Overexpression of Wild Type and Mutated Human Ferritin
H-chain in HeLa Cells

IN VIVO ROLE OF FERRITIN FERROXIDASE ACTIVITY*

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Transflectant HeLa cells were generated that expressed human ferritin H-chain wild type and an H-chain mutant with inactivated ferroxidase activity under the control of the tetracycline-responsive promoter (Tet-off). The clones accumulated exogenous ferritins up to levels 14–16-fold over background, half of which were as H-chain homopolymers. This had no evident effect in the mutant ferritin clone, whereas it induced an iron-deficient phenotype in the H-ferritin wild type clone, manifested by −5-fold increase of IRPs activity, −2.5-fold increase of transferrin receptor, −1.8-fold increase in iron-transferrin iron uptake, and −50% reduction of labile iron pool. Overexpression of the H-ferritin, but not of the mutant ferritin, strongly reduced cell growth and increased resistance to H2O2 toxicity, effects that were reverted by prolonged incubation in iron-supplemented medium. The results show that in HeLa cells H-ferritin regulates the metabolic iron pool with a mechanism dependent on the functionality of the ferroxidase centers, and this affects, in opposite directions, cellular growth and resistance to oxidative damage. This, and the finding that also in vitro H-chain homopolymers are much less efficient than the H/L heteropolymers in taking up iron, indicate that functional activity of H-ferritin in HeLa cells is that predicted from the in vitro data.

Ferritins are the major iron storage proteins, ubiquitous in mammalian cells and tightly regulated by iron. They are made of 24 subunits that assemble in an almost spherical shell to delimit a cavity where iron is accommodated and concentrated in a mineral and compact form. Mammalian ferritin are composed of two subunit types, the H- and L-chains, with −50% sequence identity and very similar three-dimensional structures made of a four-helix bundle (1). The mRNAs for the two chains have nearly identical iron-responsive elements (IREs) close to the 5’ termini that bind to the iron sensors iron-regulatory proteins (IRPs) with a mechanism that determines a tight translational iron-dependent regulation of protein expression (2, 3). Iron supplementation to cells in culture determines a strong up-regulation of both ferritin chains, whereas treatment with chelating agents such as desferrioxamine determines an almost total suppression of ferritin accumulation in about 24 h (4). Ferritin function has been studied mainly in vitro, using horse spleen ferritin first and recombinant proteins later. The recombinant ferritins have the property of being composed by a single subunit type, the H-chain, L-chain, or artificial variants (5). Although these homopolymers are virtually nonexistent in mammalian cells, the approach proved instructive and showed important functional differences between the two gene products. The recombinant L-chain homopolymers purified from Escherichia coli as iron poor proteins, induce a slow uptake of iron from Fe(II) salts, and are more stable to denaturants than the H-homopolymers (6, 7). The L-subunit has no catalytic activity on its own, but it facilitates the activity of the H-subunits by offering sites for iron nucleation and mineralization and increasing the turnover at the ferroxidase centers (8–10). Recombinant H-chain homopolymers purified from E. coli as iron-containing molecules and induce fast iron oxidation (5). This is due to the presence of ferroxidase centers buried inside the protein fold and consisting in di-iron binding sites coordinated by atoms of seven residues that are conserved in most ferritins from animals, plants, and bacteria (1, 11). These catalytic sites accelerate Fe(II) oxidation, which is a rate-limiting step in the mechanism of ferritin iron incorporation, in a reaction that consumes one dioxygen molecule/two Fe(II) ions with the production of hydrogen peroxide (10). Present understanding of the mechanism of in vitro ferritin iron uptake indicates that Fe(II) ions move inside the cavity through hydrophilic channels on the 3-fold symmetry axes and the transfer is facilitated by the presence of local metal binding sites (12). The iron then localizes in the ferroxidase centers where a fast interaction with O2 occurs with the formation of a transient peroxyferric complexes that readily decay (13), the resulting μ-oxo bridged di-iron intermediates then split, and the mononuclear Fe(III) complexes move to the cavity where they hydrolyze to build iron cores of up to 4000 iron atoms (1, 14). The vacated ferroxidase center is then available for other cycles of iron oxidation (10). In vitro, in parallel to the catalyzed reaction, spontaneous iron oxidations occur with the stoichiometry of four Fe(II) ions per O2 molecule and the formation of zyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; LIP, labile iron pool; Dox, doxycycline; MTT, dimethylthiazol tetrazolium.

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‡ The abbreviations used are: IRE, iron-responsive element; IRP, iron-regulatory protein; NTA, nitritoltriacetate; MEL, mouse erythroblastemic; DMEM, Dulbecco’s modified Eagle’s medium; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; LIP, labile iron pool; Dox, doxycycline; MTT, dimethylthiazol tetrazolium.

