Role of Ferritin in the Control of the Labile Iron Pool in Murine Erythroleukemia Cells*

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In vitro studies have shown that ferritin iron incorporation is mediated by a ferroxidase activity associated with ferritin H subunits (H-Ft) and a nucleation center associated with ferritin L subunits (L-Ft). To assess the role played by the ferritin subunits in regulating intracellular iron distribution, we transfected mouse erythroleukemia cells with the H-Ft subunit gene mutated in the iron-responsive element. Stable transfected displaying high H-Ft levels and reduced endogenous L-Ft levels, resulting in a marked change in the H:L subunit ratio from 1:1 in control cells to as high as 20:1 in some transfected clones. The effects of H-Ft overexpression on the labile iron pool were determined in intact cells by a novel method based on the fluorescent metalloprotein calcine. H-Ft overexpression resulted in a significant reduction in the iron pool, from 1.3 μM in control cells to 0.56 μM in H-Ft transfectants, and in higher buffering capacity following iron loads. A fraction of the H-Ft-associated iron was labile, available to cell-permeant, but not cell-impermeant, chelators. The results of this study provide the first in vivo demonstration of the capacity of H-Ft to sequester cell iron and to regulate the levels of the labile iron pool.

Most mammalian cells acquire iron by the receptor-mediated endocytosis of iron-transferrin complexes. After internalization of transferrin into endocytic vesicles, iron is translocated across the endosomal membrane into a cytosolic labile iron pool (LIP), from which it is distributed among various cellular compartments (1). The chemical composition and intracellular levels of the LIP have remained essentially elusive. However, the LIP is widely implicated in cellular phenomena involving generation of reactive oxidant species (2, 3), and it has been identified as the chelatable cell iron pool (4) or as the regulatory iron pool associated with the control of cell iron metabolism at both transcriptional and post-transcriptional levels (5). In most mammalian cells, iron-responsive proteins (IRPs), belonging to the aconitase family of enzymes, act as regulators of cell iron by sensing putative LIP levels and affecting either the stability or the translation of specific mRNAs coding for proteins associated with iron metabolism (6). In the native form, IRP has a high affinity for a nucleotide motif called “iron-responsive element” (IRE), which is present primarily in the 5′-noncoding region of ferritin (Ft) mRNAs and in the 3′-non-coding region of the transferrin receptor mRNA. Binding of IRP to these IREs represses Ft synthesis and stabilizes transferrin receptor mRNA. However, upon cell iron loading, IRPs undergo changes in their mRNA binding affinities (7) or in their half-life (8), thereby enabling both Ft translation and degradation of transferrin receptor mRNA to proceed. This coordinated regulation of iron uptake and storage serves as the major mechanism of cell iron homeostasis (5, 6).

An additional mode of regulation of the LIP operates through modification of the subunit composition of the Ft molecule (9). Such modifications are likely to be of physiological importance since recent in vitro studies indicated that recombinant human heavy and light subunits (H-Ft and L-Ft, respectively) play distinct roles in iron incorporation and subsequent iron core formation within ferritin molecules (10). The sequestration of the metal has been primarily attributed to a ferroxidase activity associated with H-Ft subunits (11), and formation of the iron core to a nucleation center associated with the L-Ft subunit (12). The alleged functional division of iron handling is apparent in the tissue distribution of the respective Ft subunit mRNAs and protein (13). Moreover, it is also corroborated by increased H-Ft gene transcription following cell exposure to cytokines (14), oxidative stress (15), erythropoietic differentiation (16, 17), and cellular proliferation (18). In some of these situations, the induction of H-Ft expression was assumed to confer upon cells a defensive tool against iron-mediated oxidative injury and possibly other properties.

In a recent work (9), we showed that induced overexpression of the H-Ft subunit from a transgene transfected into mouse erythroleukemia cells (MEL) leads to IRP activation and a concomitant reduction in both Hb production and endogenous Ft synthesis. This result led us to hypothesize that the regulation of H-Ft expression is a major factor in controlling cellular LIP levels and associated functions. However, it is only recently that an experimental approach for determining the LIP in intact living cells has become available (19, 20). Using the intracellular fluorescence metallosensor calcein, we attempted here to directly assess the proposed roles of H-Ft in model systems. The results presented here provide the first demonstration for the in vivo down-regulation of the LIP by
overexpression of H-Ft and the role played by this protein in the overall regulation of iron distribution in cellular compartments.

