Human Mitochondrial Ferritin Expressed in HeLa Cells Incorporates Iron and Affects Cellular Iron Metabolism*

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Mitochondrial ferritin (MtF) is a newly identified ferritin encoded by an intronless gene on chromosome 5q23.1. The mature recombinant MtF has a ferroxidase center and binds iron in vitro similarly to H-ferritin. To explore the structural and functional aspects of MtF, we expressed the following forms in HeLa cells: the MtF precursor (~28 kDa), a mutant MtF precursor with a mutated ferroxidase center, a truncated MtF lacking the ~6-kDa mitochondrial leader sequence, and a chimeric H-ferritin with this leader sequence. The experiments show that all constructs with the leader sequence were processed into ~22-kDa subunits that assembled into multimeric shells electrophoretically distinct from the cytosolic ferritins. Mature MtF was found in the matrix of mitochondria, where it is a homopolymer. The wild type MtF and the mitochondrially targeted H-ferritin both incorporated the $^{55}$Fe label in vivo. The mutant MtF with an inactivated ferroxidase center did not take up iron, nor did the truncated MtF expressed transiently in cytoplasm. Increased levels of MtF both in transient and in stable transfectants resulted in a greater retention of iron as MtF in mitochondria, a decrease in the levels of cytosolic ferritins, and up-regulation of transferrin receptor. Neither effect occurred with the mutant MtF with the inactivated ferroxidase center. Our results indicate that exogenous iron is as available to mitochondrial ferritin as it is to cytosolic ferritins and that the level of MtF expression may have profound consequences for cellular iron homeostasis.

Ferritins are ubiquitous proteins made of 24 subunits that form a spherical shell that can accommodate up to 4,000 iron atoms (reviewed in Ref. 1). In mammals, nearly all of the ferritin is found in cytoplasm, where its expression is controlled translationally by iron through an iron regulatory element in the mRNA (2, 3). This ferritin is composed of two subunit types, H and L, with ~50% sequence identity and very similar three-dimensional structures made of a bundle of four $a$-helices. H-ferritin shells have ferroxidase activity that results in the conversion of soluble ferrous ions into inert aggregates of ferric hydroxides (4–6). This ferroxidase activity is associated with di-iron binding sites coordinated by seven residues that are conserved in ferritins from animals, plants, and bacteria (1, 7). These sites catalyze Fe(II) oxidation, a rate-limiting step in iron incorporation, in a reaction that consumes one dioxygen molecule per two Fe(II) ions and produces hydrogen peroxide (1, 6, 8). The L-subunit lacks the ferroxidase center, and L-homopolymers do not incorporate iron in vivo. However, the L-subunit provides efficient sites for iron nucleation and mineralization and somehow increases turnover at the H-ferroxidase centers (4–6).

The ferroxidase activity of the H-chain is largely responsible for the biological activity of mammalian ferritins. Inactivation of H-chains in knockout mice is lethal at early stages of embryogenesis (9). Overexpression of H-chains in stable transfectants of the mouse erythroleukemic (MEL) cell line (10–12) and HeLa cells results in an iron-deficient phenotype (13). This is accompanied by reductions in heme and hemoglobin synthesis and also in proliferation rate, a reduction multidrug resistance, and a reduction of oxidative damage from free iron (10–13). These effects are abolished by iron supplementation or by the mutational inactivation of the ferroxidase center (13). Other than facilitating iron deposition, little is yet known of the biological role of L-chains. Large increases in L-ferritin levels occur as a result of mutations in the iron regulatory element. These increases cause catacausts but no apparent abnormalities in body iron metabolism (14, 15). However, a mutation in the C-terminal sequence of the L-chain causes a neurological disorder with increased deposition of ferritin and iron in the basal ganglia of the brain (16).

