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Aceruloplasminemia: Molecular characterization of this disorder of iron metabolism

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ABSTRACT **Ceruloplasmin is an abundant α_2-serum glycoprotein that contains 95% of the copper found in the plasma of vertebrate species. We report here on the identification of a genetic defect in the ceruloplasmin gene in a patient previously noted to have a total absence of circulating serum ceruloplasmin in association with late-onset retinal and basal ganglia degeneration. In this patient T_2 (transverse relaxation time)-weighted magnetic resonance imaging of the brain revealed basal ganglia densities consistent with iron deposition, and liver biopsy confirmed the presence of excess iron. Although Southern blot analysis of the patient's DNA was normal, PCR amplification of 18 of the 19 exons composing the human ceruloplasmin gene revealed a distinct size difference in exon 7. DNA sequence analysis of this exon revealed a 5-bp insertion at amino acid 410, resulting in a frame-shift mutation and a truncated open reading frame. The validity of this mutation was confirmed by analysis of DNA from the patient's daughter, which revealed heterozygosity for this same 5-bp insertion. The presence of this mutation in conjunction with the clinical and pathologic findings demonstrates an essential role for ceruloplasmin in human biology and identifies aceruloplasminemia as an autosomal recessive disorder of iron metabolism. These findings support previous studies that identified ceruloplasmin as a ferroxidase and are remarkably consistent with recent studies on the essential role of a homologous copper oxidase in iron metabolism in yeast. The clinical and laboratory findings suggest that additional patients with movement disorders and nonclassical Wilson disease should be examined for ceruloplasmin gene mutations.**

In 1948 Holmberg and Laurell (1) isolated an abundant copper-containing protein from pig serum that they termed ceruloplasmin or "the sky-blue protein." In this same year, the hepatolenticular degeneration of Wilson disease was found to be due to excess copper accumulation (2). After these discoveries, the observation was made that the serum ceruloplasmin concentration is decreased in Wilson disease, thus providing a diagnostic test for this disorder (3). Further clinical studies revealed the acute-phase nature of this protein and identified individuals with inherited differences in serum ceruloplasmin concentrations (4, 5). Analysis of one such family identified this alteration as hereditary hypoceruloplasminemia and concluded that no clinical abnormalities were associated with changes in serum ceruloplasmin to 50% of normal (6). The marked decrease in serum ceruloplasmin in Wilson disease is not a primary defect but the result of impaired copper trafficking into a common pool for biliary excretion and holoceruloplasmin biosynthesis. Consistent with this, the Wilson disease gene was recently cloned and encodes a putative copper-transporting ATPase presumably located within the secretory compartment of hepatocytes (7–9).

Ceruloplasmin is a blue copper oxidase that is synthesized in hepatocytes and secreted as a holoprotein with six atoms of copper incorporated during biosynthesis (10). Copper does not affect that rate of synthesis or secretion of apoceruloplasmin, but failure to incorporate copper during biosynthesis results in an unstable protein lacking oxidase activity (11). Although the copper ligands in ceruloplasmin are unknown, physicochemical studies reveal the presence of a trinuclear copper cluster in the carboxyl-terminal domain essential for oxidase activity (12). Amino acid sequencing of human ceruloplasmin was a *tour de force*, proving the single-chain structure of the protein and identifying triplicate repeat domains of 350 amino acids (13). Subsequently homology was identified in these domains with clotting factors V and VIII (14). Subsequent cloning of the human cDNA confirmed these sequence data and demonstrated the mechanisms of gene expression during the acute-phase response (15–18).

