Biochemical Analysis of a Missense Mutation in Aceruloplasminemia*

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Aceruloplasminemia is an inherited neurodegenerative disease characterized by parenchymal iron accumulation secondary to loss-of-function mutations in the ceruloplasmin gene. To elucidate the molecular pathogenesis of aceruloplasminemia, the biosynthesis of a missense mutant ceruloplasmin (P177R) occurring in an affected patient was examined. Chinese hamster ovary cells transfected with cDNAs encoding secreted and glycosylphosphatidylinositol (GPI)-linked wild-type or P177R human ceruloplasmin were examined by pulse-chase metabolic labeling. These experiments, as well as immunofluorescent analysis and N-linked glycosylation studies, indicate that both the secreted and GPI-linked forms of the P177R mutant are retained in the endoplasmic reticulum (ER). The P177R mutation resides within a novel motif, which is repeated six times in human ceruloplasmin and is conserved in the homologous proteins hephaestin and factor VIII. Analysis of additional mutations in these motifs suggests a critical role for this region in ceruloplasmin trafficking and indicates that substitution of the arginine residue is critical to the ER retention of the P177R mutant. Metabolic labeling of transfected Chinese hamster ovary cells with $^{64}$Cu indicates that the P177R mutant is retained in the ER as an apoprotein and that copper is incorporated into both secreted and GPI-linked ceruloplasmin as a late event in the secretory pathway. Taken together, these studies reveal new insights into the determinants of holoceruloplasmin biosynthesis and indicate that aceruloplasminemia can result from retention of mutant ceruloplasmin within the early secretory pathway.

Ceruloplasmin is an abundant serum glycoprotein, which contains >95% of the copper found in the plasma of all vertebrate species (1). This protein is synthesized in hepatocytes and secreted into the plasma as a holoprotein with six atoms of copper incorporated during biosynthesis. Although copper does not affect the rate of apoceruloplasmin synthesis or secretion, impairment of copper incorporation results in the secretion of an apoprotein that is devoid of enzymatic activity and rapidly degraded in the plasma (2, 3). Consistent with this concept, in patients with Wilson disease, the absence or dysfunction of a copper-transporting ATPase abrogates copper transfer into the secretory pathway, resulting in marked diminution in the serum concentration of ceruloplasmin (4). Extrahepatic synthesis of human ceruloplasmin has been detected in several tissues, including the retina and brain (5, 6), and recent studies in rodents suggest that in brain and testis ceruloplasmin is synthesized as a glycosylphosphatidylinositol (GPI)$^-^-$anchored form via alternative RNA splicing (7–10).

Ceruloplasmin is a member of the multicopper oxidase family of enzymes, which use the facile electron chemistry of the bound copper atoms to couple substrate oxidation to the four-electron reduction of dioxygen to water (11). Although ceruloplasmin can oxidize multiple substrates in vitro, recognition of an essential role for this protein as a ferroxidase came with the identification of patients with aceruloplasminemia (12, 13). In this disorder, affected individuals present with diabetes and neurodegeneration in association with parenchymal iron accumulation and absent serum ceruloplasmin attributable to inherited loss-of-function mutations in the ceruloplasmin gene. Studies in a murine model of aceruloplasminemia reveal an essential physiologic role for ceruloplasmin in determining the rate of iron efflux from cells with mobilizable iron stores (14). The homologous multicopper oxidase hephaestin appears to play an analogous role in iron efflux from enterocytes (15).

Despite the critical importance of copper for ceruloplasmin function, little is known about the mechanisms of copper incorporation into this protein. Furthermore, although a GPI-linked form of ceruloplasmin has been identified in the rat, no information is available on the mechanisms of synthesis or copper incorporation into this isoform. Recent studies have revealed that the delivery of copper to specific proteins within the cell is mediated by a family of proteins termed copper chaperones (16). Although it is clear that the atox1 chaperone plays a critical role in copper delivery to the secretory pathway of mammalian cells (17), the specific mechanisms of copper incorporation into any of the known copper proteins in the secretory pathway including ceruloplasmin remain unknown. In this current study, the identification of a missense mutation resulting in aceruloplasminemia provided the opportunity to investigate the molecular basis for ceruloplasmin deficiency in this disease and to use this mutation to elucidate the mechanisms of holoceruloplasmin biosynthesis.

