Cp-GPI WT, Q692K, F198S, or I9F and analyzed after a further 24 h by epifluorescence (*Fpn-GFP*) and immunostaining (*Cp*). *DIC*, differential interference contrast. *B,* Western blot analysis for Cp on silenced or nonsilenced cell extracts, and equal amounts of total protein (150 $\mu$g) were loaded per lane. Cells were silenced 48 h before preparing the cell extracts. *C,* cell extracts from C6 cells silenced for Cp-GPI and transfected with the indicated human Cp-GPI were examined for Cp by Western blot analysis, and equal amounts of total protein (30 $\mu$g) were loaded per lane. *D,* culture supernatants from C6 cells silenced for Cp-GPI and transfected with the indicated human secreted Cp were concentrated 10-fold and analyzed by nondenaturing SDS-PAGE and Western blot to evidence holo-hCp (85 kDa) and apo-hCp (130 kDa).

precipitated using anti-FLAG-agarose (Sigma) at room temperature for 4 h. Samples were eluted from beads with 2× SDS-PAGE sample buffer with dithiothreitol. Semiquantitative RT-PCR was carried out to assess mRNA levels for both endogenous Fpn and recombinant Fpn-GFP. Total RNA was extracted with TRIzol (Invitrogen) 24 h post-transfection and reverse-transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase and oligo$(dT)_{12-18}$ (Invitrogen). Transcripts were amplified using the following specific primers: mFPN_for, ttgtggtcctgatgaaaaag, and GFP_rev, atgaacttcagggtcagctt (for recombinant Fpn-GFP); and rFPN_for, aatgttgtcaataccgtcca, and rFPN_rev, accatgatgaaatgcagaag (for rat Fpn). The mRNA for $\beta$-actin was examined as the reference cellular transcript.

*Production of Recombinant Cp in Yeast*—Recombinant human secreted Cp was expressed in the yeast *Pichia pastoris*. WT and mutant secreted Cp cDNA was cloned SacI-XhoI in the integrative pIB2 vector (17) under control of the strong constitutive glyceraldehyde-3-phosphate dehydrogenase promoter and expressed in a *fet3*Δ *P. pastoris* strain, which lacks the endogenous ferroxidase Fet3p. Details of construction of the yeast strain and expression of the recombinant protein will be reported elsewhere.[4] WT and mutant Cp were partially purified from culture supernatants by chromatography on DEAE-Sephacel (GE Healthcare) with buffers containing 5 mM EDTA. Deglycosylation with endoH (PerkinElmer Life Sciences) was performed in denaturing conditions, according to the manufacturer's instructions.

## RESULTS

*Missense Aceruloplasminemia Mutants Can or Cannot Complement Silencing of Endogenous Ceruloplasmin*—Ferroxidase-competent Cp is required to maintain Fpn on the plasma membrane (9). Therefore, the ability of missense aceruloplasminemia mutants to prevent Fpn degradation represents a valid test of the functionality of the mutated protein. Because brain iron overload is a hallmark of aceruloplasminemia, we chose the rat C6 glioma cell line as our model system. Oligonucleotides selective for rat Cp were used to silence C6 cells for endogenous Cp-GPI, and cells were co-transfected with mouse Fpn-GFP and with human WT or mutant Cp-GPI (hCp-GPI). Then the presence of Fpn-GFP was assessed by epifluorescence microscopy 48 h after silencing. As already reported (9), hCp WT was able to restore Fpn on the membrane (Fig. 1*A*). On the other hand, the Cp missense mutants were found to fall into three categories as follows: fully functionally competent, partially competent, or functionally incompetent. Epi- and immunofluorescence fields obtained on selected mutants representative of the three categories are also

[4] M. C. Bonaccorsi di Patti, G. De Francesco, and G. Musci, manuscript in preparation.

shown in Fig. 1*A*. Mutants known to be enzymatically inactive (P177R, G631R, and G969S) and mutants D58H, Q692K, and R701W were unable to rescue Fpn-GFP; mutations F198S and A331D restored Fpn-GFP on 40–60% of cells, whereas mutations I9F, Q146E, W264S, G606E, and G876A fully complemented the silencing of endogenous Cp-GPI. Identical results were obtained by transfecting the corresponding soluble isoforms of hCp (data not shown). All hCp-GPI mutants except P177R, which was found in the ER, as expected (10), correctly localized to the plasma membrane, and Western blot analyses demonstrated that endogenous Cp-GPI was fully silenced under our conditions (Fig. 1*B*), its synthesis being mostly inhibited even 72 h after addition of the silencing oligonucleotides (data not shown). All recombinant hCp-GPI proteins were expressed at comparable levels (Fig. 1*C*). It is known that measurement of intracellular ferritin levels under proper conditions is an index of the iron export ability of cells, as ferritin accumulates in the cell when the iron export system is impaired (18). To this purpose, cells transfected with different hCp mutants were loaded with ferric ammonium citrate, and ferritin was measured 24 h after removal of iron from the medium. As expected, we found largely higher ferritin levels in cells transfected with mutants unable to rescue Fpn-GFP on the membrane (data not shown).

The apo/holo status of transfected hCp mutants was evaluated by nondenaturing SDS-PAGE, *i.e.* without heat treatment and in the absence of reducing agents prior to loading samples on the gel. Fig. 1*D* reports the results on the mutants depicted in Fig. 1*A*. It should be noted that the soluble isoform of the protein had to be employed in this experiment, because of instability of the GPI-linked isoform in these conditions. The results indicated that the ability of the mutants to rescue (totally or in part) Fpn-GFP was related to the presence of holo-hCp, which runs as a band at 85 kDa *versus* apo-hCp at 130 kDa (16), as exemplified by the lanes of I9F and F198S in Fig. 1*D*. On the other hand, only the apoprotein form was detected for functionally incompetent mutants Q692K, R701W (Fig. 1*D*), D58H, and G631R. The amount of secreted hCp turned out to be much higher in samples where most of the protein was holo. Because it is known that the rate of synthesis and secretion of wild type Cp is the same for the holo- and the apo-forms (11), these data suggest that secreted apo-hCp is unstable; however, the hypothesis that soluble mutants synthesized as apoprotein are poorly secreted compared with wild type cannot be ruled out.