Despite years of investigation, the functional role of ceruloplasmin has not been clarified. A direct role in copper transport is unlikely, given kinetic data that indicate that the copper is turned over at the same rate as the protein (19). In a series of biochemical investigations Osaki *et al.* (20) demonstrated ceruloplasmin ferroxidase activity, suggesting a role for ceruloplasmin in ferric iron uptake by transferrin. Consistent with this concept, the anemia that develops in copper-deficient animals is unresponsive to iron but is correctable by ceruloplasmin administration (21). After the cloning of the Wilson disease gene, we have investigated a number of patients referred to us for molecular diagnosis with neurologic degeneration and low serum ceruloplasmin. In the course of this analysis we recognized that several of these patients did not have Wilson disease and have begun to reexamine the role of ceruloplasmin in human disease. In this study, we report the genetic analysis of one such patient originally identified in Hamamatsu, Japan, and reported as a case of familial apoceruloplasmin deficiency (22).

MATERIALS AND METHODS

DNA Preparation and Analysis. DNA was isolated from peripheral blood and layered on Hypaque solution (Sigma) followed by centrifugation at 400 × *g* for 10 min. Leukocytes were removed from the buffy coat and lysed in hypotonic saline; DNA was then precipitated and purified as described (23). All samples were analyzed spectrophotometrically and shown to have an A_{260}/A_{280} ratio of >1.8. Oligonucleotides corresponding to the 5′ and 3′ ends of each exon encoding the human ceruloplasmin gene (M. L. Koschinsky and R.T.A.M., unpublished work) were synthesized and used for PCR am-

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plification of genomic DNA. Each oligonucleotide pair (see Table 1) was used at 100 pmol with 75 ng of genomic DNA for a total of 30 cycles of amplification. Reactions were done with denaturing at 96°C for 1 min, annealing at 55°C for 1.5 min, and extension at 72°C for 3 min. All reactions were completed with a final extension cycle at 72°C for 10 min. After amplification aliquots of each sample were subjected to nondenaturing electrophoresis on 10% polyacrylamide gels. Final products were analyzed by ethidium bromide staining (0.5 μg/ml) for 15 min followed by visualization with shortwave UV illumination.

For additional analysis amplified fragments were isolated in DEAE paper and subcloned into the PCRII vector (Invitrogen) by T-tail cloning (24). Individual colonies were isolated, and DNA was sequenced by the dideoxynucleotide chain-termination method (25). For Southern blot analysis genomic DNA was digested with restriction endonuclease according to specifications (Promega), electrophoresed in 0.6% agarose gels, transferred to nylon Hybond-N (Amersham) membranes, and hybridized overnight at 42°C with a ^{32}P-labeled cRNA antisense probe encoding human ceruloplasmin (17). After hybridization, Southern blots were washed and exposed to film as described (24). Ceruloplasmin concentration and oxidase activity in the sera were measured as described (10, 17). Serum ceruloplasmin was analyzed by immunoblotting using a polyclonal antibody.

RESULTS

The index patient whose DNA is examined in this study is currently a 61-yr-old woman with retinal degeneration and blepharospasm for the past 10 yr. Since her original presentation, she has also developed cogwheel rigidity and dysarthria. Her younger sister, who was asymptomatic at the time of the original presentation despite undetectable ceruloplasmin, is currently 51 yr old and has had the recent onset of retinal degeneration and basal ganglia symptoms. In each case the absence of serum ceruloplasmin is associated with mild anemia, low serum iron, and elevated serum ferritin. Magnetic resonance imaging studies reveal prominently decreased signal intensity in the basal ganglia on T_2 (transverse relaxation time)-weighted images, suggestive of significantly elevated content of iron in the brain (Y.T. and H.M., unpublished work). The daughter of the patient reported here is entirely asymptomatic and, as noted earlier, has a serum ceruloplasmin concentration that is 50% of normal, consistent with an obligate heterozygote. There is no history of consanguinity present in the family, and the number of affected members, as well as evaluation of the proband's offspring, suggest this as an autosomal recessive disorder. The clinical studies and details of iron and copper metabolism in the extended family will be presented elsewhere.