**EXPERIMENTAL PROCEDURES**

*Materials—* $^{59}$FeCl$_3$ (5 mCi/mg iron) and $[^{35}$S]methionine (400 Ci/mmol) were purchased from Amersham International. Caclene and calcium/AM were procured from Molecular Probes, Inc. SIH was a kind gift of Prof. Prem Ponka (McGill University, Montreal, Canada), and macromolecular HES-DFO (6 desferrioxamine/M, 50,000 starch molecule) was donated by Biomedical Frontiers (Minneapolis, MN).

*Plasmid Construction and Stable Transfections—*The construct, described in detail elsewhere (9), contains the entire murine H-Ft gene in which the first 2 bases of the IRE loop have been deleted. 1 kilobase of promoter region, 2.6 kilobases of 3'-flanking sequence, the Neo selection gene under control of the thymidine kinase promoter, and hypersensitive site II from $\beta$-globin locus control region (21). MEL cells were transfected by electroporation, and individual clones of transfected cells were selected in methyl cellulose in the presence of G418 (800 $\mu$g/ml).

*In Vitro Metabolic Labeling and Enzyme-linked Immunosorbent Assay—*Bioisolation of H-Ft and L-Ft was assessed by suspending $10^6$ cells in methionine-free medium supplemented with 75 $\mu$Ci of $[^{35}$S]methionine, incubating for 2 h, washing, and lysing (22). The same amount of trichloroacetic acid-prefitable radioactivity was used for immunoprecipitation with polyclonal anti-Ft antibodies followed by protein A-Sepharose. After solubilization, the samples were loaded on a 12% SDS-polyacrylamide gel, and the radioactivity associated with each Ft subunit was estimated with an Instant-Imager (Packard Instrument Co.). Enzyme-linked immunosorbent assays specific for mouse H-Ft and L-Ft were used to evaluate the ferritin content in cells (23). Microtiter plates were coated with 1 $\mu$g of polyclonal antibodies specific for the recombinant mouse H or L chain. Controls, standard ferritins, and soluble cell lysates were added to the plates, and the bound ferritin was revealed with the same antibodies labeled with horseradish peroxidase. Activity was developed with o-phenylenedia- mine dihydrochloride (Sigma).

*LIP Assays—*The LIP was assayed as recently described (20). Cells ($2 \times 10^6$ cells/ml) were incubated with 0.125 $\mu$Ci calcium/AM for 5 min at $37^\circ$C in bicarbonate-free medium containing 1 mg/ml bovine serum albumin and 20 mM HEPES, pH 7.3. After washing, the cells were resuspended in the same medium without calcium/AM and maintained at room temperature. Prior to measurements, 1 ml of the cell suspension was centrifuged; the cells were resuspended in 2 ml of prewarmed HBS and placed in a stirred, thermostated (37°C) cuvette; and fluorescence was monitored at an excitation of 488 nm and an emission of 517 nm in a Photon Technology Instrument fluorescence station. Anti-calrene fluorescence-quenching antibodies were used to ensure that all recorded fluorescence originated from within the cells (24). The calrene-loading cells have a fluorescence component ($\Delta F$) that is quenched by intracellular iron and can be revealed by addition of 100 $\mu$M SIH, a highly permeant iron chelator. The rise in fluorescence ($\Delta F$) provides a measure of the cell calrene-bound iron concentration (CA-Fe), which, together with the experimentally determined [CA-Fe] $K_d$ of 0.22 $\mu$M (20) and the mass law equation, yields the [Fe] in equilibrium with cell [CA]. LIP levels are given as [CA-Fe] + [Fe].

*Iron-buffering Capacity—*The rate of iron accumulation in the LIP was assessed by monitoring the rise of fluorescence quenching of calrene-loaded cells in the presence of 20 $\mu$M Fe(II) given as ferrous ammonium sulfate (FAS). Iron uptake was determined by addition of HES-DFO (100 $\mu$M), and cell iron [CA-Fe] + [Fe] was retrieved by addition of a 100 $\mu$M concentration of the permeant chelator SIH.