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water. These reactions predominate at high iron concentrations and high pH values and occur also inside the cavity on iron core surface after it has reached a sufficiently large size (15). Thus, in the ferritin iron is oxidized on the catalytic center and on the surface of the mineral core, but the physiological relevance of the latter is dubious. In fact, in conditions in which spontaneous iron hydrolysis does not occur, ferritin can still incorporate iron, although with lower efficiency, as in the case Fe(III) chelated to citrate or nitrilotriacetate (NTA) in the presence of ascorbate. This reaction, which is possibly closer to that occurring in the cell, necessitates of the ferroxidase activity of H-chain and the integrity of hydrophilic channels (16).

The in vivo functionality of ferritin is more complex than that predicted by the in vitro data, because of interactions with other molecules and the poor characterization of the iron forms accessible to the ferritin inside the cell. The analysis of the distinct roles of H- and L-subunits is facilitated by the bypassing of the IRE-IRPs iron-mediated regulation that equally affects the two chains. This occurs in the hereditary hyperferritinemia cataract syndrome where heterogeneous point effects the two chains. This occurs in the hereditary hyperferritinemia cataract syndrome where heterogeneous point effects the two chains.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction and Cells Culture**—The cDNAs for the human entire coding sequences of human H-chain wild type and H-222 mutant (E62K, H65G) were generated by polymerase chain reaction and subcloned into pUDH10–3 vector (24) under the control of ITR promoter to obtain pUD-HF1 and pUD-222F plasmids that encode for the entire ferritin H-chain and its mutant 222, respectively. HeLa-tet Off cells (CLONTECH) were co-transfected with 3.5 μg of pUD-HF1 or pUD-222F plasmids and with 1 μg of pTK-Hyg plasmid (5:1 molar ratio) (CLONTECH) using calcium phosphate method (25). The colonies were grown in DMEM (Life Technologies, Inc.) with hygromycin B (150 μg/ml) and doxycycline (2 μg/ml), and the surviving ones were screened for integration of ferritin cDNA by the fluorescence. Clones were further tested for protein expression by growing them up to 30 days in the absence of doxycycline and analyzing ferritin content in the cell extracts. The selected cells were maintained in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (CLONTECH), 100 μg/ml G418 (Geneticin, Sigma), 150 μg/ml hygromycin B (CLONTECH), 100 μg/ml streptomycin, 1 mM l-glutamine, and with (Dox+) or without (Dox−) 2 ng/ml doxycycline (Sigma).

**Ferritin Quantification and Immunoprecipitation**—Extracts of 10^6 cells were analyzed for ferritin content by using ELISA assays based on monoclonal antibodies specific for the H-ferritin (rH20) and the L-ferritin (LF03) calibrated on the corresponding recombinant homopolymers expressed in E. coli (26). Protein content was evaluated by BCA method (Pierce) calibrated on bovine serum albumin. In immunoblots experiments 3 μg of soluble proteins were loaded on 12% SDS-PAGE, and the nitrocellulose filters were incubated with anti-TR antibody (dilution 1:1000) (Zymed Laboratories Inc.) followed by secondary, peroxidase-labeled antibody (Envision, DAKO). Bound activity was revealed by ECL (Amersham Pharmacia Biotech).

**Metabolic Labeling and Immunoprecipitation**—Cells (5 × 10^6) were incubated for 1 h in DMEM w/o L-glutamine, methionine, and cysteine (ICN), 0.5% fetal calf serum, 50 μg/ml bovine serum albumin, and then were labeled for 18 h with 50 μCi/ml [35S]methionine (ICN) in the same medium (19). The cells were washed with phosphate-buffered saline and then lysed with 500 μl of lysis buffer (20 mM Tris-Cl, pH 8.0, 200 mM LiCl, 1 mM EDTA, 0.5% Nonidet P-40). Total radioactivity associated to the soluble proteins were determined by trichloroacetic acid precipitation. For immunoprecipitation studies, 4 × 10^6 cpm of cytosolic lysates were preclarified 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 1 min at 14,000 rpm. Then anti-ferritin L-chain antibody LF03 (30 μg) and protein A-Sepharose (30 μl) were added, the samples were incubated for 1 h at 4 °C, and the precipitates were collected. The soluble fractions were further incubated for 1 h at 4 °C with 30 μg of anti-ferritin H-chain antibody rH20 and protein A-Sepharose (30 μl) and precipitated (19). The immunobeads were washed resuspended in SDS buffer, boiled for 10 min, and loaded on 12% polyacrylamide SDS-PAGE. The gels were treated with autoradiography image enhancer (Amersham, Arlington Heights, IL), dried, and exposed. The intensity of ferritin subunit bands was quantified by densitometry. In other experiments, after 18 h labeling with [35S]methionine, the cells were washed twice with Dulbecco's phosphate-buffered saline and then grown in the complete medium. At the indicated times the cells were harvested and lysed, and 10 μg of soluble extract protein were subjected to immunoprecipitation with anti-H-ferritin antibody as above.