Genomic DNA was isolated from this 61-yr-old woman to determine directly whether a mutation in the ceruloplasmin gene might account for the decreased ceruloplasmin in the serum. Southern blot analysis of the isolated DNA using cDNA probes encompassing the entire ceruloplasmin-coding region revealed no fragment size differences between the patient's sample and normal control DNA (data not shown). To assess directly the relative size of each exon encoding the ceruloplasmin gene, we next performed PCR using the oligonucleotide primer pairs shown in Table 1. Amplification primers corresponding to exon 19 were omitted, as it seemed less likely that a mutation in the 3′ untranslated region would result in a lack of detectable ceruloplasmin. After analysis of all amplification products on polyacrylamide gels, an obvious size difference was observed in exon 7 amplified from the patient's DNA compared with controls (Fig. 1, lanes 3 and 4). The predicted size of this exon is 140 bp (see Table 1), and the size difference suggested an additional 4–5 bp. This size difference was not the result of a generalized difference in the patient's DNA because amplification products of all other exons were equivalent in size compared with controls (for example, Fig. 1, exons 6 and 8). Furthermore, this difference was unique to this patient because amplification of DNA from a patient in St. Louis with aceruloplasminemia (Z.L.H. and J.D.G., unpublished work) showed the expected 140-bp size for exon 7 (Fig. 1, lane 6). To be certain that this finding reflected a unique genetic difference, genomic DNA was isolated from the patient's daughter and subjected to similar analysis. As can be seen in Fig. 1, although amplification of exons 6 and 8 resulted in single detectable fragments of the expected 172 bp and 153 bp, PCR amplification of exon 7 in the daughter's DNA resulted in two bands (exon 7, lane 5). Inspection of these bands reveals that one corresponds to the normal 140-bp product, whereas the other is identical in size to the band observed in the patient's DNA (Fig. 1, lanes 3–5).

The results of the amplification of exon 7, including the evidence of heterozygosity in the daughter, strongly supported

Table 1. PCR primer sets used in amplification of the human ceruloplasmin gene exons 1–18

Exon	Upstream primer Name	Upstream primer (5′-3′)	Coordinates*	Downstream primer Name	Downstream primer (5′-3′)	Coordinates	PCR product size, bp
1	hCp1	ATGAAGATTTTGATACTT	1–18	hCp2	GTGTCAACAGAAATAAGT	146–129	158
2	hCp3	GGAACATTCCAATATCTA	147–164	hCp4	CCTCATGTTCCTTATAGT	394–377	248
3	hCp5	GGGCCATCTACCCTGATA	395–412	hCp6	CTTTTTTACAGATTATTA	607–590	213
4	hCp7	ATTCTCTAGATAAAGAAA	608–625	hCp8	AATACATTCTGTTACTCT	781–764	174
5	hCp9	CTGTGAATGGATACACTT	782–799	hCp10	CTTTCAGATGGTTTAGAT	1036–1019	255
6	hCp11	CCGGTTTGCAAGCCTTTT	1037–1054	hCp12	CTTCCAGGTGCTGTTAAG	1208–1191	172
7	hCp13	TGACTCAGCGGTGTTTTT	1209–1226	hCp14	CCAGGATGCCAAGATGCT	1348–1331	140
8	hCp15	GTCCTGTCATTTGGGCAG	1349–1366	hCp16	TTCTGCTCTGGGGGTTGT	1501–1484	153
9	hCp17	GTGTGCCTCCTTCAGCCT	1502–1519	hCp18	CTGTCTCCCATTTGCATG	1713–1696	212
10	hCp19	AAAGATGTAGACAAGGAA	1714–1731	hCp20	AGTGCATTTTATTAGATT	1864–1847	151
11	hCp21	CCATGAATGGATTCATGT	1865–1882	hCp22	CCTCTGTGTCAGGCCACA	2077–2060	213
12	hCp23	GGACTTTTAATGTTGAAT	2078–2095	hCp24	TTCTGCTCTTGTAAATGA	2285–2268	208
13	hCp25	TGTTTCAAATGCATTTTT	2286–2303	hCp26	CTAGAATTCCCAGATGTT	2425–2408	140
14	hCp27	GTCCACAACTTCATGCAG	2426–2443	hCp28	CTGGTAATGTTGGAGTAA	2554–2537	129
15	hCp29	GTGAAACTCTCACTTACG	2555–2572	hCp30	CTTAACTTGATCCACAGT	2661–2644	107
16	hCp31	GACCTCTACAGTGGATTA	2662–2679	hCp32	CATGCATTTTATTGCTTT	2878–2861	217
17	hCp33	CTATTAATGGAAGAATGT	2879–2896	hCp34	CTTGTATTGGAAGCTATG	3018–3001	140
18	hCp35	CACAGGGGAGTTTATAGT	3019–3036	hCp36	CTTCATTTTGTAGAACGG	3181–3164	163