$^{55}$Fe Incorporation into Cell Ferritins—*An iron solution of 263 $\mu$M was prepared by mixing $^{55}$FeCl$_3$ (1.4 mCi in 0.1 M HCl) with $^{56}$FeSO$_4$ (2 $\mu$M in 0.1 M HCl) at a molar ratio of 1:10 in the presence of 0.27 mM succrose (stock solution of 1 M) and of a 50-fold molar excess of $\beta$-mercaptoethanol. Cells ($2 \times 10^6$) were incubated for 10 min with 55 $\mu$M iron, washed, and resuspended in HBS supplemented with either 500 $\mu$M DTPA or 100 $\mu$M dialyzed HES-DFO and 1 $\mu$M bovine serum albumin. Immediately or after a 20-min incubation, the cells were washed with HBS and 1% bovine serum albumin and with HBS alone, harvested, and lysed for 20 min in 25 mM Tris, pH 7.4, 1% Triton X-100, 40 mM KCl, 0.2 $\mu$g/ml leupeptin, and 0.2 mM phenylmethlysulfonyl fluoride, followed by 5 min at 55°C. The radioactivity was counted in 2 ml of the lysate, and the same amount of radioactivity was loaded on a 7.5% nondenaturing acrylamide gel.

**RESULTS**

**Ferritin Synthesis in MEL Cells Transfected with the H-Ft Gene—*The transfection strategy using a plasmid carrying a 2-base mutated IRE was designed to generate MEL cells expressing exogenous H-Ft in a mode uncoupled from feedback regulation by iron (9). The individual transfected clones were selected and analyzed for transferrin expression by quantitative reverse transcription-polymerase chain reaction using fluorescent oligonucleotides located on either side of the mutation. Of 15 selected clones, we retained clones 6 and 12, in which the transgene mRNA reached 80% of the endogenous level, and clone 16, in which the transgene mRNA was only 20% of the endogenous level.

The biosynthesis of Ft in the various clones was followed by immunoprecipitation of metabolically $^{35}$S-labeled cells with anti-Ft antibodies and analysis on denaturing polyacrylamide gels (Fig. 1A). The biosynthetic capacity of H-Ft relative to L-Ft subunits was highest in cells from clones 6 and 12, about equal in clone 16, and lowest in untransfected cells. These apparent differences were even more pronounced when the respective Ft subunits were compared in terms of steady-state accumulation measured by enzyme-linked immunosorbent assay (Fig. 1B). In the control clone, the levels of H and L subunits were similar (190 and 188 ng of ferritin/mg of protein, respectively). In clones 6 and 12, H-Ft levels were 4–6-fold higher than in

![FIG. 1. Expression of Ft subunits in transfected cells. A, shown are the results from the metabolic labeling of Ft subunits. Cells from the control clone (C) and from transfected clones 6, 12, and 16 were incubated with $[^{35}$S]methionine for 2 h, washed, and lysed, and the same amounts of trichloroacetic acid-prefitable counts were used to load SDS-polyacrylamide gels. B, enzyme-linked immunosorbent assays were carried out on the same clones using polyclonal antibodies specific for either mouse H- or L-Ft. Data are expressed as ng of Ft/mg of total proteins.](http://www.jbc.org/Downloaded from http://www.jbc.org/)

**TABLE I**

| LIP levels in MEL cells | Control | Clone 6 | Clone 12 | Clone 16 |
|-------------------------|---------|---------|----------|----------|
| $\mu$M Fe(II)           | 1.30 ± 0.17 | 0.56 ± 0.14$^a$ | 0.65 ± 0.10$^a$ | 1.14 ± 0.13 |

$^a$ Significant difference ($p < 0.05$) from the control in both unpaired and paired t-tests.
Control of the Labile Iron Pool by H-Ft Subunits

**TABLE II**

| LIP and H-Ft levels in MEL cells following HMBA induction of erythroid differentiation |
|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|
| H-Ft LIP | Control | Clone 6 | Clone 12 | Clone 16 |
| Hb at day 2 (μg/mg protein) | | | | |
| Induction day | | | | |
| 0 | 21 | 6 | 8 | 18 |
| 1 | | | | |
| 2 | 247 | 120 ± 0.11 | 2043 | 0.37 ± 0.12* |

* Significant difference (p < 0.05) from the control at each day of induction (paired t-test analysis).

control cells, and the L-Ft levels were drastically reduced, so that 90% of the cell Ft was composed of H subunits. Relative to control cells, clone 16 showed only moderately elevated levels of H-Ft (300 ng/mg of protein) and a 2-fold reduction in cell L-Ft (90 ng/mg of protein). It is interesting to note that despite of the relatively faster synthesis of L versus H subunits, their protein levels were equivalent, possibly reflecting differences in protein stability and turnover.