**EXPERIMENTAL PROCEDURES**

*Materials—General chemicals and reagents were purchased from Sigma. DNA restriction and modifying enzymes were purchased from Promega and used according to the manufacturer’s specifications. Hybridization membranes and ECL reagents were purchased from Amer-

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oligonucleotides (21). The presence of specific mutations and the fidelity of the entire cDNA sequence were verified in each case by automated fluorescent sequencing (PerkinElmer Life Sciences).

Cloning of GPI-linked Isoform of Ceruloplasmin—A BLAST search of the human expressed sequence tag data base using the DNA sequence of exons 16–18 of human ceruloplasmin identified a candidate GPI-linked isoform (accession number BE065557) with considerable homology to the published sequence of rat GPI-linked ceruloplasmin (10). The predicted coding sequence was amplified from a human brain cDNA library by polymerase chain reaction using 5′ (CACAGGGGAGTTTAT-AGTGCTGGATCTTTCGACA) and 3′ antisense (ACCTTGGAGACGTC- TCTTGGGTAGATTTGGAATATAACATC) oligonucleotides containing an added XhoI site (underlined). This polymerase chain reaction product was digested with PmlI and XhoI and subcloned into pcDNA3Sp, generating a full-length cDNA of the GPI-linked isoform.

Cell Culture, Transfection, and Immunofluorescence—Chinese hamster ovary (CHO) and HepG2 cells were obtained from the American Type Culture Collection and maintained in either Ham’s F-12 (CHO) or Dulbecco’s modified Eagle’s medium (HepG2), each containing 10% fetal bovine serum and supplemented with glutamine and penicillin-streptomycin. Transient transfections were performed with LipofectAMINE (Invitrogen) according to the manufacturer’s instructions. For indirect immunofluorescence, transfected CHO cells were plated on coverslips, fixed in 4% paraformaldehyde, and quenched with 1 M ethanolamine. Cells were permeabilized in 0.1% Triton X-100 and analyzed with a 1:2000 dilution of anti-ceruloplasmin or a 1:200 dilution of anti-protein disulfide isomerase antisera as described previously (22). In some experiments, cells were resuspended in Dulbecco’s modified Eagle’s medium containing 600 milliunits/ml phosphatidylinositol-specific phospholipase C (Roche Molecular Biochemicals) in a volume of 4 ml and incubated for 60 min at 37 °C to release GPI-anchored proteins.

Metabolic Labeling and Immunoprecipitation—Forty-eight hours after transfection, CHO cells were pulse-labeled for 20 min with 60 μCi/ml [35S]methionine and [35S]cysteine and chased with serum-free Ham’s F-12 medium for the indicated time points, followed by collection of media and lystate for immunoprecipitation as described previously (22). In some experiments, the immunoprecipitate was eluted by boiling the presence of 5 ms Tris-HCl, pH 8.0, containing 0.2% SDS, split into two aliquots, and incubated at 37 °C overnight in the presence or absence of 0.1 milliunits/µl endoglycosidase H (Roche Molecular Biochemicals). Samples were subjected to 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed by PhosphorImager or exposure to Eastman Kodak Co. MR film. For 64Cu metabolic labeling, 48 h after transfection cells were washed in phosphate-buffered saline, pulsed for 2 h with 300 μCi/ml 64Cu in serum-free Ham’s F-12 medium, washed in phosphate-buffered saline, and chased for 2 h with fresh serum-free Ham’s F-12 medium containing 100-fold excess cold copper. Media were collected and concentrated using Centricon-30 columns (Millipore).

