A Human Mitochondrial Ferritin Encoded by an Intronless Gene*

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Ferritin is a ubiquitous protein that plays a critical role in regulating intracellular iron homeostasis by storing iron inside its multimeric shell. It also plays an important role in detoxifying potentially harmful free ferrous iron to the less soluble ferric iron by virtue of the ferroxidase activity of the H subunit. Although excess iron is stored primarily in cytoplasm, most of the metabolically active iron in cells is processed in mitochondria. Little is yet known of how these organelles regulate iron homeostasis and toxicity. Here we report an unusual intronless gene on chromosome 5q23.1 that encodes a 242-amino acid precursor of a ferritin H-like protein. This 30-kDa protein is targeted to mitochondria and processed to a 22-kDa subunit that assembles into typical ferritin shells and has ferroxidase activity. Immunohistochemical analysis showed that it accumulates in high amounts in iron-loaded mitochondria of erythroblasts of subjects with impaired heme synthesis. This new ferritin may play an important role in the regulation of mitochondrial iron homeostasis and heme synthesis.

The biological advantages of iron in redox reactions and as an oxygen carrier are offset by the interaction of ferrous ions with reactive oxygen species to produce harmful radicals that damage membranes, proteins, and nucleic acids and have been implicated in neurodegenerative diseases and in apoptosis (reviewed in Ref. 1). With the exception of yeast, most organisms rely on ferritin to buffer free cytosolic iron. This highly conserved protein consists of large multimeric shells that accommodate up to 4500 atoms of iron (reviewed in Ref. 2). Iron is taken up in the ferrous form and deposited as ferric hydroxides after oxidation by catalytic ferroxidase sites. Release of iron is effected by reducing agents without shell breakdown (3, 4). Mammalian ferritins consist of variable amounts of two subunit types, H and L, in a 24-subunit shell (5). The H chain has the ferroxidase activity that is responsible for the cytoprotective action of ferritin and its central role in cellular processes (2). Up-regulation of the H chain reduces free iron levels with a consequent reduction in proliferation rate and increased resistance to oxidative damage (6). Down-regulation increases free iron and is associated with increased apoptosis and cell proliferation and is also critical for cell transformation by c-MYC (7, 8). Inactivation of H ferritin (HF1) in knockout mice is lethal at early stages of embryogenesis (9).

Mitochondrial iron toxicity should be a particular concern in erythroid cells that have to process >80% of body iron flux, but it is not yet known how this iron is maintained in a nontoxic form. Massive increases in mitochondrial iron occur when heme synthesis is blocked in sideroblastic anemia (10–12) and yet the mitochondria survive. Much of this iron has the characteristic electron microscopic appearance of ferritin (11), but this material does not stain with antibodies to cytoplasmic H and L ferritins (12), and its form has remained an enigma. Here we describe an unusual gene that encodes a mitochondrial ferritin (MtF) that has ferroxidase activity. The levels of MtF increase dramatically in sideroblastic anemia, suggesting that this new ferritin is likely to be important in the trafficking of iron in mitochondria as well as its detoxification.

EXPERIMENTAL PROCEDURES

PCR Analyses—The following primers derived from AA469940 were used to amplify fragments from human genomic DNA or human cDNA libraries: T1, TAATTCCTTCCAGCTCAGG (372–392); T2, TGAAGATGGGCCCCGCCTAGG (672–699); TIR, GCAGGGACAGCCTGACCTGTG (824–830); and T3R, TGGGAGAATGTATAACAC (875–885). PCR conditions were 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s. Genomic Southern—Southern analysis of DNA from normal human lymphocytes digested with EcoRI or XbaI was performed as described (13). The DNA was probed with the c.345-P-labeled 4′UTR probe and with the c.299-P-labeled 3′UTR probe (MtF) generated from AA469940 by PCR amplification using primers T2′T3R.

RNA Analyses—A human poly(A)+ RNA Northern blot from Origene (Rockville, MD) was hybridized with the 3′-UTR probe (above). After overnight hybridization using the ultrahyb buffer (Ambion) at 66 °C, the blot was washed twice with 0.5× SSC, 0.1% SDS at 68 °C and exposed to Kodak Biomax Ms for 8 h.