We have recently identified a new human ferritin, MtF,¹ that is encoded by an intronless gene on chromosome 5q23.1 and a mouse ortholog (17). Human MtF is synthesized as a 242-amino acid precursor with a long N-terminal sequence for mitochondrial import (17). Experiments with transfectant cells showed that this precursor is efficiently targeted to mitochondria and processed into typical ferritin shells. The amino acid sequence of the predicted mature protein overlaps the H sequence with 77% identity and contains all the residues of the

¹ The abbreviations used are: MtF, mitochondrial ferritin; T-MtF, truncated MtF; Mt, mitochondrial ferritin; HF, H-ferritin; LF, L-ferritin; DMEM, Dulbecco’s modified Eagle’s medium; FAC, $[^{55}]$FeFerric ammonium citrate; ELISA, enzyme-linked immunosorbent assay; DFO, desferrioxamine.
ferroxidase center. The mature protein produced in Escherichia coli incorporated iron in vitro, indicating that it has ferroxidase activity (17). As judged from mRNA levels, MtF is expressed at low levels in most cells except testis. MtF is present at a low level in normal erythroblasts, but this level increases dramatically in iron-loaded erythroblasts from patients with sideroblastic anemia (17). This increased expression does not appear to be due to the typical translational control since MtF mRNA lacks the classical stem-loop iron regulatory element.

The function and regulation of this new ferritin have not been established. Mitochondria are exposed to a heavy traffic in iron for the synthesis of heme and Fe/S clusters. Mitochondria are also the major sites of reactive oxygen species production (18–20) and presumably must have efficient mechanisms to sequester Fe(II) from reactive oxygen species (particularly H₂O₂) to prevent the production of highly toxic hydroxyl radicals in Fenton-type reactions. Iron homeostasis in mitochondria also differs from that in the cytoplasm. Iron deprivation affects mitochondrial iron enzymes less than cytosolic iron enzymes (21). By contrast, excess iron is not usually deposited in mitochondria but is deposited in the cytosol as ferritin.

Although iron does not normally accumulate in mitochondria, defects in its transport or utilization in mitochondria can result in mitochondrial iron loading. Visible granular iron deposits are formed inside the mitochondria of erythroblasts with defective heme synthesis as in subjects with sideroblastic anemia (22, 23). Much of this iron is probably present as MtF (17). Iron also accumulates in the mitochondria of patients with Friedreich’s ataxia resulting from defects in the synthesis of frataxin (24) or in sideroblastic anemia with ataxia from defects in the Fe/S transporter ABC7 (25). The form of this iron is not known, but the iron overload is associated with a decrease in respiratory chain and aconitase activity, probably from iron-induced oxidative damage (26).

Very little is yet known about how iron is delivered to mitochondria and whether it is normally accessible to MtF. It is also not known whether MtF responds to changes in cellular iron or whether its level affects the partitioning of cellular iron. This report explores some of these issues through analyses of different forms of MtF and H-ferritins transfected into HeLa cells. We show that MtF readily incorporates iron inside mitochondria by a process similar to that of H-ferritins. Unlike cytoplasmic ferritins, the levels of MtF are not increased by exogenous iron. However, when increased by transfection, MtF retains a high proportion of available iron, and cells show signs of iron deficiency. We conclude that iron is potentially as accessible to MtF as it is to cytosolic ferritin and that the control of MtF levels may offer a powerful method for regulating cellular iron homeostasis.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—The vector pcDNA3.1 was purchased from Invitrogen. Monoclonal antibodies, rH02 and LF03, prepared against human ferritin H- and L-chains, respectively, have been described previously (27, 28) as has the rabbit antisera, anti-r9MtF elicited by a truncated form of MtF corresponding to residues 10–182 of the H-chain (17). A more potent antisera, anti-MtF, was elicited in mice by injecting the full mature form of recombinant MtF. Monoclonal rH02 recognizes H- but not L-ferritins and also cross-reacts with MtF (17), whereas LF03 is specific for L-ferritins. Both antisera recognized MtF, as well as the mature forms of recombinant MtF and H-ferritins transfected into HeLa cells. Monoclonal rH02 monoclonal antibody was purchased from Zymed Laboratories Inc. (San Francisco, CA).