*Substitution of Arginine 701 with Tryptophan Results in the Synthesis of an Apo-Cp*—The next step was to investigate whether mutants synthesized as apo-Cp lacked copper because of structural defects. To this purpose, *in situ* reconstitution of secreted hCp with the Cu(I)-GSH complex, which has been shown to remetallate apo-Cp *in vitro* (15), was attempted. Cells were silenced for endogenous Cp-GPI and transfected with the soluble form of hCp WT, D58H, Q692K, or R701W. Consistent with previous data (9), transfected soluble hCp WT was able to rescue Fpn-GFP. Cu(I)-GSH was ineffective on untransfected silenced cells and on cells transfected with hCp D58H or Q692K; the latter is shown in Fig. 2. On the other hand, addition of Cu(I)-GSH to the medium restored Fpn-GFP on about 30–40% of cells transfected with secreted hCp R701W (Fig. 2),

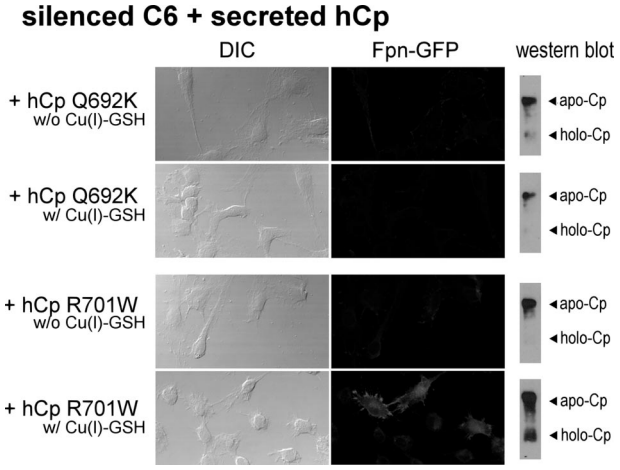


FIGURE 2. **The copper-glutathione complex reconstitutes a functional hCp R701W.** Cells were silenced for Cp-GPI, transfected with Fpn-GFP and secreted hCp R701W or Q692K, and then examined by epifluorescence after 24 h in the absence or presence of 50 $\mu$M Cu(I)-GSH complex in the medium. The corresponding Western blot analysis run after nondenaturing SDS-PAGE to distinguish apo- and holo-Cp is shown beside each fluorescence set. *DIC*, differential interference contrast.

suggesting that in this case the secreted protein lacks copper but retains the ability to bind it. Consistently, Western blot analysis confirmed that, at variance with Q692K, apo-hCp R701W was partly converted to holo-hCp R701W by Cu(I)-GSH (Fig. 2). The amount of secreted apo-Cp appeared to be higher in samples where Cu(I)-GSH was present, irrespective of the mutant used. This could be due to a general stabilizing effect of added copper or to a positive effect of the complex on Cp secretion. Human Cp D58H appeared to be partially reconstituted by Cu(I)-GSH, despite lack of Fpn-GFP recovery. Therefore, hCp R701W is unique in that it does not possess a structural defect, which makes it intrinsically unable to bind copper, and that an active enzyme can be produced under proper conditions.

Quite strikingly, hCp R701W was dominant over Cp WT. This was first assessed on nonsilenced cells, as shown in Fig. 3*A*. Clearly, Fpn was degraded when cells were transfected with hCp-GPI R701W, despite the presence of endogenous Cp (Fig. 3*A*). The same result was obtained when nonsilenced cells were transfected with the soluble form of hCp R701W (data not shown). The dominance of R701W mutation over the wild type protein was maintained when cells were silenced for endogenous Cp and transfected with hCp WT (both soluble and GPI-linked). Again, no Fpn was observed in the presence of hCp R701W irrespective of the simultaneous presence of the WT protein (Fig. 3*B*). R701W was the only natural mutation found to have this property. As a matter of fact, rescue of Fpn-GFP by hCp WT was not affected by co-expression of any other functionally incompetent mutant, as exemplified by the Q692K mutant in Fig. 3*B*.

The effect of mutation R701W was not on the stability of Fpn mRNA. Levels of Fpn mRNA (both endogenous Fpn and recombinant Fpn-GFP) were assessed by RT-PCR, and they were found to be comparable in untransfected cells and in cells transfected with hCp-GPI WT or hCp-GPI R701W (data not shown).