All amplifications were as described; annealing temperature was 42°C for exons 3 and 4; 50°C for exons 1, 13, and 17; 55°C for exons 10, 11, and 12; and 58°C for the remaining exons.

*Coordinates are based upon nucleotide sequence of cDNA (14).

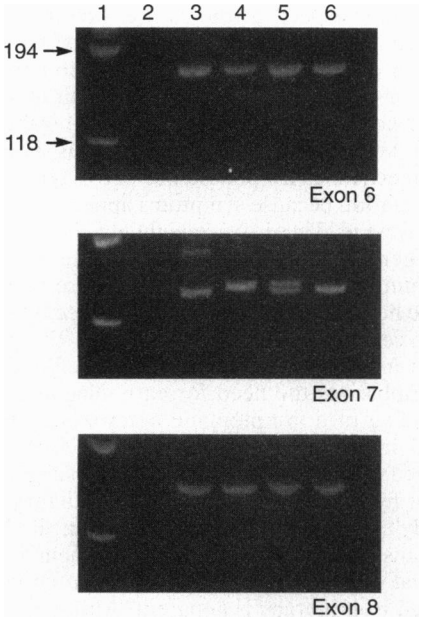

FIG. 1. Electrophoretic analysis of PCR-amplified fragments from the ceruloplasmin gene. Fragments derived from exon 6–8 are shown with DNA from normal (lane 3), patient (lane 4), patient's daughter (lane 5), and a second patient (St. Louis) with aceruloplasminemia (lane 6). Expected exon sizes: exon 6, 172 bp; exon 7, 140 bp; and exon 8, 153 bp. Lane 1 contains ΦX174 *Hae* III-restricted DNA. An additional band is observed in all lanes from exon 7 (182 bp) which is a nonspecific product of PCR amplification observed with this primer set.

the idea that this exon contained an insertional mutation that might account for the aceruloplasminemia. To determine this possibility directly, the amplified exon 7 products from each of the samples shown in Fig. 1 were subcloned and sequenced. As can be seen in Fig. 2, direct analysis of the sequence in this region confirmed the presence of a 5-bp insertion at amino acid 410. The sequence reaction from normal control DNA did not contain this insertion and was identical to the nucleotide sequence of ceruloplasmin in this region reported previously. This insertion was not the result of a PCR artifact because sequence analysis of some of the individual clones isolated from the daughter's amplified exon 7 revealed the identical insertion (data not shown). This result was specific to this

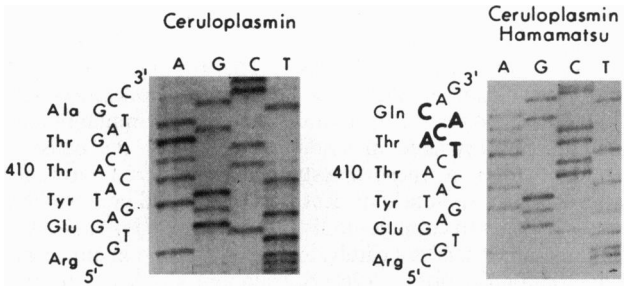

FIG. 2. Sequence ladders from normal ceruloplasmin DNA and ceruloplasmin Hamamatsu depicting a region from exon 7. The boldface type represents the 5-bp insertion in the ceruloplasmin Hamamatsu DNA.

family, as the sequence of exon 7 from the St. Louis patient with aceruloplasminemia was entirely normal (data not shown).