Expression in HeLa Cells—The DNA encoding the entire precursor protein was cloned into pcDNA3 vector (Invitrogen). The DNA fragment encoding the first 67 N-terminal amino acids was subcloned and linked to the 3′-UTR from MtF

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1 The abbreviations used are: HF, H ferritin; LF, L ferritin; Mt, mitochondrial; MtF, mitochondrial ferritin; r, recombinant; PCR, polymerase chain reaction; UTR, untranslated region; GFP, green fluorescent protein; contig, group of overlapping clones; PAGE, polyacrylamide gel electrophoresis.
microscope (Zeiss) with a 565-nm filter for rhodamine and with a 516-nm filter for MitoTracker Green.

Expression in Erythroid Cells—Erythroid cells from normal marrow donors and patients with sideroblastic anemia were analyzed for cytoplasmic HF. Using a monoclonal mouse antibody as described (12) and for MtF, with polyclonal rabbit antibody anti-rMtF antisera. Bound antibody was detected by an immunofluorescent method. For negative controls nonimmune mouse or rabbit serum was substituted for the primary antibody.

Expression in Escherichia coli—rMtF was produced in E. coli by subcloning the DNA encoding amino acids 70–242 of the predicted precursor into pet expression vector (Novagen). Cell transformation and protein expression were performed as described (15). Antibodies to electrophoretically pure rMtF were raised in rabbits.

Protein Labeling—The pcDNA3MtF was transcribed and translated in the TNT T7/T3-coupled reticulocyte lysate system L5010 obtained from Promega (Madison, WI) in the presence of [35S]methionine, according to the manufacturer’s instructions. Ferritin was labeled in vivo by incubating transfected HeLa cells with [35S]methionine for 18 h and was isolated as described (14).

In Vitro Iron Uptake—Apoferritins (1 μM final concentration) were incubated aerobically with 1 μM ferrous ammonium sulfate in 0.1 M HEPES buffer, pH 7.0, for 2 h at room temperature. The proteins were separated on 7% native polyacylamide gels and stained for protein or iron (14).

RESULTS

Gene—BLAST searches of the GenBank™ human EST data base with the cDNA for human HF, pHF16 (13), identified clones AA469940, AI024273, and AI149710 from a testis library with about 80% homology to human HF. The extended and corrected sequences indicated that all represent the same mRNA and have the same predicted C terminus as HF. Although none has a complete coding sequence, that in AI49710 extended above the N terminus of HF. BLAST searches of the high throughput gene sequence data base of GenBank™ with this cDNA contig identified an identical 754-nt sequence in the BAC clone (AC011181), which is now contained in Hs5_23264 mapping at 5q21.3. The initiating codon in this genomic sequence was predicted to be 60 residues above that of HF and was located about 30 nt downstream from the initiation site of transcription of the H gene. Two mouse cDNAs (AK0105400 and AK015346) from a testis library have recently been reported that are similar to the new human ferritin (see below).

There is no corresponding gene yet in public mouse data bases. Amplification of human genomic DNA with primers T1/T1R from the cDNA produced a single 452-base pair fragment with the same sequence as the published genomic sequence. Southern analyses of EcoRI and XbaI digests of human genomic DNA with a probe from the predicted 3′-UTR gave single hybridizing bands of about 5.5 and 10.5 kb, respectively (Fig. 1A), consistent with the restriction map of the BAC. These results confirm that this is a single copy intronless gene.