Plasmid Construction and Cell Culture—The pcDNA3MtF vector, encoding the entire precursor MtF protein, was described in Ref. 17. The MtF₀₂₂₂ mutant (E62K, H65G, H-chain numbering) with an inactivated ferroxidase activity was produced by oligonucleotide-directed mutagenesis of pcDNA3MtF. The chimera Mt-HF was constructed by fusing the mitochondrial leader peptide of MtF (residues 1–60) to the full human H-ferritin chain sequence. The plasmid for the truncated MtF (T-MtF) was constructed by subcloning into pcDNA3 the sequence encoding residues 2 to 182 (H-chain numbering). To obtain stable transfectants, the full coding regions of MtF and of the MtF₀₂₂₂ mutant were subcloned into pUDH10–3 vector (CLONTECH) (29) under the control of the tTA promoter to obtain pUD-MtF and pUD-MtF₀₂₂₂ plasmids.

HeLa cells were transfected with calcium phosphate as in Ref. 30 and grown in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM l-glutamine. Typically in transient experiments, 10⁶ cells were transfected with 10 μg of pcDNA3 plasmid containing the ferritin cDNA or with the pcDNA3 vector for control. Transfection efficiency was monitored by immunofluorescence staining with anti-9MtF antisera and ranged between 20 and 30% of cells. A stable HeLa-tet Off cell line was generated and selected as described in Ref. 13. The HeLa-tet Off cells (CLONTECH) were co-transfected with 3.5 μg of pUD-MtF or pUD-MtF₀₂₂₂ plasmids and with 1 μg of pTK-Hyg plasmid (5:1 molar ratio) (CLONTECH). Clones expressing MtF and MtF₀₂₂₂ were selected and maintained in DMEM supplemented with 10% fetal bovine serum, 100 μg/ml G418 (Geneticin, Sigma), 150 μg/ml hygromycin D (CLONTECH), 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM l-glutamine. In the presence of doxycycline (2 ng/ml, Sigma), the protein synthesis was repressed, whereas in its absence, the synthesis was induced.

Ferritin Evaluation and Immunoblotting—The levels of cytosolic ferritins were assayed in extracts of 10⁶ cells with ELISA assays using the monoclonal antibody rH02 calibrated on the recombinant homopolymer (28). Purified recombinant MtF was not recognized by L-ferritin ELISA, but it gave a signal in the H-ferritin ELISA that corresponded to 1% of the ferritin content and was also recognized by this antibody in Western blots. Protein concentration was evaluated by the BCA method (Pierce).

Cellular ⁵⁵Fe Incorporation—In experiments with transient transfectants, the cells (2 × 10⁵) were transfected with 2 μg of DNA plasmid and grown for 30 h in complete medium. Stable transfectants were induced to express ferritin by omitting doxycycline for 7 days. The cells were then incubated for 18 h, or the indicated time, with 2 μCi/ml [⁵⁵Fe]ferrous ammonium citrate (FAC) (ratio 1:2), 200 μM ascorbic acid, or 1 μM Fe-labeled transferrin in DMEM, 0.5% fetal calf serum, 0.5% bovine serum albumin. The cells were washed and lysed in 0.3 M ascorbic acid buffer. After centrifugation, 10 μl of the soluble fraction were mixed with 0.3 ml of Ultima Gold (Packard) and counted for 1 min in a scintillation counter (Packard). The soluble proteins were analyzed also by PAGE in 7% non-denaturing gels. Nitrocellulose filters from the blotted gel were incubated with rabbit anti-9MtF antisera (dilution 1:2,000) or rH02 monoclonal antibody (dilution 1:1,000) followed by peroxidase-labeled antibody (Sigma). The bound peroxidase was revealed by ECL (Amersham Biosciences).

Mitochondrial Enrichment—Transfectant cells were grown for 18 h in the presence of 2 μCi/ml FAC (ratio 1:2), 200 μM ascorbic acid, and mitochondrial fraction enriched as described previously (31). Briefly, the cells were washed twice in phosphate-buffered saline and lysed on the plate using 0.007% digitonin in 0.25 M sucrose, 10 mM Hepes, pH 7.4, 0.15 M bovine serum albumin. Unbroken cells and nuclei were first cleared by centrifugation at 1,000 × g for 10 min, and the mitochondria were precipitated by a further centrifugation at 3,000 × g for 10 min at 4 °C. The cytosolic supernatants (post-mitochondrial fractions) and the mitochondrial pellet (mitochondrial fractions) were analyzed directly or heated at 75 °C for 10 min for ferritin enrichment. The heat-stable proteins were separated by PAGE in 7.5% non-denaturing gels and exposed to autoradiography.