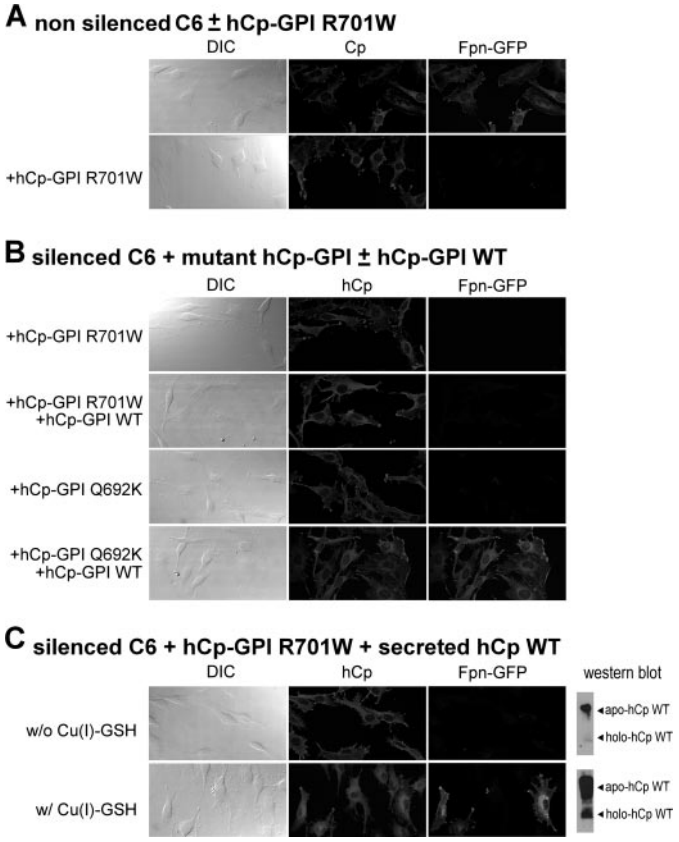


FIGURE 3. **Human Cp R701W is dominant over Cp WT.** *A,* C6 cells were co-transfected with Fpn-GFP and hCp-GPI R701W and then examined after 24 h by epifluorescence (*Fpn-GFP*) and immunostaining (*Cp*). *DIC*, differential interference contrast. *B,* C6 cells were silenced for Cp-GPI and co-transfected with Fpn-GFP and hCp-GPI R701W or Q692K. Dominance of the mutation was assessed by further co-transfection with hCp-GPI WT. Cells were examined after 24 h by epifluorescence (*Fpn-GFP*) and immunostaining (*Cp*). *C,* C6 cells were silenced for Cp-GPI and co-transfected with Fpn-GFP, hCp-GPI R701W, and secreted hCp WT. Cells were examined by epifluorescence (*Fpn-GFP*) and immunostaining (*Cp*) after 24 h in the absence or presence of 50 $\mu$M Cu(I)-GSH complex in the medium. The corresponding culture supernatants were concentrated and analyzed by nondenaturing SDS-PAGE and Western blot to distinguish apo- and holo-Cp. Quite similar results were obtained when secreted hCp R701W and hCp-GPI WT were employed.

The mechanism of dominance of Cp R701W on WT was then investigated. To this purpose, the effect of Cu(I)-GSH was first studied. Silenced cells were co-transfected with hCp-GPI R701W and secreted hCp WT, and the protein recovered in the medium was analyzed by Western blot after nondenaturing SDS. As shown in Fig. 3*C*, *upper panel*, the secreted hCp WT was mostly in the apo-form, which could be partially converted to holo-form in the presence of Cu(I)-GSH (*lower panel*). The fluorescence analysis confirmed that a significant fraction of cells recovered Fpn-GFP in the presence of Cu(I)-GSH. Altogether, these data indicate that the presence of the mutant R701W impairs copper loading of co-transfected hCp WT and that copper loading is partially restored by Cu(I)-GSH, with consequent recovery of Fpn-GFP. Moreover, the efficiency of reconstitution of Cp by the Cu(I)-GSH complex is similar for both Cp WT and R701W (*cf.* the Western blots in Figs. 2 and Fig. 3*C*), leading to a similar extent of recovery of Fpn-GFP. Consistently, the same effect of Cu(I)-GSH was observed when silenced cells were co-transfected with secreted hCp R701W and hCp-GPI WT. These findings are particularly relevant because R701W is found in a heterozygous atypical very young patient. Thus, this mutant was further investigated, to better elucidate the origin of the failure of copper loading leading to the dominant negative phenotype.

*The Presence of hCp-GPI R701W Does Not Affect Cellular Localization of hCp-GPI WT*—First, we analyzed the subcellular localization of WT and R701W hCp-GPI in co-transfected cells. Differently tagged derivatives of hCp-GPI were produced by inserting either a Myc or a FLAG epitope in a surface-exposed loop, replacing arginine 481. Neither epitope affected the functionality of the protein, as tagged hCp-GPI WT fully complemented the silencing of endogenous Cp-GPI (shown in supplemental Fig. S1 for the Myc derivative). Immunofluorescence analysis on cells co-transfected with hCp-GPI WT (Myc) and hCp-GPI R701W (FLAG) demonstrated that both proteins were simultaneously present at the plasma membrane (supplemental Fig. S1), indicating that the R701W mutant did not cause aberrant localization of the WT protein. Failure to co-immunoprecipitate tagged hCp-GPI WT and R701W suggested that the dominant negative phenotype of the R701W mutation could not be explained assuming Cp is dimeric (data not shown).

*Substitution of Arginine 701 with Tryptophan Impairs ATP7B-dependent Copper Loading Process*—When secreted hCp WT and R701W were expressed in the yeast *P. pastoris* and partially purified from culture medium, they both showed robust oxidase activity, as shown by nondenaturing SDS and staining with *o*-dianisidine (Fig. 4*A*, *left panel*). This indicates that both hCp WT and R701W can be obtained as holo-proteins by heterologous expression in yeast. The recombinant proteins were hyperglycosylated, as demonstrated by the electrophoretic shift observed after treatment with endoH (Fig. 4*A*, *right panel*). It should be noted that the *right panel* of Fig. 4*A* was run under denaturing conditions, and therefore the electrophoretic mobilities cannot match those observed in the *left panel*. Addition of the recombinant proteins produced in yeast, either WT or R701W, to the medium of C6 cells silenced for Cp-GPI completely rescued Fpn-GFP (Fig. 4*B*), unequivocally indicating that oxidase-active hCp R701W is functional and that the ferroxidase activity of Cp is sufficient to stabilize Fpn. Consistently, iron scavenging from Fpn by addition of either purified native hCp, purified yeast ferroxidase Fet3p, or the iron chelator BPS (9) to cells silenced for endogenous Cp-GPI and transfected with hCp-GPI R701W stabilized Fpn at the plasma membrane (supplemental Fig. S2), also confirming that hCp-GPI R701W on the plasma membrane does not *per se* impair Fpn or hCp WT.