The genomic sequence has a short poly(A) sequence in the 3′-UTR in the same position as in the cDNA. This is immediately followed by a sequence, AAGTTTGGCCCA, which has a possible counterpart, TCGATTCCCCCA, 25 nt above the initiating ATG and close to the transcription initiation site of the H gene. Both features are characteristics of a processed pseudogene (18, 19). However, the mouse cDNA has a similar sequence TCGATTCCCCCT in the same position above the ATG. This region therefore seems more likely to be part of the human transcript than a flanking genomic repeat. There is no apparent iron-responsive element (IRE) for translational control by iron (20) in the 400-nt region above the coding region or in the mouse orthologs. However, there is weak homology with the IRE of the H mRNA in the Mt sequence immediately after the initiating AUG (AUGCUGUCCGUCCGCGCCUCUCUCAGGCACATC versus GGGGUGUCCUCCUCUCUCUCAGCAATCGCUUUGGACCGGAACC). 10 of the first 14 bases of the 5′ stem are conserved, but the canonical CAGUG loop (underlined) and the

3′ stem region are largely substituted. This result suggests that much of the 3′-UTR of an H-like sequence, including the IRE, mutated to form a leader sequence in MtF. The lack of an apparent IRE indicates that the expression of MtF will be not be translationally controlled by iron.

Transcript—Northern analysis of mRNA from different human tissues using the 3′-UTR probe showed that the polyadenylated mRNA corresponding to MtF was slightly above 1 kb (Fig. 1B). This is similar in size to H- and L-mRNAs (21). However, in contrast to the ubiquitous H- and L-mRNAs, MtF has a different expression pattern. As expected from EST representation, the transcript is expressed in testis, but levels are low in iron storage organs such as the liver and spleen and in other tissues represented in this blot.

Protein—in overlapping coding regions, MtF is 79% identical to HF and 63% to LF. The seven amino acids responsible for the ferroxidase activity in HF are conserved in MtF (Fig. 2). In the coding region, the mouse ortholog is 84% identical to human MtF and 78% identical to mouse HF and human HF and also has a conserved ferroxidase center. The predicted protein leader of 56 residues compares with that of 57 for human MtF.

Mitochondrial Localization—the long N-terminal extension suggested a leader sequence. A structural model in α helical formation with the Insight II program (Molecular Simulations) predicted four equally spaced arginines on one face, a characteristic of a mitochondrial targeting sequence. The Mitoprot II program (22) also indicated mitochondrial targeting and predicted a cleavage site at residue 57, three residues before the
A Mitochondrial Ferritin Encoded by an Intronless Gene

42439

FIG. 3. Mitochondrial localization. A, HeLa cells were transfected with a construct encoding the first 67 residues of the MtF fused to GFP and its distribution (i) compared with that of mitochondrial marker, MitoTracker II orange (ii). B, distribution of MtF in HeLa cells expressed from a construct, pcDNA3-MtF, encoding the complete MtF precursor and analyzed 24 h after transfection. 1, stain with anti-r9MtF antibody followed by rhodamine-labeled secondary antibody. ii, the same field stained with MitoTracker Green FM. The two stains overlap.

FIG. 4. Immunological properties, processing, and ferroxidase activity of MtF. A, specificity of anti-r9MtF antibody. Purified rHF, rLF, and r9-MtF were run in duplicate sets on 7% nondenaturing PAGE. One portion of the gel was stained with Coomassie Blue, and the other was immunoblotted, probed with anti Δ9-Mt antibody (1/2000 dilution), and developed with ECL. Samples: r9-MtF (lanes 1 and 4), rHF (lanes 2 and 5), and rLF (lanes 3 and 6). All wells were loaded with 1 μg of purified protein, except for lane 1, which was loaded with 0.2 μg. Minor bands in rHF and rLF represent higher polymers (23). B, processing of MtF. SDS-PAGE analyses of cytosolic and mitochondrial ferritins. HeLa cells were transfected with pcDNA3-MtF and metabolically labeled with [35S]methionine for 18 h. Extracts were first cleared of cytosolic ferritins by precipitation with antibodies to human L ferritin. HeLa cells were transfected with pcDNA3-MtF and metabolically labeled with [35S]methionine, immunoprecipitated with anti-r9MtF, and the precipitate separated on SDS-PAGE. This product was compared with that obtained by in vitro translation of mRNA from the same construct and in the absence of mitochondria. These experiments showed that the 30-kDa precursor protein was processed in cells to a 22-kDa peptide. The processed subunit was slightly larger than the H subunit and was not recognized by antibodies to human L ferritin that precipitated the cytoplasmic H and L ferritins (Fig. 4B). We conclude that the precursor for the new ferritin (MtF) is targeted to mitochondria and is appropriately processed.