**Cellular 55Fe Incorporation**—Freshly harvested and exposed 2 × 10^5 cells were incubated for 18 h in the presence of 1 μM 55Fe-thetaferrin in DMEM, 0.5% fetal calf serum, and 50 μg/ml bovine serum albumin. The cells were washed and lysed in 0.5 ml of lysis buffer, and after centrifugation 10 μl of the soluble fraction was mixed with 0.3 ml of Ultima Gold (Packard) and counted for 1 min in a scintillator counter (Packard). The soluble proteins were analyzed also on nondenaturing PAGE (7% polyacrylamide) directly or after immunoprecipitation (19). Gels were dried and exposed to autoradiography. In the experiments to evaluate the effect of cell integrity on ferritin iron.
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Fig. 1. Time course of ferritin expression in Hwt- and H222-tTA-clones. The cells grown in the presence of 2 ng/ml doxycycline were transferred to a medium without doxycycline (dox−) and harvested at the indicated days. The cellular homogenates were analyzed for H-ferritin content by ELISA assay. Control cells were maintained in 2 ng/ml doxycycline (dox+). The results are expressed as ng of ferritin/mg of soluble protein. Values are the means ± S.D. from three independent experiments.

uptake, the cells in DMEM, 0.5% fetal calf serum, 0.5% bovine serum albumin or in lysis buffer were added to 2 μCi/ml 55Fe-NTA (1:10), 200 μM ascorbic acid and incubated for 3 h at 37 °C, and the protein was separated in nondenaturing PAGE.

Analysis of Cell Growth—To evaluate the rate of cell growth, the cells were counted, and 10⁶ of them were plated in 96 wells culture plates (Greiner) in octuplicate in normal medium. They were grown for 18 h at 37 °C in DMEM, then MTT was added, and after 3 h the developed color was read at 570 nm (28), following manufacturer’s instructions (MTT assay, Sigma). In the experiments in which the cytotoxic effect of H2O2 was studied, the cells were cultured at different concentrations (1.5 × 10³ of the induced Hwt-tTA cells and 10⁴ of the other cells) to obtain analogous reading at 570 nm after 18 h growth at 37 °C. Then cells were washed with phosphate-buffered saline, incubated for 1 h in serum free medium, added of different concentrations of H2O2, and further incubated for 2 h. Finally, the plates were washed twice, and cellular viability was measured by incubating the cells in MTT for 3 h as above.

RESULTS

Ferritin Expression—The cDNAs for human H-ferritin wild type and for its mutant named 222 in which ferrooxidase activity was inactivated by the substitutions E62K/H65G (29) were subcloned into the pUHDL1-3 vector under the control of inducible tetracycline responsive promoter (24). The constructs were used to transfet HeLa cells, and among the stable transfecant clones obtained we selected the ones that expressed the highest levels of ferritin after induction. The clones were named Hwt-tTA and H222-tTA, respectively. Ferritin levels in the cell homogenates were measured by specific ELISA assays, and we found that the ferritin expression was maximally repressed at doxycycline (Dox) concentrations >2 ng/ml and fully derepressed with Dox concentrations <0.015 ng/ml (not shown). Upon withdrawal of the drug (from 2 ng/ml Dox), the H-ferritin concentration increased steadily in the H222-tTA clone up to day 7 and then leveled off, whereas H-ferritin accumulation in Hwt-tTA clone showed a biphasic pattern with a maximum around day 4 followed by a decrease (Fig. 1). The maximum H-ferritin concentrations reached in the two clones were similar and 14–16-fold higher than that of untransfected parent cells (Table 1). In the repressed Dox− H222-tTA clone H-ferritin level was analogous to that of untransfected HeLa cell, whereas in the Hwt-tTA clone it was slightly higher possibly for some leakiness. The concentration of the partner L-ferritin was consistently higher in the repressed Dox− than in the derepressed Dox− clones (Table 1). For a qualitative analysis of the isoferritins the cells were metabolically labeled with [35S]Met, and the homogenates were immunoprecipitated first with anti-ferritin L-chain and then with anti-H-chain antibodies, a system that separates ferritin heteropolymers from the H-homopolymers (20). The H-chain homopolymers were undetectable in the untransfected and repressed cells, as expected, whereas they were highly represented in both derepressed clones (Fig. 2, lanes 5 and 9). Densitometry showed that the ferritin heteropolymers in derepressed Dox− cells (Fig. 2, lanes 2 and 7) had a H- to L-chain ratio of about 20 to 1, whereas in the repressed or untransfected cells, the ratio was in the range of 4–5 to 1 (Fig. 2, lanes 1, 3, and 8).