Copper loading of hCp R701W can thus take place efficiently in yeast, but it is critically perturbed in mammalian cells with a dominant effect over co-expressed hCp WT. Copper delivery in the secretory pathway requires the copper-transporting ATPases ATP7A and ATP7B in mammalian cells (3) and their homologue Ccc2p in *S. cerevisiae* (19). The homologous transporter of *P. pastoris* has not been characterized so far. Studies on the biosynthesis of Cp demonstrate that the protein acquires copper from ATP7B in the secretory pathway in an "all-or-none" process (3, 20 and references therein).

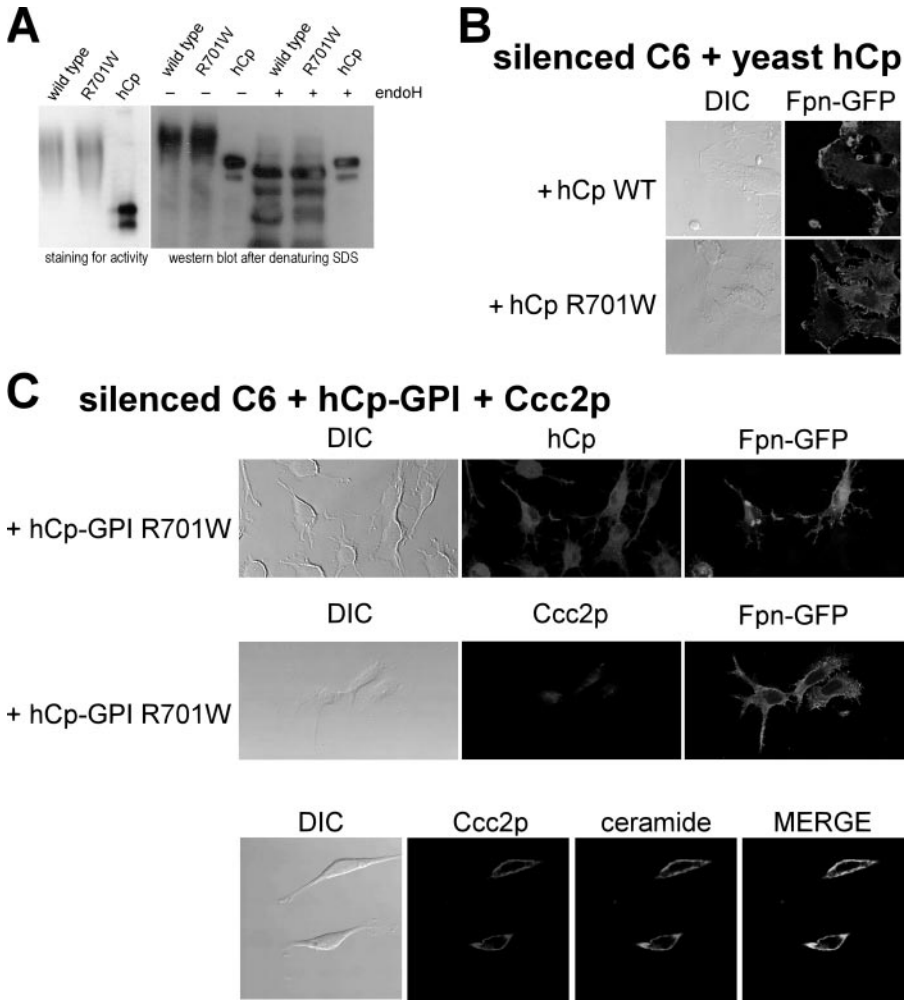


FIGURE 4. **Copper incorporation in hCp R701W is promoted by yeast Ccc2p.** *A,* secreted human Cp WT and R701W produced in yeast were partially purified by anion exchange chromatography on DEAE-Sephacel and analyzed by nondenaturing SDS-PAGE and staining for oxidase activity with *o*-dianisidine (*left panel*) or by Western blot in denaturing conditions before and after deglycosylation by endoH (*right panel*). *B,* cells were silenced for Cp-GPI, transfected with Fpn-GFP, and recombinant secreted human Cp WT or R701W (0.1 $\mu$M) produced in the yeast *P. pastoris* was added to the medium. After 24 h cells were examined by epifluorescence. *DIC,* differential interference contrast. *C, upper* and *middle panels,* cells silenced for Cp-GPI were transfected with Fpn-GFP, human Cp-GPI R701W, and *S. cerevisiae* Ccc2p-FLAG. After 24 h cells were examined by epifluorescence (*Fpn-GFP*) and immunostaining (*Cp, Ccc2p*). *Lower panel,* cells transfected with *S. cerevisiae* Ccc2p-FLAG were stained with NBD-$C_6$-ceramide and observed by epifluorescence (Golgi) and immunostaining (Ccc2p).

was unequivocally demonstrated by using NBD-C6-ceramide, a well established Golgi marker. As shown in the *bottom panel* of Fig. 4*C*, Ccc2p completely co-localized with ceramide. In this case, focusing was set across the nuclear plane, and the perinuclear localization of the ATPase is much more evident. Ccc2 was also found to co-localize with both the trans-Golgi TGN-38 and the cis-Golgi GM130 (data not shown).