To explore its structural properties, the fragment 70–242, corresponding to residues 10–182 of the H chain, was expressed in E. coli. The protein (r9MtF) accumulated in the soluble fraction as an assembled ferritin (see below) and could be purified with procedures (14) used for recombinant H and L ferritins (rHF and rLF). It was reversibly denatured in acidic 8 M guanidine HCl, and, when renatured together with H or L subunits, formed heteropolymers (not shown).

Iron Uptake—The r9MtF isolated from E. coli stained with Prussian blue, indicating that it incorporated iron in vivo, an index of ferroxidase activity (2). To compare this activity with HF and LF, purified r9MtF was made iron-free and incubated at pH 7.0 with ferrous iron in the presence or absence of equimolar amounts of iron-free rHF and rLF. Prussian blue staining of the PAGE-separated shells showed that r9MtF incorporated similar amounts of iron to rHF and much more than rLF (Fig. 4C), consistent with the conservation of residues Glu47, Tyr34, Glu43, Glu62, His105, Glu107, and Gln141 that act as iron ligands in the ferroxidase center of HF (2).

Expression in Erythroid Cells—MtF sequences were identified in PCR products amplified with primers T1/T1R from a cDNA library from the erythroblastic cell line K562 cells. This finding, together with the electron microscopic evidence from erythroid cells (11), suggested that MtF might be expressed in erythroid cells. We therefore examined levels of MtF in bone marrow smears from healthy donors and from individuals with X-linked sideroblastic anemia (XLSA) arising from mutations in the erythroid-specific 5-aminolevulinic acid synthase. This mutation causes both anemia and mitochondrial iron loading in marrow erythroblasts (12). As shown in Fig. 5A, normal erythroblasts showed very light staining. By contrast,

start of the H chain. The DNA fragment encoding the first 67 amino acids was therefore fused to GFP cDNA and the construct expressed in HeLa cells. Fluorescent imaging of cells transfected with the parent GFP under the same promoter but without the leader sequence showed a diffuse cytoplasmic stain (not shown). By contrast the construct with the attached leader showed accumulation of GFP in filamentous intracellular bodies characteristic of mitochondria, and this pattern overlapped completely with that of a specific mitochondrial stain (Fig. 3A). This localization was confirmed by transfecting a construct containing the entire coding region of MtF into HeLa cells and examining the distribution of the expressed protein with anti-r9MtF, prepared against, and specific for, the new ferritin. This antiserum recognized r9Mt MtF but not rHF or rLF (Fig. 4A) and showed (Fig. 3B) that the distribution of the expressed MtF coincided with that of the mitochondrial marker.

Processing—To determine the size of mature MtF, transfected HeLa cells were metabolically labeled with [35S]methionine, immunoprecipitated with anti-r9MtF, and the precipitate separated on SDS-PAGE. This product was compared with that obtained by in vitro translation of mRNA from the same construct and in the absence of mitochondria. These experiments showed that the 30-kDa precursor protein was processed in cells to a 22-kDa peptide. The processed subunit was slightly larger than the H subunit and was not recognized by antibodies to human L ferritin that precipitated the cytoplasmic H and L ferritins (Fig. 4B). We conclude that the precursor for the new ferritin (MtF) is targeted to mitochondria and is appropriately processed.

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erythroblasts from patients with XLSA showed a dense granular, perinuclear staining (Fig. 5B). This pattern is consistent with a mitochondrial localization and is very similar to that given by ringed sideroblasts by iron staining (11, 12). Staining with anti-HF antibodies showed a diffuse cytoplasmic distribution (not shown) as reported previously (12). These results strongly suggest that much of the deposition of iron in mitochondria is present in this new ferritin.