Metabolic Labeling and Immunoprecipitation—After transient transfection, the cells (5 × 10⁶) were grown for 30 h, or the stable clones were grown for 7 days in the absence of doxycycline. Then, they were incubated for 1 h in DMEM, methionine, and cysteine-free (ICN) 0.5% fetal calf serum, 0.5% bovine serum albumin and labeled with 18 μCi/ml [⁵⁵S]methionine, [⁵⁵S]cysteine (SL-15) in the same medium (13). The cells were washed with phosphate-buffered saline and then lysed with 500 μl of lysis buffer (20 mM Tris-HCl, pH 8.0, 200 mM LiCl, 1 mM EDTA, 0.5% Nonidet P-40). Total radioactivity associated with the soluble proteins was determined by trichloroacetic acid precipitation. For immunoprecipitation studies, 4 × 10⁶ cpm of cytosolic lysates were precladated by incubation with 30 μl of protein A-Sepharose 50% v/v (Sigma) for 1 h at 4 °C with gentle shaking and centrifuged for 10 min at 500 × g before use.
Characterization of Transfected MtF—The cDNA for the human MtF precursor was subcloned into pcDNA3 vector to transiently transfet HeLa cells. MtF protein expression was first analyzed using anti-MtF antibodies by Western blot after separating cell extracts on non-denaturing PAGE. No MtF was detected in the untransfected HeLa cells, but high levels were found in the transfectants (Fig. 1A, lanes 1 and 2). The single band in the transfectants had a similar, but slightly slower, mobility than that of the cytoplasmic ferritin shown in Fig. 1A (lane 3), indicating that MtF has a similar multimeric structure. To explore iron uptake into MtF, cells were incubated with the 55Fe label, supplied as FAC, for 18 h. Cells and organelles were lysed with 0.5% Nonidet P-40, and the proteins in supernatant fractions were separated on non-denaturing gels. To confirm the identity of the bands, cytosolic ferritin heteropolymers were first precipitated from the cell extracts with polyvinylpyrrolidone and then brought to 1.86 M sucrose and 20% polyacrylamide by sucrose. Then, anti-ferritin L-chain monoclonal antibody was added, incubated for 1 h followed by protein A-Sepharose (30 μl). The immuno complexes were washed, resuspended in SDS buffer, boiled for 10 min, and loaded on 12% SDS-polyacrylamide gel. The gels were treated with autoradiography image enhancer (Amplify, Amer sham Biosciences), dried, and exposed.

RESULTS

Characterization of Transfected MtF—The eDNA for the human MtF precursor was subcloned into pcDNA3 vector to transiently transfet HeLa cells. MtF protein expression was first analyzed using anti-MtF antibodies by Western blot after separating cell extracts on non-denaturing PAGE. No MtF was detected in the untransfected HeLa cells, but high levels were found in the transfectants (Fig. 1A, lanes 1 and 2). The single band in the transfectants had a similar, but slightly slower, mobility than that of the cytoplasmic ferritin shown in Fig. 1A (lane 3), indicating that MtF has a similar multimeric structure. To explore iron uptake into MtF, cells were incubated with the 55Fe label, supplied as FAC, for 18 h. Cells and organelles were lysed with 0.5% Nonidet P-40, and the proteins in supernatant fractions were separated on non-denaturing gels. To confirm the identity of the bands, cytosolic ferritin heteropolymers were first precipitated from the cell extracts with polyvinylpyrrolidone and then brought to 1.86 M sucrose and 20% polyacrylamide by sucrose. Then, anti-ferritin L-chain monoclonal antibody was added, incubated for 1 h followed by protein A-Sepharose (30 μl). The immuno complexes were washed, resuspended in SDS buffer, boiled for 10 min, and loaded on 12% SDS-polyacrylamide gel. The gels were treated with autoradiography image enhancer (Amplify, Amer sham Biosciences), dried, and exposed.