The dominant negative effect of hCp R701W could then be due to abnormal interaction of the transporter with the ferroxidase, which could possibly make ATP7B unavailable even for hCp WT. However, attempts to co-immunoprecipitate hCp-GPI WT or R701W with ATP7B were unsuccessful. This could be due to inefficient capturing by the polyclonal antibody or to disruption of the interaction in the experimental conditions required. Therefore, the subcellular localization of ATP7B was directly assessed by immunofluorescence. For this experiment, hCp-GPI tagged with the FLAG epitope was employed to allow use of different conjugated secondary antibodies (*i.e.* anti-mouse *versus* the monoclonal anti-tag antibodies and anti-rabbit *versus* anti-ATP7A/B) for simultaneous visualization of both proteins. The analysis showed that ATP7B is expressed in C6 cells with a diffuse perinuclear staining and that the subcellular localization of the transporter is unaffected by transfection with hCp-GPI WT (Fig. 5). Silencing of endogenous Cp did not affect ATP7B localization as well (data not shown). On the other hand, ATP7B partially relocates to more peripheral vesicular compartments in cells expressing hCp-GPI R701W (Fig. 5). At variance with what was observed with hCp-GPI WT, merging of ATP7B and hCp-GPI R701W immunofluorescence was apparently significant, suggesting a stronger interaction with the transporter in the latter case. Changes are specific for ATP7B, because ATP7A did not change its distribution within the cell (data not shown).

Co-expression of *S. cerevisiae* Ccc2p in C6 cells transfected with hCp-GPI R701W revealed that Fpn-GFP was very reproducibly rescued in about 30% of the cells (Fig. 4*C*). Similar results were obtained on C6 cells silenced for ATP7B and co-transfected with *S. cerevisiae* Ccc2p and hCp-GPI WT or hCp-GPI R701W (data not shown). These findings indicate that the *S. cerevisiae* copper transporter can directly, although partially, complement the defect caused by hCp R701W on the mammalian copper-ATPase and that the efficiency of copper delivery is about equal for the WT and the mutant protein. The *middle panel* of Fig. 4*C* focalizes on cells where Fpn was rescued by overexpression of Ccc2p in the presence of hCp R701W. As expected, cells where Fpn was rescued were positive for Ccc2p, and the yeast ATPase displayed a diffuse perinuclear staining suggestive of localization in the Golgi compartment. It should be noted that the focal plane was in this case chosen to maximize the observation of Fpn-GFP. The localization of Ccc2p

Higher magnification images of the subcellular localization of ATP7B are shown in Fig. 5*B*. Here, cells silenced for endogenous Cp and transfected with either hCp-GPI WT or R701W were stained for both the trans-Golgi network marker TGN-38 and ATP7B. The merged fields show that the ATPase invariably co-localizes with the trans-Golgi network marker, and that the presence of the mutant protein apparently induces a significant

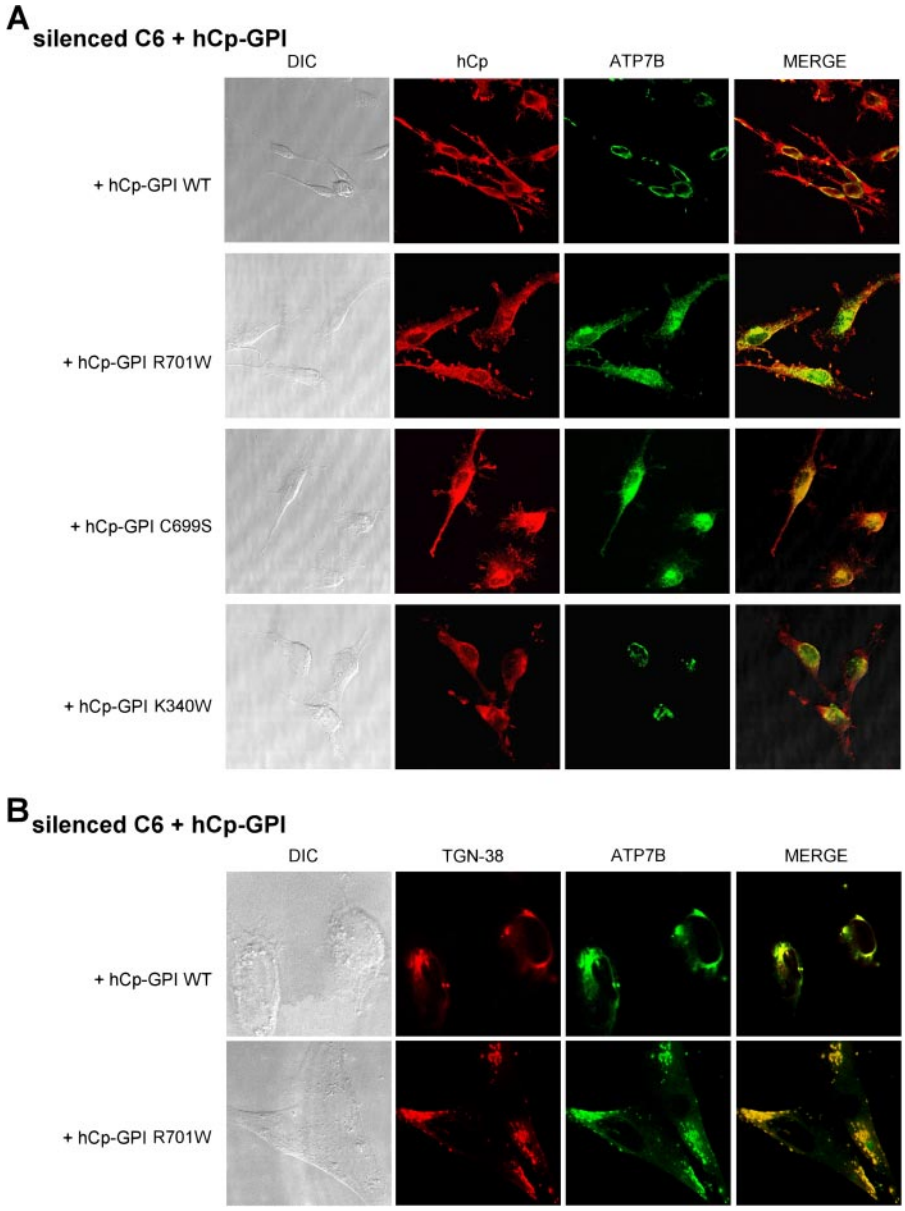


FIGURE 5. **Human Cp-GPI R701W and C699S induce relocalization of ATP7B.** *A,* cells silenced for Cp-GPI were transfected with FLAG-tagged human Cp-GPI WT or mutant, as indicated, and immunostained for the FLAG tag and for ATP7B. *B,* higher magnification images of cells silenced for Cp-GPI, transfected with human Cp-GPI WT or R701W, and immunostained for TGN-38 and ATP7B. *DIC,* differential interference contrast.

fragmentation of the Golgi apparatus and the formation of vesicles originating from the trans-Golgi network itself (Fig. 5*B*).