**DISCUSSION**

We describe a new human ferritin (MtF) that is expressed as a precursor, targeted to mitochondria, and then processed into a functional protein with a structure similar to the ferritins in cytoplasm. Like the cytosolic HF, MtF incorporates iron in vivo and in vitro, consistent with its conserved ferroxidase center. The major difference is in the quaternary structure: the cytosolic ferritins are heteropolymers, while those in mitochondria are homopolymers, since the subunits assemble only after processing in the mitochondria. The discovery of MtF solves some old puzzles. About 40 years ago, iron deposits in mitochondria were identified as ferritin cores by electron microscopy (11), but this view was challenged because of negative immunological findings and because it lacked a molecular basis for mitochondrial targeting. Our finding of an immunologically distinct ferritin with a targeting leader sequence resolves both paradoxes.

This is the first example of a mammalian ferritin that is specifically targeted to an organelle, although it has parallels with organelle-specific ferritins in other organisms such as the plastid ferritin in plants, hemolymph ferritin in insects, and yolk ferritin in snails (2). Compared with the ubiquitous HF and LF, MtF appears to have a limited tissue distribution. Its relatively high expression in testis does not seem to reflect the levels of mitochondria, since other mitochondria-rich tissues, such as brain, have low MtF levels. Nor does its level correlate with cytosolic iron content, since MtF is also low in the liver and spleen. Further experiments in animal models would be helpful in elucidating expression patterns in different physiological states.

Our findings may be relevant to iron trafficking in erythroid cells. Ferrous iron is efficiently incorporated into heme by ferrochelatase. If this reaction is temporarily blocked, the iron accumulated in the mitochondrial matrix is available for heme synthesis when the block is removed (24). Although this iron seems likely to be in ferritin. Iron flux through mitochondrial ferritin may therefore, in some situations, play a role in regulating heme synthesis, a major determinant in the overall control of hemoglobin synthesis (24). Since iron uptake into ferritin requires oxygen and its release requires reductants, the trafficking of iron to ferrochelatase or to ferritin may be affected by the redox state of the organelle.

The finding of high levels of MtF in sideroblasts but not in normal erythroblasts suggests that MtF expression increases with mitochondrial iron loading. The response to iron is usually attributed to increased rates of translation due to removal of a protein that binds the IRE in the ferritin mRNA (20). However, there is no apparent IRE in the gene for the mitochondrial ferritin, and some other mechanism, possibly transcriptional, seems likely. The absence of an IRE in the transcript for MtF but not for HF or LF has parallels with other differentially expressed proteins of iron metabolism. The response of MtF and others to iron overload is not uniform, and it has been suggested that frataxin substitutes for ferritin to detoxify mitochondrial iron in mammalian cells (16). It will be interesting to determine whether levels of MtF are also increased in this disorder and whether the two proteins are metabolically related.

The origin of the MtF gene and its evolutionary relationship to H and L genes remain a puzzle. The human genome contains multiple copies of H- and L-like sequences, but only one member of each family is known to be functional. Both have introns with similar arrangements (21). The other members seen by Southern analyses with the H- and L-cDNAs are intronless and appear to be processed pseudogenes (19). None has a counterpart in cDNA databases, and all are therefore presumed to be nonfunctional. Although the MtF gene has some characteristics of a processed pseudogene, it is clearly not an inactive relic whose expression can be artificially forced by transfection, since we show regulated expression of the protein in untransfected cells. It could conceivably be derived from a putative bacterial precursor of mitochondria, but its sequence is more similar to mammalian ferritins (>75% identity) than to any known bacterial ferritin (<30% identity). This suggests that it is more likely to have arisen from an ancient H-like sequence that acquired a functional promoter. The presence of MtF in rodents indicates that it is evolutionarily relatively old and arose before the divergence of primates and rodents about 112 million years ago (17). Preliminary analyses of the putative promoter region show potential binding sites for erythroid-specific factors such as GATA and NFE2, but the functionality of these remains to be determined. In conclusion, the finding of different ferritins in different cellular compartments, and under different regulation, offers exciting prospects for selectively modulating cellular iron homeostasis.

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