Cells were metabolically labeled with [35S]Met for 18 h, and then the ferritins immunoprecipitated at various times with anti-H-chain antibody. Ferritin remained detectable up to 24 h after labeling and disappeared at similar rates in the untransfected parent cells and in the transfected cells, repressed or not (Fig. 3). The H to L ratio did not change throughout the experiments, indicating that the two chains degrade at similar rates and that the homo- and hetero-polymers of Hwt or H222 mutant have similar stability. The calculated half time of about 18–24 h is in agreement with that found in human fibroblasts (30).

Table I

| Cells          | H-Ferritin | L-Ferritin |
|----------------|------------|------------|
| Hwt-tTA dox−  | 39.0 ± 3.2 | 32.0 ± 2.9 |
| Hwt-tTA dox+  | 33.7 ± 2.1 | 27.0 ± 2.0 |
| H222-tTA dox− | 38.0 ± 2.4 | 31.0 ± 2.3 |
| H222-tTA dox+ | 32.0 ± 2.2 | 25.0 ± 2.1 |

Ferritin Overexpression and Cellular Iron Metabolism—Electromobility shift assays for IRE binding activity of the IRPs were carried out on the cell extracts. The activity, indicated by the intensity of the IRE-IRP complex, was analogous in the repressed and derepressed H222-tTA clone, whereas it was about 5-fold higher in the derepressed than the repressed Hwt-tTA clone (Fig. 4A). In derepressed Hwt-tTA clone, the IRP activity was down-regulated by iron supplementation, although less so than in the corresponding repressed cells, and it was only marginally up-regulated by desferrioxamine treatment, likely because already highly activated (Fig. 4B). The effect of IRP up-regulation in the derepressed Hwt-tTA clone was manifested by a 2.5-fold higher accumulation of transferrin receptor, detected by Western blotting, compared with the other cells (Fig. 4C).

The influence of Hwt or H222-ferritin overexpression on cellular LIP was measured by a fluorescent permeable metal sensor calcein-AM (23). Cells were loaded by 15 min of incubation with 0.250 μM calcein-AM at 37 °C, and after extensive washing, the highly permeable iron chelator isonicotionyl salicylaldehyde hydrazone was added. The increase in fluorescence secondary to isonicotionyl salicylaldehyde hydrazone sequestration of calcein bound iron, which cause dequenching, provides a reliable index of labile iron concentration. The variation of fluorescence was analogous in the derepressed and repressed H222-tTA clone (Fig. 5B), whereas in the repressed Hwt-tTA clone it was about 2-fold higher than in the dere-
pressed conditions, implying that H-ferritin overexpression reduced iron available to calcein binding (Fig. 5A).

Cells were incubated for 18 h with $^{55}$Fe-Tf, and, after washing, the radioactivity associated to the soluble fraction of cell homogenates or to ferritins was determined. Total cellular iron uptake was analogous in the parent HeLa cells and in the transfected and repressed clones, whereas it increased of about 4-fold higher in the derepressed than in the repressed Hwt-tTA clone (Fig. 6A). Autoradiography of $^{55}$Fe-labeled homogenates was immunoprecipitated with saturating amounts of anti-H-ferritin antibody (aH). The precipitates were analyzed on 12% polyacrylamide SDS-PAGE under denaturing conditions and exposed to autoradiography.

Fig. 3. Analysis of ferritin lifetime. The untransfected and transfected HeLa cells were grown in the presence or absence of doxycycline for 4 (Hwt-tTA) or 7 days (H222-tTA), and then they were metabolically labeled for 18 h with 50 μCi/ml $[^{38}]$Smethionine, $[^{38}]$Systeine for 18 h and then lysed, and 4 × 10$^6$ cpm of the homogenates were precipitated with saturating amounts of anti-L-ferritin antibody (aL). The soluble fraction was precipitated again with saturating amounts of anti-H-ferritin antibody (aH). The precipitates were analyzed on 12% polyacrylamide SDS-PAGE under denaturing conditions and exposed to autoradiography.

Fig. 5. LIP assays. The derepressed transfected clones were grown to reach maximum ferritin accumulation, then 2 × 10$^6$ cells were loaded with 250 nm of calcein-AM for 15 min at 37 °C. Cells were washed and resuspended in HBS medium, and the increase in fluorescence emission at 517 nm was monitored after addition of the permeant chelator isonicotionoyl salicylaldehyde hydrazone (100 μM). Results are representative of three independent experiments.

for about 18% of the total in the derepressed Hwt-tTA clone (Fig. 6C, lane 8) and <5% in the derepressed 222-tTA clone (Fig. 6C, lane 4). In the repressed clones no ferritin iron remained in solution after immunoprecipitation, because of the absence of H-homopolymers.