Overexpression of H-Ft Down-regulates the Intracellular LIP—A fluorescence method was applied to the various transfectants for *in situ* detection of cell iron and estimation of their LIP levels (24). The results of 12 independent measurements done in parallel on each individual clone are summarized in Table I. They indicate that whereas clone 16 cells had steady-state LIP levels comparable to those of control cells (1.3 and 1.14 μM, respectively), clones 6 and 12 showed a significant (~50%) reduction in their LIP levels. To confirm that those differences in LIP levels associated with the different clones were directly related to H-Ft accumulation, we also followed H-Ft expression and LIP levels during 2 days of HMBA induction. The presence of an erythroid-specific enhancer (hypersensitive site II) in the construct increases the production of exogenous H-Ft mRNA, although to a variable extent in the different clones (data not shown). The HMBA treatment resulted in a progressive (up to 2-fold) accumulation of H-Ft and a reduction in LIP levels and hemoglobin synthesis, particularly in clones 6 and 12 (Table II). These studies clearly indicate an inverse relationship between H-Ft expression and the steady-state LIP levels in the various clones expressing different levels of H-Ft, whether before or following induction by HMBA.

Overexpression of H-Ft Subunits Enhances the Iron-buffering Capacity of Cells—To assess the possible role of H-Ft in buffering the LIP following imposed (acute) iron loads, we used an exogenous source of Fe(II) and followed iron diversion into Ft and into the LIP. The various cell clones were acutely loaded with 55Fe(II) sulfate for 10 min, washed with the impermeant chelator HES-DFO, and lysed either immediately or after a subsequent incubation in the presence of HES-DFO or DTPA (500 μM). Cytoplasmic extracts were loaded directly on a 7.5% nondenaturing polyacrylamide gel in parallel with recombinant mouse H-Ft (recomHFT) previously incubated *in vitro* with 55Fe sulfate. The dried gel was autoradiographed, and the radioactivity of the bands was counted in an Instant-Imager.

![Fig. 2. 55Fe(II) incorporation into ferritin.](image-url)

Fig. 2. 55Fe(II) incorporation into ferritin. The control clone (C) and clones 6, 12, and 16 were incubated with 55Fe(II) sulfate for 10 min at room temperature, washed, and resuspended in HBS and 1% bovine serum albumin supplemented with 100 μM HES-DFO or 500 μM DTPA. Immediately or following 20 min of incubation, cells were washed with HBS alone and lysed, and the cytoplasmic extracts were loaded on a 7.5% nondenaturing polyacrylamide gel in parallel with recombinant mouse H-Ft (recomHFT) incubated *in vitro* with 55Fe sulfate (Fig. 2). In clones 6 and 12, the major radioactive band migrated at exactly the same position as the *in vitro* labeled recombinant H subunit, differing from the radioactive band in the control clone. Interestingly, in clone 16, Ft migrated as a relatively broader band, suggesting a more heterogeneous subunit composition of the Ft molecules than in the other clones. Clones 6 and 12 took up the highest amount of radiolabeled iron into Ft, virtually all in a form unavailable to the impermeant iron chelator HES-DFO. However, in the presence of excess DTPA, which slowly but demonstrably permeates into MEL cells, 2 a sizable amount of 55Fe could be dissociated from the Ft in a 20-min incubation period (Fig. 2). This might indicate that, at the early phase of iron incorporation into H-Ft, the metal can be labile, inasmuch as it can be recruited by permeant iron chelators.

The parallel rise in LIP levels following acute iron loads was monitored in terms of time-dependent changes in the fluorescence induced by exposure of cafein-loaded cells to FAS (Fig. 3). Entry of the quenching metal into cells was stopped by addition of the impermeant iron chelator HES-DFO. The permeant iron chelator SHH was added subsequently, and the ensuing recovery of fluorescence was converted into cafein-bound iron concentrations ([ICA-Fe]), as depicted in Fig. 3 (bar graph). The data indicate that clones displaying the lowest levels of H-Ft (control and clone 16) were precisely those that showed the greatest increase in LIP levels, whereas clones 6 and 12, which express the highest H-Ft levels, had not only a constitutive lower LIP level, but also a better capacity for maintaining lower LIP levels following imposed iron loads.