Analysis of cell lystate and media for 64Cu-labeled ceruloplasmin (holo-ceruloplasmin) was carried out as described previously (23). Samples were electrophoresed in 4–20% Tris-HCl gradient gels without denaturation before loading and then analyzed by PhosphorImager. 35S and 64Cu pulse-chase analyses of HepG2 cells were carried out in an identical fashion with the exception that the pulse period for each was only 5 min.

RESULTS

A cDNA encoding human ceruloplasmin was transfected into a series of mammalian cell lines followed by metabolic labeling
and immunoprecipitation. In transfected CHO cells, the kinetics of ceruloplasmin synthesis and secretion (Fig. 1A) was found to be identical to that observed for endogenous ceruloplasmin in the hepatoma cell line HepG2 (23). Using a polyclonal antiserum known to recognize hamster ceruloplasmin, no endogenous ceruloplasmin synthesis was detected in these CHO cells (data not shown). Furthermore, incubation of transfected CHO cells with 64Cu revealed copper incorporation into ceruloplasmin equivalent to that observed in HepG2 cells and primary hepatocytes (see Fig. 6). Taken together, these results suggested that transfected CHO cells could be used to investigate the mechanisms of serum ceruloplasmin deficiency resulting from the P177R missense mutation identified in an affected patient. Accordingly, a ceruloplasmin cDNA encoding this mutant was transfected into CHO cells, followed by pulse-chase analysis. These studies revealed marked intracellular accumulation of newly synthesized P177R ceruloplasmin with no apparent degradation or secretion of this mutant protein throughout the time course of the experiment (Fig. 1A). Because ceruloplasmin is a secreted glycoprotein, the data in Fig. 1A suggest that the P177R mutant protein may be improperly trafficked within the secretory pathway of the cell.

Recent studies in the rat have revealed that alternative splicing can result in the synthesis of ceruloplasmin as a plasma membrane-anchored GPI-linked isoform (10). If a similar situation occurs in humans, the presence of a GPI-linked isoform would have important implications for our understanding of the biosynthesis and trafficking of ceruloplasmin as well as the effects of any patient mutations. To determine whether there is a GPI-linked isoform of human ceruloplasmin, a BLAST search of the human expressed sequence tag data base was performed. This approach identified a ceruloplasmin cDNA with a carboxyl terminus highly homologous to rat GPI-linked ceruloplasmin, and subsequent analysis of human genomic DNA revealed the presence of a separate downstream exon encoding this putative GPI-linked sequence (Fig. 1B). Analysis of RNA using a specific probe derived from this exon revealed that a transcript encoding this putative GPI-linked ceruloplasmin is expressed in a variety of human tissues in vivo (data not shown).

To characterize this isoform of ceruloplasmin, a full-length cDNA was generated by polymerase chain reaction of a human brain library and transfected into CHO cells. Immunofluorescence analysis of transfected cells with and without prior permeabilization using anti-ceruloplasmin antisera revealed cell surface expression (Fig. 1C). Treatment of transfected cells with phosphatidylinositol-specific phospholipase C abrogated this staining, suggesting that the expressed ceruloplasmin was anchored to the plasma membrane via a GPI linkage. Pulse-chase analysis in transfected CHO cells revealed that the GPI-linked form of wild-type ceruloplasmin remained in the cellular fraction for the duration of the experiment, with the gradual appearance of a second higher molecular weight band corresponding to the mature, fully glycosylated membrane-anchored protein (Fig. 1D). As anticipated, expression of the GPI-linked isoform of P177R ceruloplasmin revealed no secretion of this mutant into the media. However, the pattern of intracellular synthesis was distinct from that of the wild-type GPI-linked ceruloplasmin in that no higher molecular weight band was detected during the later part of the chase period (Fig. 1D).