Iron Uptake—To compare iron uptake into the transfected ferritins, the soluble fractions of the lysates of transfecant cells labeled with FAC were separated on non-denaturing PAGE, blotted, and probed with anti-MtF antibodies by Western blot. The transfected ferritins were identified with monoclonal rH02 that reacts with human H-ferritin and also with antibody essentially eliminated the upper band specific to anti-L-ferritin antibody (al). Bound radioactivity was revealed by autoradiography. A, lanes labeled with 55Fe as described in panel A were lysed with digitonin, and the mitochondrial fraction (MF) was separated from the post-mitochondrial fractions (PMF) by sequential centrifugation. The two fractions were heated at 75 °C, and 10 μg of the heat-stable proteins were resolved on non-denaturing PAGE and exposed to autoradiography. The arrow indicates the mobility of MtF and cytosolic ferritin (H/LF).

Analyses of MtF Mutants—To explore structural and functional elements for iron uptake into MtF, different constructs were expressed in HeLa cells. MtFex22 has Glu-62 → Lys and His-65 → Gly (H-chain numbering), which inactivate the ferroxidase activity of human H-ferritin (13). T-MtF represents the predicted mature protein lacking the mitochondrial targeting sequence and starting at position 2 (H-chain numbering). Finally, Mt-HF has the N-terminal MtF sequence (residues 1–60) fused to the H-chain and predicted to be cleaved at residue 58. Transfected ferritins were identified with monoclonal rH02 that reacts with human H-ferritin and also with MtF (17). Western analyses of cell lysates showed that all four transfected ferritins were detected with this antibody and are therefore expressed. MtF, MtFex22, and Mt-HF had a similar mobility, whereas T-MtF was faster and co-migrated with the cytosolic ferritin (Fig. 2A). In addition, this bloting and that of Fig. 2C (bottom panel) indicate that the transfected ferritins accumulate in the cells at levels much higher than those of the endogenous cytosolic H-ferritins. In the absence of an ELISA assay, it could not be quantitated.
Mitochondrial Ferritin Expression in HeLa Cells

To determine whether TfF expression affected cellular iron metabolism, we

1. **Expression and iron incorporation of TfF mutants.**
   - HeLa cells (2 × 10⁶) were transiently transfected with 2 μg of the plasmids encoding for MtF (Mt), its mutant with substitutions E62K and H65Q (H-chain numbering) to inactivate the ferroxidase center (MtE62K, MtH65Q) and the chimeric construct for the mitochondrial leader sequence fused to H-subunit (MtH). Soluble proteins from cell homogenates were analyzed on non-denaturing gels as described in the legend for Fig. 2. A, cells were harvested 48 h after transfection. 10 μg of the soluble proteins were resolved by PAGE, and the proteins were revealed by blotting with rH02 antibody, which recognizes HF and MtF using ECL development. B, after transfection, the cells were metabolically labeled for 18 h with ³²Fe as described in the legend for Fig. 1, A, and homogenized, and 10 μg of protein soluble extracts were resolved by PAGE and exposed to autoradiography. C, control; H/LF, cytosolic ferritin. C, the homogenates of T-MtF transfected cells metabolically labeled with ³²Fe were analyzed on non-denaturing PAGE before (–) or after incubation with an excess of anti-L-ferritin antibody (± L) to sequester cytosolic ferritins. Ferritin-bound iron was revealed by autoradiography (upper) and ferritin protein by blotting with the rH02 antibody (lower).