Cells can be iron loaded by incubation with $^{55}$Fe-NTA (2 μCi/ml) in the presence of 200 μM ascorbate, conditions that can be used to load in vitro ferritins with ferroxidase activity (16). Thus, we performed experiments in which equal amounts of cells of the Hwt-tTA clone were incubated for 3 h with the same solution of $^{55}$Fe-NTA ascorbate either before and after cell lysis with Nonidet P-40. Then the homogenates were run on nondenaturing gel electrophoresis, and the ferritin-bound iron was detected by autoradiography. In the Dox− and Dox+ clones, ferritin iron incorporation was very similar, whereas in the lysed cells ferritin iron incorporation was about 4-fold higher in the derepressed than in the repressed Hwt-tTA clones (Fig. 6D). Controls of untransfected parent HeLa cells gave results analogous to the repressed cell (not shown).

Ferritin Overexpression and Cellular Growth—The Hwt-tTA and H222-tTA clones could be maintained in culture in the
to autoradiography and evaluated by densitometry. D, samples of soluble cell homogenates containing 3 μg of protein were loaded on nondenaturing PAGE and exposed to autoradiography. Ferritin mobility is indicated by the arrow. C, samples of 250 μg of the soluble protein homogenates were immunoprecipitated with an amount of H-ferritin antibody to remove the ferritin heteropolymer isoferritin population. The resulting soluble fractions (10 μg/well, lanes 2, 4, 6, and 8) that contain all isoferritin populations. The gels were exposed to autoradiography and run in parallel with the corresponding nonimmunoprecipitated samples (10 μg/well, lanes 1, 3, 5, and 7) that contain all isoferritin populations. The gels were exposed to autoradiography and evaluated by densitometry. D, cells (2 × 10^6) of the repressed or derepressed Htw-tTA clone were incubated with 2 μCi/ml of ^55^Fe-NTA, 200 μM ascorbate for 3 h in serum free DMEM (± lysis) or 0.5% Nonidet P-40, 20 mM Tris–HCl, pH 8.0, 200 mM LiCl, 1 mM EDTA (± lysis). Equal amounts of soluble homogenates (3 μg) were loaded on nondenaturing PAGE and then exposed to autoradiography.

FIG. 6. ^55^Fe incorporation. The cells grown for 4 days (Hwt dox−) or 7 days (H222 dox−) for maximal accumulation of ferritin were incubated for 18 h in serum-free DMEM supplemented with 1 μM ^55^Fe-transferrin, and then cells were washed, lysed on the plates, and analyzed. A, radioactivity of total soluble homogenates expressed as pmol of radioactive iron/mg of soluble protein, an index of cellular iron uptake. Means and S.D. of three independent experiments. B, samples of soluble cell homogenates containing 3 μg of protein were loaded on nondenaturing PAGE and exposed to autoradiography. Ferritin mobility is indicated by the arrow. C, samples of 250 μg of the soluble protein homogenates were immunoprecipitated with saturating amount of anti-H-ferritin antibody to remove the ferritin heteropolymer isoferritin population. The resulting soluble fractions (10 μg/well, lanes 2, 4, 6, and 8) that contain all isoferritin populations. The gels were exposed to autoradiography and run in parallel with the corresponding nonimmunoprecipitated samples (10 μg/well, lanes 1, 3, 5, and 7) that contain all isoferritin populations. The gels were exposed to autoradiography and evaluated by densitometry. D, cells (2 × 10^6) of the repressed or derepressed Htw-tTA clone were incubated with 2 μCi/ml of ^55^Fe-NTA, 200 μM ascorbate for 3 h in serum free DMEM (± lysis) or 0.5% Nonidet P-40, 20 mM Tris–HCl, pH 8.0, 200 mM LiCl, 1 mM EDTA (± lysis). Equal amounts of soluble homogenates (3 μg) were loaded on nondenaturing PAGE and then exposed to autoradiography.