All the other aceruloplasminemia Cp mutants were not dominant when co-expressed with Cp WT, and they did not induce a change in localization of ATP7B, irrespective of their ability to bind copper (data not shown).

*Possible Role of Solvent-exposed Loops in Aceruloplasminemia*—To gain further insight on the origin of the defect carried by hCp R701W, the structure of the protein was analyzed. Cp is a multidomain protein made up of six plastocyanin-like domains with ternary pseudosymmetry (Fig. 6). Domains 1 and 2, 3 and 4, and 5 and 6 interact with each other through extensive, highly packed hydrophobic interfaces, whereas polar interactions and loosely packed interfaces are present between domains 2 and 3 and 4 and 5, with the interface between domains 6 and 1 hosting the catalytically essential trinuclear copper cluster. Residue Arg-701 is located in a large solvent-exposed loop connecting domains 4 and 5 and comprising residues 700–708; a corresponding loop which connects domains 2 and 3 is found at positions 339–350. Domains held together by hydrophobic interactions are also connected by surface loops. Despite a low degree of sequence homology, all these loops start with a C*X*(R/K) motif, with the cysteine residue stabilizing the loop by forming a disulfide bridge. The position of the five loops and the identity of the basic residue within the C*X*(R/K) motif are also shown in Fig. 6.

The role of the 700–708 loop was investigated by extensive mutagenesis. Residue Arg-701 was either deleted or substituted with glutamine (R701Q), glutamic acid (R701E), or phenylalanine (R701F) to test whether it is specifically the bulky hydrophobic tryptophan side chain that gives rise to a nonfunctional Cp or any substitution at position 701 is deleterious. Mutants R701Δ, R701Q, R701E, and R701F were unable to rescue Fpn-GFP, yet they were not dominant and did not result in relocalization of ATP7B. Representative images for the R701Q mutant are shown in supplemental Fig. S3. Mutants Q702M, S703A, E704M, and D705M were functional and rescued Fpn-GFP in Cp-GPI silenced cells, whereas substitutions that were definitely nonconservative and structurally more challenging (R700W, Q702W, E704W, and D705P) yielded proteins unable to complement the loss of endogenous Cp; all proteins were localized at the plasma membrane, and the inactive mutants were not dominant. Disruption of the disulfide bridge in hCp C699S, on the other hand, produced a nonfunctional protein that was dominant over Cp WT and quite interestingly caused relocalization of ATP7B similarly to that observed with hCp R701W (Fig. 5).

The possible role of other loops in copper incorporation into hCp was tested by mutating Lys-340 and Arg-883, the basic residues corresponding to Arg-701 and located on the loops connecting domains 2 and 3 and 5 and 6. Human Cp mutants K340M, K340W, R883M, and R883W were nonfunctional but not dominant, and they did not alter the localization of ATP7B, as shown in Fig. 5 for hCp-GPI K340W. As with R701W, both

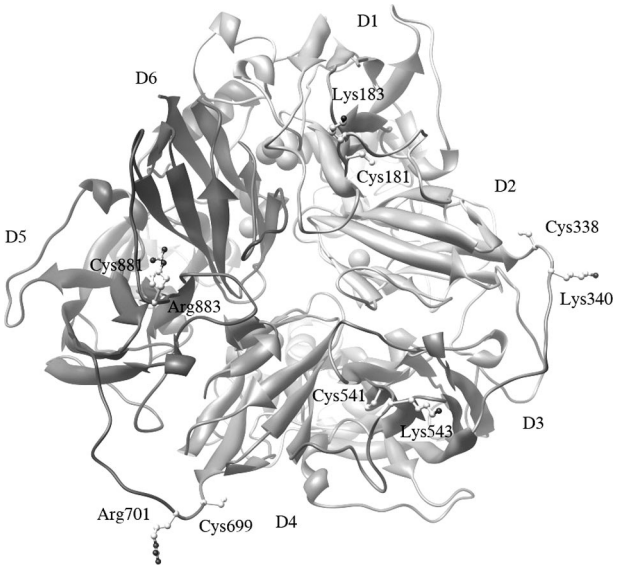


FIGURE 6. **Schematic representation of the three-dimensional structure of Cp.** For clarity, only the residues making up the Cys-*X*-(Arg/Lys) motifs located on the loops connecting Cp domains (Cys-181 and Lys-183, Cys-338 and Lys-340, Cys-541 and Lys-543, Cys-699 and Arg-701, Cys-881 and Arg-883) are shown in *ball-and-stick* representation. Copper ions are indicated by *spheres*. Domains 1 and 2, 3 and 4, 5 and 6 are light grey, grey, and dark grey respectively and indicated by the labels *D1–D6*. The figure was produced with Chimera (40).

soluble K340W and R883W were enzymatically active when produced in *P. pastoris* (data not shown). Not surprisingly then, addition of Cu(I)-GSH to the culture medium of C6 cells silenced for Cp-GPI and transfected with secreted hCp C699S, K340W or R883W rescued Fpn-GFP in about 30–40% of the cells, as observed with the hCp R701W mutant (*cf.* Fig. 2).