2. **Immunoprecipitation of the transient transfectant cells.** After transient transfection, the cells (5 × 10⁶) were grown for 30 h and then metabolically labeled for 18 h by incubation in medium containing 50 μCi/ml [³⁵S]methionine and [³⁵S]cysteine. Aliquots of the soluble fractions containing 4 × 10⁶ cpm were sequentially precipitated first with a saturating amount of anti-L-ferritin antibody (¹° L) to collect cytosolic ferritins and then with saturating amounts of rH02 antibody (²° rH02) to precipitate the remaining ferritins. The precipitates were separated on 12% SDS-polyacrylamide gels and exposed to autoradiography. The arrows indicate the mobility of MtF and of the cytosolic H- and L-ferritin subunits. T-MtF mutant deleted of the mitochondrial leader sequence; MtH, chimeric construct for the mitochondrial leader sequence fused to H-subunit.

3. **Cell Localization.** Transfected cells were examined by in situ immunostaining using rH02 antibody. In addition to a faint background from cytosolic ferritins, MtF, MtF₂₂₂, and Mt-HF transfecteds all showed strong staining of filamentous and perinuclear intracellular bodies in 10–20% of the cells, whereas the T-MtF transfecteds showed a strong diffuse and cytosolic staining (Fig. 4A) similar to that obtained with H-ferritin wild type transfectants (not shown). For a more precise localization, the MtF transfecteds were frozen, sliced, and subjected to immunostaining with the anti-MtF-specific antibody followed by immunogold secondary antibody. Electron microscopic imaging showed that most of the signal accumulated inside the mitochondria with sparse background signals elsewhere, possibly from damage during slide preparation (Fig. 4B). The gold granules in the mitochondria appeared to be in the soluble matrix and were not associated with the cristae or membranes.

**MtF Expression and Cell Iron Metabolism.** To analyze whether MtF expression affected cellular iron metabolism, we...
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first studied iron incorporation from FAC and $^{55}$Fe-labeled transferrin in the transiently transfected cells. The MtF transfectants incorporated similar amounts of iron from FAC or from $^{55}$Fe-labeled transferrin (not shown) over an 18-h incubation as the control cells (empty vector transfected). Uptake of iron from FAC into both MtF and cytosolic ferritin was detectable after only a 10-min incubation as shown by non-denaturing PAGE analyses (Fig. 5). In all experiments with transient expression, MtF accounted for ~20% of the total ferritin iron. Since only 20–30% of cells express MtF, the retention of iron as MtF in transfected cells was presumably much higher than 20%.

For a more reliable assessment of the effect of MtF expression on cellular iron metabolism, we produced stable transfectants expressing MtF and MtF$^{222}$ under the regulation of the tetracycline promoter. Clones MtF-tTA and MtF$^{222}$-tTA expressed the highest level of transferrin protein in the absence of doxycycline repressor as judged by Western blot with anti-MtF antibody and were selected for further study. A representative time course of accumulation of MtF and MtF$^{222}$ is shown in Fig. 6. No MtF was detected in the transfected cells in the presence of doxycycline. After withdrawal of the repressor, MtF was expressed with the highest accumulation occurring between days 5 and 10 (Fig. 6, upper panel). The increased expression of MtF was followed by a marked increase in the expression of transferrin receptor (Fig. 6, lower panel). By contrast, the expression of MtF$^{222}$ had no detectable effect on transferrin receptor expression (Fig. 6, lower panel). Since the expression of transferrin receptor is inversely related to the level of free iron in the cytosol, the results in Fig. 6 suggested that the expression of MtF in mitochondria reduces the level of free iron in the cytosol. To test this hypothesis, we examined the effect of MtF expression on cytosolic ferritins whose synthesis is also largely controlled by the levels of free iron. This was done by metabolic labeling of proteins with $^{35}$S-methionine followed by autoradiography of the labeled endogenous and transfected ferritin chains. As expected, only the cytosolic H- and L-ferritins were labeled in the presence of the doxycycline repressor in both cell types. Doxycycline withdrawal induced MtF and MtF$^{222}$ expression in the two clones, as indicated by labeling in the Mt chain. The extent of labeling was about 20% of that in the H-chain in the repressed cells as judged by densitometry. However, the synthesis of H- and L-chains was greatly reduced (a range of 50–80% in different experiments) in the MtF clone. On the other hand, the synthesis of H- and L-chains was unaffected in the clone expressing MtF$^{222}$ that does not incorporate iron (Fig. 7A). This reduced synthesis of ferritin following the induction of MtF was reflected in the amount of H- and L-ferritin protein. As shown in the ELISA assays in Fig. 7B, the level of the cytosolic H-ferritin in cells expressing MtF$^{222}$ was about half of that of the control cells or of the MtF$^{222}$ cells, even after 18 h of incubation with 3 µM FAC (Fig. 7B).