2 S. Epstzejn, W. Breuer, and Z. I. Cabantchik, unpublished observations.
Taken in toto, the H-Ft-overexpressing MEL cells have a demonstrably higher capacity for scavenging incoming iron and limiting the rise in LIP levels, following an acute iron load. This property is referred as the iron-buffering capacity of the cell.

**DISCUSSION**

In this paper, we provide direct evidence supporting the functional role of H-Ft in regulating the cytoplasmic LIP. The study was based on the generation of clones of MEL cells expressing various levels of H-Ft (9) and the advent of a novel method for tracing metals in living cells (19). The clones selected for this study carried a stable transfected mouse H-Ft gene mutated in the IRE (for iron-independent expression). They showed 4–6-fold increased accumulation of the H-Ft subunit, resulting from higher rates of H-Ft synthesis and repression of endogenous L-Ft levels. The total amount of Fe increased from 380 ng/mg of protein in control cells to 900–1200 ng/mg of protein in transfected cells, whereas the H:L subunit ratio rose from 1:1 in control cells to 4–20:1 in transfected cells.

To assess the functional consequences of H-Ft overexpression, we had to overcome the problem associated with the determination of the LIP, whose chemical nature and physiological properties have remained largely elusive. This is particularly the case for highly differentiated erythroid cells, which utilize most of the transferrin-derived iron for mitochondrial heme synthesis, allegedly without a direct participation of the LIP (25). However, most analytical assays previously used to assess the LIP have been applied to cell lysates rather than to intact cells (25–27), and they might have yielded "artifacts occurring during the homogenization process" and erroneous measures of "the original iron complex in vivo" (28). We have therefore applied in this work a non-invasive method for continuous monitoring of the LIP in living cells using the metal-losensing properties of a fluorescent chelator (19, 20).

In support of the previously observed activation of IRP and down-regulation of endogenous L-Ft expression (9), H-Ft overexpression was shown here to reduce LIP levels, from 1.3 μM in control cells to 0.56 μM in the highest overexpressing clones. The cell LIP measurements were accurate within the ±0.1 μM Fe(II) range and therefore could not resolve minor differences between relatively poor H-Ft-expressing clones and wild-type cells. Nevertheless, the 2-fold increase in H-Ft levels in clone 16 gave consistently lower LIP levels than in the parent cells. In addition, in all the clones analyzed, there was a parallel reduction in LIP levels and endogenous L-Ft levels. These properties were also reflected in the iron-buffering capacity associated with H-Ft, both under resting conditions and following acute iron loads. However, despite the higher iron absorptive capacity and reduced LIP levels resulting from higher expression of H-Ft, the total amount of iron in the cells remained at a constant level of 350–400 mM (data not shown). Given the relatively high expression of H-Ft in some of the clones, those results indicate that a major fraction of ferritin might be iron-poor. The form of iron that is associated with H-Ft shortly after its entry into cells is apparently accessible to cell-permeant chelators, but its chemical properties remain to be elucidated. Such reversible association of the metal with H subunit-rich Ft might reflect a transitory state of the bound metal, which, in the absence of sufficient L subunits, might fail to mineralize (10). Thus, an apparent fine-tuning of H and L gene expression might be necessary for correct handling and processing of cell iron and appears to be a key factor in controlling the labile iron pool and IRP activity (Fig. 4). On the other hand, tissue accumulation of nonfunctional L-Ft homopolymers, which results from the presence of a mutation in the IRE of L-Ft mRNA in patients with hereditary hyperfer-
ritinemia-cataract syndrome, has apparently limited pathological implications (29, 30). In these patients, the aberrant expression of L-Ft subunits affects the transparency of the lens with progressive onset of cataracts, but not the major parameters associated with intracellular iron metabolism. Finally, this study opens the possibility of manipulating ferritin levels independently of iron and of assessing the proposed roles of the LIP in controlling cellular functions, including regulation of gene expression and generation of reactive oxygen species.

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