We next examined the effects of such an insertion on the open reading frame of ceruloplasmin. As shown in Fig. 3 the insertion results in a frame-shift at amino acid 411 that terminates 34 amino acids downstream, predicting a protein of 445 amino acids in contrast to the normal 1046-amino acid ceruloplasmin. Although we have termed this truncated protein ceruloplasmin Hamamatsu, the deletion of the carboxyl-terminal copper-containing trinuclear cluster would predict that were any protein translated, it would lack oxidase activity and be unstable in the plasma after secretion. The polyclonal antisera used in these studies is able to recognize determinants in the NH_2 terminal domain, and thus the failure to detect a truncated protein in the plasma suggests impaired secretion or rapid turnover. These data are entirely consistent with the aceruloplasminemia observed.

DISCUSSION

The data presented here indicate that the clinical findings of abnormal iron metabolism and neurologic degeneration previously observed in this patient are the result of a mutation in the ceruloplasmin gene. The evidence of heterozygosity for this exon-7 mutation in the proband's daughter, the family pedigree, and the failure to detect any deletions in this region by Southern blotting of the patient's DNA support the concept that despite a lack of consanguinity in the family history, the patient reported here is homozygous for this particular mu-

Ceruloplasmin

410 420
.....Arg Glu Tyr Thr Asp Ala Ser Phe Thr Asn Arg Lys Glu Arg Gly Pro Glu Glu Glu
.....CGT GAG TAC ACA GAT GCC TCC TTC ACA AAT CGA AAG GAG AGA GGC CCT GAA GAA GAG

430 440
His Leu Gly Ile Leu Gly Pro Val Ile Trp Ala Glu Val Gly Asp Thr Ile Arg Val Thr
CAT CTT GGC ATC CTG GGT CCT GTC ATT TGG GCA GAG GTG GGA GAC ACC ATC AGA GTA ACC

450
Phe His Asn Lys Gly Ala.....
TTC CAT AAC AAA GGA GCA.....

Ceruloplasmin Hamamatsu

410 420
.....Arg Glu Tyr Thr Thr Gln Met Pro Pro Ser Gln Ile Glu Arg Arg Glu Ala Leu Lys
.....CGT GAG TAC ACT ACA CAG ATG CCT CCT TCA CAA ATC GAA AGG AGA GAG GCC CTG AAG

430 440 445
Lys Ser Ile Leu Ala Phe Trp Val Leu Ser Phe Gly Gln Arg Trp Glu Thr Pro Ser Glu
AAG AGC ATC TTG GCA TCC TGG GTC CTG TCA TTT GGG CAG AGG TGG GAG ACA CCA TCA GAG

446
Stop
TAA

FIG. 3. DNA sequence and predicted amino acid sequence of exon 7 from normal ceruloplasmin and ceruloplasmin Hamamatsu. The underlined nucleotides indicate the region of the 5-bp insertion. The numbering above the line refers to the amino acid residue in the mature ceruloplasmin.