absence of Dox for up to 6 weeks without evident signs of toxicity. However, we observed that Hwt-tTA reached confluence more readily in the presence of Dox than in its absence, whereas the drug had no evident effects on cellular growth of the H222-tTA clone. More quantitative data were collected by seeding an equal number of cells of the different clones and analyzing them after 18 h growth under the same conditions. Cell protein content and cell viability monitored by MTT assay was not affected by the presence or the absence of Dox in the H222-tTA clone, although the values were reduced compared with the untransfected parent HeLa cells (Table II). In contrast, both protein content and total cell viability was more than 2-fold higher in the Hwt-tTA clone grown in the presence than in the absence of Dox (Table II). Analysis of cells from different induction experiments and collected at different times of induction showed an almost linear and negative correlation between the growth rate monitored by MTT assay and H-ferritin content when above 2,000 ng/mg protein (Fig. 7). The observed differences in cell growth were evidently not caused by a direct effect of doxycycline, and in fact its presence or absence did not affect cell mortality detected by trypan blue exclusion (Table II). To assess whether the H-ferritin effect was related to iron availability, the cells were grown for 4 days in the presence of 100 μM ferric ammonium citrate and then split, and cell growth was analyzed with the MTT assay. The treatment had no detectable effect on parent untransfected cells and on the repressed Hwt-tTA clone, while restoring the derepressed Hwt-tTA cell growth to the level on the controls (Table II).

**Ferritin Overexpression and Oxidative Stress**—To assess the resistance to oxidative stress, cells were incubated with various concentrations of H_2O_2_ and then their viability was monitored by the MTT assay. Fig. 8A shows that H_2O_2_ toxicity to HeLa cells becomes evident at concentrations above 300 μM to reach a plateau at 600 μM, where cell viability decreases to 30–40% of the untreated ones. The viability plots were analogous for the HeLa parent cells and repressed or derepressed H222-tTA clone, indicating that doxycycline had no major effect on the system. However, Hwt-tTA clone, which behaved as the other ones when repressed showed to be completely viable when exposed to the H_2O_2_ concentration of 600 μM, which was highly toxic to the other cells. To assess whether the protective effect in the derepressed Hwt-tTA clone was related to iron availability, the cells were incubated for 4 days with 100 μM ferric ammonium citrate and reanalyzed. The treatment did not modify the resistance to H_2O_2_ of the other clones, although it fully reverted the protective effect observed in the derepressed Hwt-tTA clone, which became as sensitive as the repressed one to 600 μM H_2O_2_ (Fig. 8B).

**DISCUSSION**

We generated two transfected HeLa clones that express exogenous ferritin under the inducible tetracycline responsive promoter and accumulate analogous levels of ferritin, up to 14–16-fold over background, under the same conditions. The two ferritins differ for two amino acid substitutions which are essential for ferroxidase activity, the H-chain wild type induces a fast iron oxidation in vitro, whereas the mutant 222 does not (11, 16). The proteins are antigenically identical, and in vitro they equally co assemble with the L-chain (9). The two clones seemed adequate to study the cellular effects of high levels H-ferritin accumulation (>5-fold higher than the ones obtained by iron induction) and to analyze how these effects are related to ferroxidase activity.

The two clones showed a number of similarities; in the presence of doxycycline the exogenous ferritin expression was low or undetectable, and the cells behaved similarly to the untransfected parent HeLa cells in all the analyses we performed. After withdrawal of the drug the ferritin synthesis in both clones was accompanied by a similar shift of the H:L ratio in the heteropolymeric fraction of the isoferritins and by the accumulation of an amount of H-homopolymers comparable with that of the
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Cells of the indicated clones were plated at 2 x 10^4 for trypan blue and protein assays or 10^4 for MTT assay. After 18 h of growth cell mortality was monitored by trypan blue exclusion method, the protein content were quantified by BCA assay, and total cell viability was determined by MTT assay also on cells grown for 4–7 days in 100 μg ferric ammonium citrate (+ FAC). Means and S.D. of at least three independent experiments. ND, not determined.

| Cells       | Trypan blue viability (– FAC) | Protein (– FAC) | MTT assay (– FAC) | Relative variation (– FAC) | MTT assay (+ FAC) | Relative variation (+ FAC) |
|-------------|-------------------------------|----------------|------------------|---------------------------|------------------|---------------------------|
| HeLa        | 87                            | 0.59 ± 0.1     | 2.0 ± 0.129      | 100                       | 1.96 ± 0.18      | 98                        |
| Hwt dox+    | 75                            | 0.56 ± 0.06    | 1.78 ± 0.15      | 89                        | 1.60 ± 0.06      | 80                        |
| Hwt dox−    | 81                            | 0.21 ± 0.01    | 0.73 ± 0.12      | 37                        | 1.96 ± 0.07      | 98                        |
| H222 dox+   | 85                            | 0.22 ± 0.05    | 1.43 ± 0.07      | 71                        | ND               | ND                        |
| H222 dox−   | 71                            | 0.21 ± 0.03    | 1.50 ± 0.17      | 75                        | ND               | ND                        |

Results are expressed as the ratio of unstained cells to the number of cells counted (%).