The biosynthetic data shown above suggest that the P177R mutation results in impaired trafficking of both the secreted and GPI-linked isoforms of ceruloplasmin. Previous studies have revealed that the secreted form of ceruloplasmin is modified by N-linked glycosylation during biosynthesis (23). To determine whether GPI-linked ceruloplasmin is also modified in this fashion and to further examine the possibility of aberrant trafficking of the P177R mutant, N-linked glycosylation of ceruloplasmin was analyzed in CHO cells transfected with the GPI-linked form of either wild-type or P177R ceruloplasmin. Although in both cases an endoglycosidase H-sensitive form of ceruloplasmin was detected throughout the chase period, maturation to a higher molecular weight, endoglycosidase H-resistant form was observed only in the lysates from cells transfected with wild-type ceruloplasmin (Fig. 2). These data indicate that the GPI-linked form is modified by N-linked glycosylation, a modification that accounts at least in part for the higher molecular weight band observed in Fig. 1D. Similar results with endoglycosidase H were obtained when these experiments were repeated using the wild-type and P177R-secreted isoforms of ceruloplasmin (data not shown). Taken together, these data suggest that trafficking of the P177R mutant is blocked before the modification of N-linked oligosaccharides in the cis- or medial-Golg.

To more directly assess the intracellular location of the P177R mutant, indirect immunofluorescence was performed in CHO cells transfected with the wild-type and P177R GPI-linked constructs. As noted previously, GPI-linked ceruloplasmin displays a staining pattern consistent with cell surface localization when transfected into CHO cells (Fig. 3A). In contrast, the GPI-linked form of P177R ceruloplasmin is detected in an intracellular compartment, which colocalizes with the endoplasmic reticulum (ER) resident protein disulfide isomerase (Fig. 3, B and C). Using these same methods, ER retention of P177R ceruloplasmin was also observed with the secreted isoform of this mutant, and this location was not altered for either mutant isoform after growth of transfected cells at 31 °C or pretreatment with the chemical chaperone glycerol (data not shown). Conditions previously shown to rescue trafficking defects of other proteins within the secretory pathway (24).

Further inspection of the amino acid sequence of human ceruloplasmin (25) revealed that the P177R mutation resides within a five-amino acid motif G(FLI)(LI)GP, which is repeated six times throughout the protein (Fig. 4A). This motif is also present and repeated in the homologous multicopper oxidase hephestin as well as coagulation factor VIII (Fig. 4B). Of interest, an analogous proline to arginine mutation in this motif has been identified in the factor VIII gene in a patient with hemophilia A and undetectable serum factor VIII (28), suggesting a possible role for this motif in protein structure (Fig. 4B). Consistent with this possibility, structural modeling reveals that in wild-type ceruloplasmin, proline 177 projects into a tightly packed hydrophobic pocket, closely surrounded by the nonpolar residues Leu24, Ile72, Ile159, Val199, and Phe267 (Fig. 4C). These data suggest that the repeat G(FLI)(LI)GP motif may be critical for proper folding and subsequent trafficking of human ceruloplasmin.

To further examine this hypothesis, site-directed mutagenesis was used to create an analogous proline to arginine mutation in the third motif, resulting in a P432R mutant ceruloplas-
min (Fig. 4A). Pulse-chase analysis of CHO cells transfected with the secreted isoform of P432R ceruloplasmin revealed a pattern of synthesis nearly identical to that seen for the P177R mutation, with little or no intracellular degradation or secretion during the chase period (Fig. 5). Similar results were obtained using the GPI-linked isoform, and additional experiments with both isoforms revealed that the P432R mutant is also retained in the ER (data not shown). The aberrant trafficking observed with the P177R and P432R mutations could result either from loss of the conserved proline residue or from insertion of the arginine residue. To distinguish between these possibilities, a P177A ceruloplasmin was generated and expressed in CHO cells. As can be observed in Fig. 5, the P177A mutation does not impair the biosynthesis or secretion of ceruloplasmin under these conditions, suggesting that the ER retention of the arginine-substituted mutants may primarily result from introducing a polar, basic guanidino moiety into a highly hydrophobic environment, leading to protein misfolding.

The retention of the P177R mutant in the ER provides the opportunity to examine the effect of this mislocalization on copper incorporation into ceruloplasmin. To accomplish this, transfected CHO cells were metabolically labeled with $^{64}$Cu and chased in media containing excess methionine and cysteine for 20 min and chased in media containing excess methionine for the indicated periods. Ceruloplasmin was immunoprecipitated from cell lysates (IC) and media (EC) and analyzed by 12% SDS-PAGE as described under “Experimental Procedures.”