## DISCUSSION

Aceruloplasminemia is generally associated with complete absence of serum Cp because of homozygous mutations of the Cp gene, although some patients are heterozygous for the causative mutation. The few disease-associated mutants that have been characterized up to now invariably lack ferroxidase activity and fall into one of two categories as follows: truncated mutants are generally retained in the ER and cause ER-associated stress and activation of the "unfolded protein stress response" (21, 22); missense mutants display a folding defect that leads to retention in the ER (I9F and P177R) or they are secreted as apoproteins (G631R and G969S). Residues Gly-631 and Gly-969 are close to type 1 copper sites in domains 4 and 6, and their substitution introduces a structural defect that makes the protein unable to bind copper, as also demonstrated by the finding that it cannot be reconstituted by copper *in vitro* (11, 12). Apo-Cp is unstable, and it is degraded in serum with a half-life of hours compared with days for the holo-protein (23), explaining the failure to detect Cp in the serum of patients.

The simplest model links the iron overload observed in the brain of aceruloplasminemia patients to lack of a ferroxidase-active Cp-GPI on the plasma membrane of astrocytes. In line with this model, inactive mutants P177R, G631R, and G969S are unable to prevent the internalization and degradation of Fpn in C6 cells silenced for Cp-GPI. Also mutant Q692K is nonfunctional. This is not surprising because Gln-692 is located close to Met-690, a type 1 copper ligand in domain 4, in a highly conserved region sandwiched between Lys-691 and Lys-693 and oriented toward the protein interior. Thus, all these mutations affect the ability of Cp to bind copper.

A novel mechanism causing aceruloplasminemia is defined by mutations on Cp that do not abrogate the ability of the protein to bind copper but impair the copper loading process requiring the copper-ATPase ATP7B. Mutants D58H and R701W are secreted as apoproteins, but at variance with all other missense aceruloplasminemia mutants, they can be reconstituted by the Cu(I)-GSH complex. This complex seems to exert a general stabilizing effect on secreted Cp, as higher quantities of the protein are present in the medium of cells transfected with any mutant and treated with Cu(I)-GSH. The alternative hypothesis that Cu(I)-GSH enhances secretion of hCp cannot be ruled out at this stage; however, it should be reminded that Cp can bind extra copper ions besides the canonical six metal atoms, and that these extra ions could help to slow down protein degradation.

Our findings can be taken as an indication that the intracellular copper loading process is disturbed by some Cp mutants. Data in support of this hypothesis include the functional complementation afforded by externally added holo-Cp WT or R701W produced in yeast and the recovery of Fpn-GFP in the presence of Cp R701W if the missense Cp mutant is co-expressed with the yeast copper transporter Ccc2p. Misfunctioning of the copper loading process is accompanied by a gross rearrangement of the Golgi apparatus, which appears fragmented in the presence of Cp R701W.

Expression in yeast can offer information on the intrinsic capacity of the Cp mutants to bind copper. As a matter of fact, all mutants that were inactive in the functional complementation assay were also enzymatically inactive when produced in yeast. Only Cp R701W, C699S, K340W, and R883W were active in yeast and inactive in mammalian cells. Our data indicate that this is because of impairment of the copper-loading process involving the mammalian copper-transporting ATPase ATP7B. The mechanism of coupling between copper transport and copper incorporation into target proteins is not understood, although it does not seem to involve additional proteins (24). Precise fit of the transporter and acceptor protein is probably not essential for copper delivery, because the mammalian copper-ATPases can load the yeast multicopper oxidase Fet3p (25, 26), and vice versa the yeast transporter Ccc2p can deliver copper to human Cp, although efficiency is lower in both cases. In this respect, it is both intriguing and puzzling that the efficiency of rescue of Fpn is partial with Cu(I)-GSH or Ccc2p (*i.e.* only some cells show the presence of Fpn-GFP, although all cells are exposed to hCp reconstituted by Cu(I)-GSH), as if the amount of holo-Cp produced must reach a cell-specific threshold to produce functional complementation.

In the six-domain structure of Cp, K340, R701 and R883 are located in long surface loops which connect domains 2 and 3, 4 and 5 and 5 and 6. Substitution of these basic residues in the three loops of Cp leads to defective incorporation of copper in mammalian cells. Because R701, K340, and R883 are located in a flat region at the bottom of the Cp structure, it is tempting to speculate that copper delivery takes place through an interac-

tion with ATP7B mediated by this region. Mutation R701W, however, is peculiar in that it exerts a dominant negative effect that appears to go beyond the simple impairment of the ATPase.

The loop containing Arg-701 seems to have a critical role, as also confirmed by the fact that the effect of structurally challenging substitutions leading to an inactive protein could be relieved by co-expression with Ccc2p (as is the case for Cp E704W; data not shown). Disruption of the disulfide bridge Cys-618 to Cys-699 preceding Arg-701 by replacement of Cys-699 with serine also generates a dominant Cp mutant, highlighting the importance of this loop.

Both Cp R701W and C699S appear to cause a change in localization of the ATPase and "functional silencing" of ATP7B. We use this term not to imply that ATP7B is necessarily dysfunctional, but simply to indicate that the ATPase is not ready for copper delivery to Cp. Indeed, in the presence of either Cp C699S or Cp R701W a significant dispersal of the Golgi apparatus takes place. In this respect, relocalization of ATP7B could be part of a general phenomenon involving most of the secretory compartment. The observed redistribution of TGN-38 implies that the mutant Cp R701W has much more global effects in the cell than just the mislocalization of ATP7B and thus is doing more than impairing the copper loading machinery. However, as far as Fpn rescue is concerned, the ability of the yeast pump Ccc2p to load copper into Cp R701W indicates that, despite the general effect on the Golgi apparatus, Cp R701W retains the ability to acquire copper under proper conditions and, in turn, to prevent degradation of Fpn that would occur in the absence of the oxidase.