Results are expressed as the protein concentration after cell lysis in the same volume of buffer (mg/ml).

Results are expressed as the absorbance at 570 nm after three hours incubation with MTT.

Results are expressed as the percentages of the absorbance of the MTT assay relative to the untreated HeLa cells.

Fig. 7. H-ferritin content and cell proliferation. Derepressed Hwt-tTA cells in different experiments and containing different concentrations of H-ferritin as determined by ELISA assays were plated (10^4 cells) in 96-well culture plates and grown for 18 h. Then cell growth was determined by MTT assay. The values were plotted as percentages of the absorbance relative to control repressed cells. Shown are the means of at least two independent experiments in triplicate.

Heteropolymers. In various experiments with both clones in which total H-ferritin concentration ranged between 2,000 and 5,000 ng/mg, we found that the amount of H-homopolymers varied, whereas the L- to H-ratio in the heteropolymers was consistently ~20–22:1, i.e. about one L-chain/molecule. The shift in the isoform compositions caused by H-chain overexpression was indirectly confirmed by the lower apparent accumulation of the L-ferritin type detected by the ELISA assay, which is sensitive to the quaternary structure of the protein (26). The data demonstrate that in the transfected HeLa clones the exogenous ferritin subunits co-assemble with the endogenous ones, at variance with the transient expression system in COS cells where the exogenous and endogenous ferritins did not coassemble and provided little indication of the effects of ferritin overexpression (20). The slower accumulation of the exogenous ferritins in the HeLa clones (Fig. 1) compared with the faster one in the transient transfection system (up to 1000-fold over background in 48 h) may allow the pools of endogenous and exogenous ferritin subunits to equilibrate. This result and the finding that exogenous and endogenous ferritins degrade with the same kinetics indicated that HeLa cells do not distinguish the exogenous subunits from the endogenous ones and that the system is adequate to analyze ferritin functionality in vivo.

Ferritin and Cellular Iron Metabolism—The derepressed Hwt-tTA clone showed increased IRPs activity (about 5-fold), increased transferrin receptor (~2.5-fold), and increased uptake of iron-transferrin (~1.8-fold). This is fully consistent with the analysis of MEL transfected cells, where a lower up-regulation (3–5-fold over background) of ferritin H-chain induced a similar iron-deficient phenotype with increased IRP activity (21). The finding that in the transfected HeLa clone the IRPs activity was modulated by iron supplementation with ferric ammonium citrate and iron chelation with deferoxamine indicated that the IRE-IRP system is not directly affected by the ferritin overexpression but rather responds to a deprivation of the cellular iron availability. This was confirmed by experiments to measure the LIP using the calcinein method; in the H derepressed clone the LIP was about 50% smaller than in the repressed ones (Fig. 5) in agreement with results on transfected MEL cells (23).

The data confirm that the size of LIP is regulated by the amount of H-ferritin accessible to it probably for a chemical equilibrium between labile iron and the ferritin (22, 23), as predicted by models of IRE-IRPs machinery where IRPs sense the iron in the LIP and direct ferritin synthesis to sequester the amount in excess (2, 3). We add the demonstration that the functionality of the H-ferritin in the HeLa cells is totally dependent on its ferroxidase activity, because the overexpression of the mutant 222 chain in H222-tTA clone did not modify IRP activity, transferrin receptor accumulation, iron-transferrin uptake, or the size of the LIP. Its effect on cellular iron metabolism was negligible, as that found for the ferritin L-chains in cell lines forms hereditary hyperferritinemia cataract syndrome subjects (19).

A question we addressed was whether the human H-chain homopolymers are competent to incorporate iron when inside the cell. The recombinant H-ferritin can take up iron in prokaryotes, because it purifies from E. coli with 200–300 iron atoms per molecule (8); however, cellular models to verify whether the same occurs in mammalian cells, which have different regulations of iron, are missing, because H-chain homopolymers are not found in nature, and the transient transfected COS cells did not demonstrate to be informative (20). In 55Fe-labeled derepressed Hwt-tTA cells ferritin heteropolymers were subtracted with anti-L-chain antibody precipitation and the resulting soluble H-homopolymers were found to contain only 18% of the total ferritin iron, although they accounted for about 50% of total ferritin protein (Fig. 6C). This indicates that the heteropolymers were about 5-fold more efficient than the H-homopolymers in incorporating iron. More intriguing were the results with the H222-tTA cells where the overex-
pressed 222 subunit can associate with the endogenous H- or L-chains. The soluble ferritin fraction after immunoprecipitation was composed by heteropolymers of H- and 222 mutant and contained <5% of total ferritin iron (Fig. 6C). This indicates that the 222/H heteropolymers are much less efficient than H-homopolymers in iron incorporation. The data are in good agreement with the in vitro results showing that the L-chain contributes substantially to the efficiency of ferritin iron incorporation by promoting iron nucleation mineralization, whereas the 222 mutant subunit is the least efficient, because of the lack of both ferroxidase and iron mineralization capacity (16, 31).