**Fig. 5.** P177A and P432R ceruloplasmin biosynthesis in transfected CHO cells. CHO cells transfected with wild-type (wt), P177A, or P432R ceruloplasmin were incubated with $[^{55}]$methionine and $[^{55}]$cysteine for 20 min and chased in media containing excess methionine for the indicated periods. Ceruloplasmin was immunoprecipitated from cell lysates (IC) and media (EC) and analyzed by 7.5% SDS-PAGE as described under “Experimental Procedures.”
media containing 300 and linked P177R ceruloplasmin (lanes 3 and vector (lanes 1 and 10), GPI-linked wild-type ceruloplasmin (lanes 5 and 11), and GPI-linked P177R ceruloplasmin (lanes 6 and 12) were incubated with media containing 300 μCi/ml 64Cu for 2 h and chased for 2 h in fresh serum-free Ham’s F-12 medium containing a 100-fold excess of cold copper. Lysate (IC; lanes 1–6) and media (EC; lanes 7–12) samples were then analyzed on 4–20% Tris-HCl gradient gels as described under “Experimental Procedures.” B, CHO cells transfected with vector (lanes 1 and 6), the secreted form of wild-type ceruloplasmin (lanes 2 and 7), GPI-linked wild-type ceruloplasmin (lane 3), the secreted form of P177A ceruloplasmin (lanes 4 and 7), and GPI-linked P177A ceruloplasmin (lane 5) were incubated with media containing 300 μCi/ml 64Cu for 2 h and chased for 2 h, after which lysate (IC; lanes 1–5) and media (EC; lanes 6–8) were analyzed on 4–20% Tris-HCl gradient gels as described under “Experimental Procedures.”

GPI-linked ceruloplasmin (10) and presumably reflects the addition of the GPI anchor to this isoform. In each case, the copper-labeled ceruloplasmin observed in CHO cells transfected with wild-type ceruloplasmin was similar to that observed for endogenous ceruloplasmin in HepG2 cells. In contrast, during this same period of 64Cu labeling, neither secreted nor GPI-linked holoceruloplasmin was detected in cell lysate or media from CHO cells transfected with the P177R mutant (Fig. 6A, lanes 4, 6, 10, and 12). Examination of the P177A mutation using these same methods revealed copper incorporation into ceruloplasmin comparable with that observed with wild-type ceruloplasmin, a finding consistent with the normal kinetics of copper incorporation into ceruloplasmin occurs as a late event within the secretory pathway.

**DISCUSSION**

Although more than a dozen unique mutations have been identified in the ceruloplasmin gene in patients with aceruloplasminemia, the majority of these are insertions or deletions predicted to result in a frameshift or termination (1). This current study reports the first functional analysis of a missense mutation in aceruloplasminemia. The kinetics of synthesis, secretion, and copper incorporation into ceruloplasmin in transfected CHO cells (Figs. 1 and 6) are similar to those observed for endogenous ceruloplasmin in hepatocytes and glia (23, 27), a finding that supports the validity of this experimental system. In this regard, the finding that both secreted and GPI-linked P177R ceruloplasmin are retained in the ER of transfected CHO cells (Figs. 2 and 3) is consistent with the clinical observation of absent serum ceruloplasmin and ferrooxidase activity in an affected individual with this mutation. Although in vivo the Wilson ATPase is required for copper incorporation into ceruloplasmin in hepatocytes, previous studies have revealed that CHO cells express the Menkes ATPase (28). Because copper is incorporated into ceruloplasmin in transfected CHO cells (Figs. 6 and 7), these data support the concept that these ATPases use common biochemical mechanisms to effect cellular copper homeostasis (22).