The decreases in synthesis and the levels of cytosolic ferritins resulting from MtF expression seemed likely to be due to a redistribution of free iron from cytosol to mitochondria. This conclusion was confirmed by examining the distribution of exogenous iron between MtF and in these cells. As shown in Fig. 5, exogenous iron appears in cytosolic and mitochondrial ferritin after only 10 min of incubation. However, more iron was found in MtF in the stable line at all periods. After only 30

![Fig. 4](image-url)

**Fig. 4.** Immunostain of the transient transfectant cells. A, the HeLa cells transfected with the plasmids encoding the four different ferritins were grown for 30 h, fixed, and permeabilized. The preparations were overlaid with rh02 antibody followed by secondary fluorescein isothiocyanate antibody, and the fluorescence image was captured. The filamentous bodies stained in MtF, MtF$^{222}$ (Mt222), and Mt-HF (Mt-H) cells are analogous to those described in Ref. 17 and identified as mitochondria, whereas the diffuse cellular stain of T-MtF (T-MtF) cells is consistent with a cytosolic distribution. B, immunogold staining of the untransfected (C) and MtF-transfected HeLa cells (MtF) with anti-$^{55}$MtF antiserum for MtF recognition followed by secondary anti-IgG-coated gold particles. Cryosections were then examined by electron microscopy.

![Fig. 5](image-url)

**Fig. 5.** Cellular iron incorporation in transient and stable MtF transfectant HeLa cells. Cells of transfected MtF-tTA clone (Mt-stable) were grown for 7 days in the absence of doxycycline to induce MtF expression and then incubated with FAC for the indicated time in parallel with untransfected HeLa cells (Control), and transiently MtF transfected cells (Mt-trans). The cell extracts (3 µg of total proteins) were separated on non-denaturing PAGE followed by autoradiography to monitor ferritin-bound $^{55}$Fe label. Densitometric quantitations of ferritin bands in arbitrary units are shown on the right side of each panel (empty squares, cytosolic ferritin (H/L); solid squares, MtF). The arrows indicate the mobility of MtF and cytosolic ferritin (H/LF).

![Fig. 6](image-url)

**Fig. 6.** Time course of ferritin and transferrin receptor expression in MtF-tTA and MtF$^{222}$-tTA clones. Cells of the MtF-tTA and MtF$^{222}$-tTA clones grown in 2 ng/ml doxycycline were transferred to a medium without doxycycline and harvested at indicated days. After homogenization, the soluble protein extracts (30 µg) were separated and analyzed by blotting using secondary horseradish peroxidase-labeled antibodies and ECL development. In the upper panel, the samples were resolved by non-denaturing PAGE and blotted with mouse anti-MtF antibody, and in the lower panel, the samples were loaded on 12% SDS-polyacrylamide gels and blotted with anti-human transferrin receptor antibody. The arrows indicate the position of MtF and transferrin receptor (TfR), and the empty arrow indicates the origin of the gel.
Mitochondrial Ferritin Expression in HeLa Cells

We next evaluated the rates of release of this newly bound radioactive iron in the ferritins in cytosol and mitochondria by removing the exogenous radioactive iron and by chelating free cytosolic iron with desferrioxamine (DFO). After 24 h of treatment with DFO, cells were lysed, the ferritins were resolved by non-denaturing PAGE, and their retention of labeled iron was assessed by autoradiography. These experiments showed that essentially all of the newly incorporated iron was lost from cytosolic ferritin in control cells. This occurred even without DFO treatment. By contrast, very little iron was lost from MtF in this 24-h period, and chelation of cytosolic iron by DFO treatment did not greatly affect the retention of iron in MtF (Fig. 8A).