tation. The identification of such a mutation in conjunction with the clinical course provides direct evidence of an essential role for ceruloplasmin in humans. This finding completes a story that began in 1928 when Hart and colleagues (26) obtained the first evidence of a role for copper in mammalian biochemistry. Engaged in studies to elucidate the cause of pernicious anemia, these investigators discovered serendipitously that the anemia that develops in rats fed cow's milk is responsive only to copper, leading to "the orthodox suggestion that the copper acts as a catalyzer for some reaction concerned in hemoglobin building" (26). Subsequent studies in copper-deficient swine established that copper deficiency results in a disorder of iron storage and that ceruloplasmin administration could correct this situation (27). Osaki *et al.* (28) then demonstrated a direct role for ceruloplasmin in the mobilization of iron from the perfused liver and proposed that the ferroxidase activity of this protein establishes a concentration gradient for iron movement from "storage sites to the capillary system". The patients reported here demonstrate clear evidence of iron accumulation in both the parenchymal and reticuloendothelial system, indicating the aceruloplasminemia should be added to the list of disorders that result in secondary iron overload (29). It is of some interest to compare these findings to atransferrinemia, another genetic cause of secondary iron overload. In this disorder, the absence of transferrin results in an increase in iron absorption with sequestration of this metal in the hepatic parenchyma and a resultant severe anemia responsive to transferrin administration (30). Although ceruloplasmin and transferrin may be linked in the process of iron transport from tissues, this is complex because patients with atransferrinemia do not have decreased iron uptake from the intestine and have no evidence of iron accumulation in the brain. Although the patients reported here have only kinetic and magnetic resonance imaging studies as evidence of iron accumulation, autopsy examination of a different patient with aceruloplasminemia revealed abundant accumulation of iron in the basal ganglia (N. Shimizu, personal communication). Interestingly, a similar process of iron accumulation and tissue damage in the basal ganglia has been observed in Parkinson disease, although the relationship of this finding to abnormalities in copper metabolism in this disorder is unexplored (31). Although ceruloplasmin oxidase activity is also decreased in patients with Wilson disease, no abnormalities of iron metabolism are observed. Because previous studies have demonstrated evidence of ceruloplasmin biosynthesis and secretion in extrahepatic tissues, it seems likely that additional copper transport ATPases may permit the product of holoceruloplasmin in extrahepatic tissues in such patients and that this may be sufficient to mediate iron metabolism (32). This concept is consistent with observations in an animal model of Wilson disease where holoceruloplasmin can be detected in extrahepatic tissues despite a complete absence of the Wilson ATPase (33).

The data presented here indicate that ceruloplasmin has no essential role in copper transport because enzymatic and copper kinetic studies in this patient were normal (22). In addition, the observation that the patient's offspring are normal suggests that there is no essential role for maternal ceruloplasmin in placental/fetal copper or iron transport. This conclusion is consistent with previous studies indicating that the exchange rate of copper in the holoprotein is not sufficient to permit a function as a transport protein (19). Alternatively, the data presented here do support an essential role for ceruloplasmin in iron metabolism, and this hypothesis is strengthened by recent studies in yeast identifying a ceruloplasmin homologue (fet 3) that functions as an essential copper oxidase and mediates ferrous iron uptake (34, 35). Taken together with the findings reported here, these studies demonstrate a remarkable evolutionary conservation of the mechanisms for eukaryotic copper metabolism.

Finally, some aspects of the clinical presentation of aceruloplasminemia deserve further discussion. Although the mechanisms of late-onset neurologic degeneration are not known, this delay in presentation is consistent with that observed in other degenerative diseases of the basal ganglia, such as Parkinson and Huntington diseases. This late onset is a potential mechanism for persistence of this recessive mutation in the population because symptoms appear after the onset of reproductive age. These issues highlight the potential importance of genetic screening, which despite the presence of multiple mutations may be possible because both the heterozygote and homozygote state can be detected by a simple assay for serum ceruloplasmin oxidase activity. The potential role for dietary manipulation to prevent or ameliorate this disease further emphasizes the need for early diagnosis, as does our recent finding of a symptomatic heterozygote who received prolonged iron therapy in childhood (Z.L.H., and J.D.G., unpublished work). This latter observation suggests an analogy to familial hypercholesterolemia, where dietary intervention can greatly affect phenotypic expression in the heterozygote state. Because patients with aceruloplasminemia will appear to have typical iron-deficiency anemia, the potential problem of misdirected iron therapy is apparent. Although the incidence of this disease is not known, in the past 4 mo since initiating this work, we have become aware of more than a dozen families in different parts of the world. Accepting the incidence observed in the original studies on hereditary hypoceruloplasminemia (now believed to be the heterozygote state), these observations and the clinical and laboratory presentations suggest that additional screening of patients with uncharacterized movement disorders or atypical Wilson disease is warranted. The potential role for treatment of such patients with ceruloplasmin is also clearly an issue for future study.

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