It should be noted that fragmentation of the Golgi apparatus appears to be a general hallmark of neurodegenerative disease. Earlier examinations of human brain tissues and animal models have shown that fragmentation of the Golgi apparatus is found in Alzheimer disease, amyotrophic lateral sclerosis, Creutzfeldt-Jacob disease, multiple system atrophy, Parkinson disease, spinocerebellar ataxia type 2, and Niemann-Pick disease type C (Ref. 27 and references therein). This has led to the hypothesis that the morphological status of the Golgi apparatus may be a reliable index of activity of degeneration (28). In this context, it is not surprising that the mutation R701W, which causes severe neurological symptoms, can induce fragmentation of the Golgi apparatus. From a molecular point of view, it is difficult at this stage to explain how a single amino acid substitution on Cp might lead to the dramatic effect observed on the Golgi apparatus. This observation, however, is not unprecedented. Some mutations of superoxide dismutase that are involved in the onset of familial amyotrophic lateral sclerosis are associated with fragmentation of the Golgi apparatus in spinal cord motor neurons (29, 30). Although the molecular mechanism still awaits to be unraveled, it is clear that interactions between mutant proteins and any of one or more proteins involved in the maintenance of the structure of the Golgi apparatus might interfere with its structure and function (31).

It is well known that ATP7B changes its subcellular distribution depending on copper availability (3); however, copper does not induce fragmentation of the Golgi apparatus. Moreover, the fact that Cp mutants that do not bind copper (such as G631R or Q692K) do not induce relocalization of ATP7B seems to exclude that an increase in the local copper concentration is responsible for this phenomenon. Lack of ATP7B cannot be fully compensated by ATP7A in many tissues that express both proteins (3). This appears to be true also for glioma cells, suggesting that Cp-GPI expressed in astrocytes is strongly dependent on ATP7B for acquisition of copper.

We believe that failure to incorporate copper in WT Cp together with aberrant localization of ATP7B and significant fragmentation of the Golgi apparatus are critical determinants of the severity of the phenotype observed in the heterozygous patient carrying the Cp R701W mutation. It is worth noting that R701W is the only mutation known so far that leads to severe neurological symptoms at a young age, despite the heterozygous genotype with the presence of the WT allele (14). It should be added that the father of this patient is also heterozygous for the R701W mutation, yet he is asymptomatic (14). This is consistent with our observation that the R701W mutation can be overcome under proper conditions (*e.g.* through increased extracellular copper bioavailability), and suggests that the severity of some forms of aceruloplasminemia is probably modulated by modifier genes and/or metabolic status. In addition, the molecular analysis of ATP7B could provide additional information, *e.g.* specific isoforms of the ATPase might be responsible for the different phenotypes.

Asp-58 is a solvent accessible residue located on the protein surface, and replacement of this residue with histidine changes the charge and polarity of this region. It has been suggested that this may cause aberrant incorporation of copper or trafficking of Cp (32). Our results indicate that aberrant incorporation of copper because of impairment of the interaction with ATP7B may be the best explanation; reconstitution with Cu(I)-GSH would lead to a protein that contains copper but is inactive.

A final remark must be made on those mutants that appear to be partially (F198S and A331D) or fully (I9F, Q146E, W264S, G606E, and G876A) functional in our system. This finding implies that the protein retains (at least partially) ferroxidase activity. It is worth noting that aceruloplasminemia is a late onset pathology, and these mutations were identified in heterozygous patients with mild neurologic symptoms (I9F and G876A) or also suffering from a multiple system atrophy (G606E) (33–35). Q146E is found in compound heterozygosity with a Cp truncated at residue 983 (36), and W264S is homozygous (37), with both patients exhibiting neurologic symptoms. The position of these mutations in the structure of Cp suggests that the protein can retain ferroxidase activity. This assumption is supported by the presence of holo-Cp in the supernatants of transfected cells shown in Fig. 1*D*. Recombinant Cp-GPI I9F was found to be prevalently retained in the ER (13); however, it is reasonable to assume that the fraction of protein that escapes can complete its maturation generating holo-Cp. Cp mutants F198S and A331D show partial complementation. These are two homozygous mutations not associated with neurologic deficits, possibly because of the age of the patients (38, 39). The impact of these two substitutions on the structure of Cp appears to be more serious; Phe-198 is located in the same hydrophobic pocket where Pro-177 is found, suggesting a folding defect; Ala-331 is close to the type 1 copper site of domain 2,

and substitution with a negatively charged amino acid could cause defective copper incorporation. Actually, replacement of Phe-198 or Ala-331 with other residues (F198Y/F198T and A331V/A331N) produced the same results in the functional complementation assay as the variants detected in the patients (data not shown), strongly implying that it is the position of the mutation in the Cp structure that is critical, irrespective of the substituted amino acid.

It should be reminded that in our model system the missense mutants (and Fpn-GFP) are overexpressed under control of the strong cytomegalovirus promoter; therefore, it is possible that even if the specific ferroxidase activity of the mutant protein is low, the amount of recombinant protein produced is sufficient to guarantee "survival" of Fpn at the plasma membrane. Clearly, it would be necessary to measure the specific activity of these mutants to verify the full impact of the amino acid substitution on Cp. Preliminary data obtained on the missense mutants expressed in yeast suggest that specific activity is lower than that of the WT and that a correlation between functional complementation in mammalian cells and oxidase activity exists (data not shown).

In conclusion, our results show for the first time that some forms of aceruloplasminemia are in principle reversible, as the responsible missense mutation leads to a protein that is inactive because of lack of copper, yet has the potential of acquiring it, with noteworthy consequences on possible therapeutic strategies.

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# Dominant Mutants of Ceruloplasmin Impair the Copper Loading Machinery in Aceruloplasminemia

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