The relative stability of the ferritin proteins in this 24-h period following iron removal or DFO treatment was assessed by labeling cells with [35S]methionine and [35S]cysteine in methionine and cysteine-free medium. After labeling, the cells were collected at times 0 and 24 h (of soluble protein) were resolved by non-denaturing PAGE and exposed to autoradiography for detection of ferritin-bound iron. The arrows indicate the mobility of MtF and of cytosolic endogenous ferritin.

Discussion

In a previous study, we identified a new ferritin, MtF, that is targeted to mitochondria (17). The possible roles of this ferritin are not clear. It is expressed at very low levels in most normal cells except reticulocytes at very high levels in erythroblasts with disrupted heme synthesis. Since it was impractical to use either type of cell, we transfected HeLa cells with cDNAs for MtF and some engineered variants of MtF and HF-ferritins to explore some aspects of the metabolism of this new protein.

Our results showed that a construct of the H-ferritin with an attached MtF leader sequence is processed like MtF into mitochondria. This result, along with previous experiments with green fluorescent protein fusions (17), demonstrates that this leader sequence is sufficient for mitochondrial targeting. The demonstration that H-sub-
units with this leader are also targeted to mitochondria and assembled into functional ferritin shells indicates that MtF has no specific structural properties for uptake and processing in the organelle. In addition, the assembly of the processed subunits into shells does not seem to require other mitochondrial components since a construct of MtF lacking the leader and expressed in cytosol also assembled into multimeric shells. We did not detect the MtF precursor by immunoprecipitation in any cell extract (Fig. 3), and in vitro translation data showed that the precursor does not assemble in ferritin shells (not shown). These observations suggest that MtF accumulates only inside the mitochondria.

Western analyses and cellular labeling experiments with the $^{55}$Fe label confirmed that MtF is not present at detectable levels in normal HeLa cells. However, transfected MtF took up iron and in similar amounts as H-ferritin targeted to mitochondria. This activity of MtF depended on residues identified previously as critical for H-ferrisodixase activity. These observations suggest that MtF and H-ferritin can use and process similar iron substrates.

The finding that T-MtF shells transiently expressed in the cytosol do not incorporate iron is puzzling in view of the efficiency of both HF and MtF in incorporating iron when expressed transiently in mitochondria. However, H-ferritin also fails to incorporate iron and does not form hybrids with L-ferritin when transiently expressed in HeLa cells but does both in stable transfectants (13, 30). The reasons for these anomalies are not known. Perhaps cytosol has limiting amounts of required cofactor(s) or only heteropolymers function in cytosol. More important for the present work is the observation that the ferritins take up and retain iron more efficiently when inside the mitochondrion than in the cytosol. In stable transfectants, MtF homopolymers accounted for more than 70% of the total ferritin iron (Figs. 5 and 8A), whereas previous studies showed that transfected H-ferritin homopolymers incorporated only a minor amount of the total ferritin iron (13). This suggests that the double membrane of mitochondria does not reduce the diffusion of iron in the organelle. Perhaps the mitochondrion offers more favorable conditions for ferritin iron uptake such as a more suitable redox status.

The activity of MtF has a profound effect on cellular iron homeostasis. In the transient experiments, in which only 20–30% of the cells were transfected, MtF incorporated about 20% of the total ferritin iron (Figs. 5 and 8A), whereas previous studies showed that transfected H-ferritin homopolymers incorporated only a minor amount of the total ferritin iron (13). This supports the idea that the double membrane of mitochondria does not reduce the diffusion of iron in the organelle. Perhaps the mitochondrion offers more favorable conditions for ferritin iron uptake such as a more suitable redox status.

The avidity of MtF for iron may be relevant to other conditions resulting in increases in mitochondrial iron, such as the heart or brain mitochondria of subjects with Friedreich’s ataxia (24). The excess iron, particularly the filterable mitochondrial iron, is thought to disturb mitochondrial function (35), but it is not known whether the levels of MtF are increased in these disorders. If not, up-regulation of MtF might prove a useful therapeutic approach to avoid iron toxicity.

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