Another question we tried to address is whether in the cells the rate of ferritin iron incorporation is limited by the rate of cellular iron transfer through cell membrane under conditions of iron loading. Normally, Fe(II) salts are used to study ferritin iron uptake in vitro and the more stable Fe(III) chelates are used for cellular iron uptake; thus the rates of ferritin and cellular iron incorporations cannot be compared. We reasoned that $^{55}$Fe-NTA, in presence of ascorbate, is taken up by cells and is also specifically incorporated into ferritins in vitro (16), and thus an analysis of ferritin iron incorporation in the absence of the presence of intact cell membranes may provide an indication on the limiting step of the transfer of iron from the medium to the ferritin. The finding that in the repressed Hwt-tTA cells the amount of iron associated to ferritin was the same in the lysed and intact cells suggested that the cell membrane transport was not the limiting step; however, in the derepressed cells, ferritin iron incorporation was 4-fold higher in the absence than in the presence of the membrane, indicating that the ferroxidase activity exceeded cell capacity to transfer iron. The finding may help to explain why the excess ferritin in the derepressed cells induces iron deficiency.

Ferritin, Cell Proliferation, and Oxidative Damage—The most evident phenotype associated to H-ferritin overexpression was a reduced growth rate; after withdrawal of the doxycycline the Hwt-tTA clone started growing slowly without evident modification of cell morphology or sign of toxicity. All the parameters we analyzed confirmed and extended the observation to the point that we noticed a correlation between cell H-ferritin content and cell growth (Fig. 7). This has not been described before, likely because in transfected MEL cells the level of H-ferritin accumulation was lower than that obtained in HeLa cells (21), and the experimental limits of the transient transfections did not allow to study cell growth (20). The finding explains why in various experiments we could not obtain stable cell lines that constitutively expressed high level of H-ferritin (not shown). The derepression of H222-tTA clone did not modify cell growth, and the suppressive effect of H-ferritin in the derepressed Hwt-tTA clone was totally reverted by prolonged preincubation with iron (Table II) but not by short 2-h incubations with iron (not shown). Iron further up-regulates the expression of the endogenous ferritins and only when supplied long enough to replenish the labile iron pool it abolished H-ferritin suppressive effect. The data demonstrate that the negative effect of H-ferritin on cell growth in HeLa cells was mediated by the ferroxidase activity and is secondary to the iron deficiency induced by it. Thus, ferritin acts as an iron chelator, possibly by reducing iron available to ribonucleotide reductase (32).

We found also that overexpression of the H-chain significantly increased cell resistance to H$_2$O$_2$; for instance only 30–40% of the repressed Hwt-tTA cells remained viable after incubation with 600 $\mu$M H$_2$O$_2$ compared with 100% of the same derepressed cells (Fig. 8). Similar, but not as strong, antioxidant effect was observed also in the transfected MEL cells (33). We add that the effect was absent in the derepressed H222-tTA clone and that it was reverted by prolonged preincubation with iron. This seems to parallel the suppressive effect, being mediated by the ferroxidase activity and its iron withholding in the cell.

In conclusion the data demonstrate that the activity of the H-ferritin overexpressed in HeLa cells is similar to that observed in vitro: (i) it sequesters iron with a mechanism that necessitates the integrity of the ferroxidase center, (ii) one or few H-chains/molecule are sufficient to confer the capacity to sequester iron, (iii) the activity is strongly enhanced by the co-presence of the L-chain in the same molecule, and (iv) H-homopolymers are functional, although less so than the H/L heteropolymers. In addition they show that all the phenotypes induced by H-ferritin overexpression are iron-mediated implying that in HeLa cells the only evident activity of the protein is to modulate iron availability. The results do not support the hypothesis that the association with ceruloplasmin is necessary for H-ferritin functionality (34). The data point also to an intriguing role of H-ferritin; if it leaves too little iron available for cellular needs, then proliferation is reduced and at the same the resistance to oxidative stress is increased. This fits well with present understanding of iron function and toxicity and with the hypothesis that iron homeostasis needs to be tightly regulated; it is also in agreement with the observation that proliferating cells have little ferritin and much transferrin receptor, whereas resting cells up-regulate ferritin to be more protected (35). The availability of two subunit types that cooperate in iron incorporation may be particularly useful to expand cell iron storage compartment (i.e. number of ferritin caviities) without affecting the capacity of iron sequestration (linked to the number of ferroxidase centers) (19).

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