Alternative splicing of the rat ceruloplasmin gene results in a GPI-linked ceruloplasmin isoform abundantly expressed in the central nervous system (10). The data in this current study reveal that a human GPI-linked ceruloplasmin isoform arises from splicing of a previously unidentified downstream exon (Fig. 1). This finding is of importance for mutation screening of affected patients and raises the possibility that if the GPI-linked isoform is critical for iron homeostasis in the human brain, individuals may be identified with the phenotype of aceruloplasminemia and normal serum ceruloplasmin. Although no GPI-linked ceruloplasmin was detected in the media of transfected CHO cells (Fig. 1), cleavage of the plasma membrane GPI anchor can result in secretion of GPI-linked proteins (29), raising the possibility that ceruloplasmin secretion observed previously in glial cells (27) may result from GPI cleavage. Because these data clearly reveal ER retention of both the secreted and GPI-linked P177R ceruloplasmin in transfected CHO cells (Figs. 1 and 2), further studies will be warranted to identify the sites of expression of human GPI-linked ceruloplasmin and to define the precise role of this isoform in iron homeostasis.

Although the copper transport ATPases required for copper delivery to the secretory pathway are localized to the trans-Golgi network, the precise location within this pathway of copper incorporation into ceruloplasmin is unknown (1). Because the P177R mutation does not involve any of the six copper binding sites in ceruloplasmin, the lack of copper incorporation into this ER-retained mutant (Fig. 6) suggests that retrograde transport of copper to the ER does not occur. Such a finding is consistent with observations that retention of tyro-
sinase in the ER of melanoma cells abrogates copper incorporation into this protein (30) and that copper incorporation into Fct3, the yeast homologue of ceruloplasmin, occurs in a late compartment of the secretory pathway (31). An important caveat to this conclusion is that the P177R mutant may be incapable of binding copper regardless of cellular location as a result of misfolding. Although this possibility could not be directly tested in these studies, further kinetic analysis of copper incorporation into newly synthesized ceruloplasmin in HepG2 cells also suggests that this process occurs only at a point very late in the secretory pathway, immediately before secretion or membrane anchoring (Fig. 7). These metabolic studies clearly reflect newly synthesized holoceruloplasmin, because previous studies have shown that no copper is incorporated into previously synthesized apoceruloplasmin (23). The observation that GPI-linked ceruloplasmin also incorporates copper is consistent with studies demonstrating oxidase activity of this isoform in the rat (7, 9). Interestingly, recent studies revealed that GPI-anchored proteins are sorted from other proteins within the ER early in the secretory pathway (32). Because copper incorporation into both the secreted and GPI-linked isoforms occurs at a point late in the secretory pathway, these data suggest that each isoform may be trafficked to a common compartment for copper incorporation.

The ER plays an essential role in the folding and maturation of newly synthesized proteins in the secretory pathway through the activity of numerous soluble and membrane-bound enzymes and chaperones (33). The G(FIL)LI(GP) motif identified in this study may play a role in packing of hydrophobic side chains and protein folding and assembly in the ER, perhaps via direct interactions with chaperones. Such a role is supported by the finding of ER retention of the P432R mutant (Fig. 5) as well as identification of this same mutation in a nonsecretory mutant of factor VIII. Regardless of the mechanisms, the data in this study indicate that aceruloplasminemia should be added to the list of human diseases that can result from the retention of a mutant protein within the ER (24). Neither decreased temperature nor chemical chaperones stimulated movement of P177R ceruloplasmin from the ER, and no appreciable degradation of this mutant protein was observed during the chase periods examined (Fig. 1). These data raise the possibility that ER accumulation of mutant ceruloplasmin may contribute to neuronal cytotoxicity in aceruloplasminemia either through activation of ER stress pathways, as observed in Perlizauers-Merzbacher disease (34) and Charcot-Marie-Tooth syndrome (35), or via protein aggregation, as seen in a number of sporadic and inherited neurodegenerative diseases, including Huntington's disease, amyotrophic lateral sclerosis, and prion-mediated encephalopathies (36). Further neuropathological studies in aceruloplasminemic patients with specific genotypes will be useful in addressing this issue and may provide for novel therapeutic approaches to modulating ER protein export or degradation in this disease.

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