Release of iron from ferritin requires lysosomal activity

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Kidane, Theodros Z., Eric Sauble, and Maria C. Linder. Release of iron from ferritin requires lysosomal activity. Am J Physiol Cell Physiol 291: C445–C455, 2006.—How ferritin-Fe becomes available for cell functions is unknown. Our previous studies with rat hepatoma cells indicated ferritin had to be degraded to release its Fe. In these studies, we investigated whether this occurs in other cell types and whether lysosomes are required. Release of ferritin-Fe was induced with desferoxamine (DFO) in ⁵⁹Fe-preloaded hepatoma, Caco2, and erythroid K562 cells and measured by rocket immunoelctrophoresis and autoradiography. The half-lives for ferritin-⁵⁹Fe and protein were parallel (23, 16, and 11 h for the hepatic, Caco2, and K562 cells, respectively). Co-treatment with 180 μM Fe, leupeptin, chymostatin, or chloroquine markedly decreased rates of ferritin-Fe release and ferritin degradation. Lactacystin had no effect except for a small one in erythroid cells. Fractionation of hepatoma cell lysates on iodixanol gradients showed rapid depletion of cytosolic ferritin by DFO treatment but no accumulation in lysosomes. We conclude that regardless of cell type, release of Fe from ferritin occurs mainly through lysosomal proteolysis.}

degradation: proteasomes

FERRITIN IS A LARGE multisubunit molecule found in virtually all mammalian cell types and in most other species (except yeasts) (2, 28, 40, 41). Its main function is the sequestration of excess iron in innocuous form. Despite considerable differences in the amino acid sequences of subunits and species, the overall and secondary structure of ferritins is highly conserved (5, 6). It consists of a globular shell, made up of dimers of subunits arranged in 432 symmetry, surrounding a hollow core within which the iron is deposited as compact mineral crystallites resembling ferrhydrite (2, 5, 6, 9, 21). Each subunit (H or L type) contains a long (5 nm) four-helix bundle with a fifth helix across one end and a loop on the other end. Twelve pairs of subunits binding head to foot form the 24 subunit ferritin protein “shell.” The result is great stability in the face of exposure to heat and acid (21, 28). The highly symmetrical arrangement of the subunits of ferritin results in eight hydrophilic, threefold intersubunit channels. As shown in vitro, iron enters into the core of ferritin through these channels, in part after being oxidized at peripheral (H subunit) ferroxidase sites and being transferred from there to carboxylate nucleation sites on the interior surface (especially of the L subunits). This leads to formation of the iron crystallites (2, 5). Storage of iron inside ferritin is very efficient, the average content in animal and human ferritins being 2,000–2,500 atoms/molecule (5), but that of individual molecules is highly variable (28).

Although only ~1 mg of iron is absorbed daily from the diet, there is a great deal of within-body transport and incorporation into proteins, especially hemoglobin. About 20–22 mg of iron cycle into and out of the red blood cell pool daily by processes involving many cell types, but especially the bone marrow, liver, and spleen (22). Unique to this element is the fact that most of the iron is in blood. Thus, if blood is lost or donated, iron is lost at a rate of ~0.5 mg/ml of blood and must be rapidly replaced. Replacement does not come from intestinal absorption but from ferritin stores. Different organs have different relationships to iron metabolism and storage. Erythroid cells need it principally to support the production of hemoglobin. Liver and spleen cells (both parenchymal and reticuloendothelial) are major sites for red blood cell processing and iron storage. Intestinal cells are concerned with iron absorption and the regulation of overall body iron homeostasis, reducing absorption when body stores are high, and “selflessly” enhancing transport from diet to blood when supplies are low.

It has always seemed reasonable that not just the entry but also the release of iron from ferritin occurs through the threefold channels, after penetration of small effector molecules (like ascorbate or flavin mononucleotide) to reduce and chelate the mineral in the core. This does happen in vitro when ferritin is treated with mercaptoacetic acid (and bipyridyl), at pH 5.5, to produce apoferritin (23, 43) [which has little or no effect on the overall structure of the protein “shell” (24)]. Theil et al. (25) have postulated that such a mechanism also operates in vivo, and they have shown in vitro that Fe release is accelerated when ferritin is mutated to make larger pores. It was thus a surprise to us when we determined that inhibition of ferritin proteolysis also prevented release of its iron (45), at least in the case of cultured rat hepatoma cells. Exposure of cells to the chelating agent, desferoxamine (DFO), accelerated ferritin protein turnover (loss of ⁵⁸S-methionine labeling) with a half-life of 22 h, and release of iron from ferritin (pretagged with ⁵⁹Fe) had the same kinetics. Treatment with iron (as ferric ammonium citrate; FAC) completely prevented ferritin protein turnover and completely stopped release of ferritin iron. These results strongly implied that mobilization of iron from ferritin did not occur as a result of the penetration of the ferritin shell by reducing and chelating agents, but rather that the protein shell of ferritin had to be degraded for its iron to be released. This led us to carry out the present studies, which addressed the question of whether this phenomenon might be a peculiarity of hepatic cells, which store excess iron and can produce a great deal of ferritin.

We also addressed the question of where in the cell, and how, the ferritin might be degraded for this purpose. Here we considered earlier studies indicating that lysosomes might be

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involved. Following a proposal of Roberts and Bomford (31), Konijn and colleagues (46) reported that inhibitors of lysosomal proteolysis (leupeptin, chymostatin, and chloroquine) reduced the rate of iron accumulation in hemoglobin in primary human erythroid cells and the reciprocal fall in ferritin concentration otherwise observed. In K562 cells, the same inhibitors reduced ferritin losses and the rate of recovery of the “labile iron pool” depleted by treatment with the chelator SIH.

Fig. 1. Changes in ferritin protein and release of ferritin Fe upon exposure to desferoxamine (DFO) in the three cell types. A: representative data showing autoradiographs for sets of immunoelectrophoresis rockets from extracts of hepatoma, intestinal (Caco2), and erythroid (K562) cells, on treatment with 100 μM DFO for the various lengths of time indicated. Cells had been preloaded overnight with iron as 59Fe-labeled ferric ammonium citrate (FAC; 180 μM). B: summary of data from multiple studies, showing changes in ferritin protein (ng/mg cell protein) (left) and 59Fe in ferritin (radioactivity as percentage of 2-h control, based on 59Fe in ferritin/mg cell protein) (right), for hepatoma cells (top), Caco2 cells (middle), and erythroid K562 cells (bottom) over time. Points are means ± SD (N = 8). Half-lives calculated from best line through the data are given above each curve. t1/2, half life.
Iron treatment inhibits loss of $^{59}$Fe from $^{59}$Fe-prelabeled ferritin in three cell types

<table>
<thead>
<tr>
<th>Ferritin</th>
<th>Hepatoma Cells (48 h)</th>
<th>Caco2 Cells (48 h)</th>
<th>K562 Cells (24 h)</th>
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<td>$^{59}$Fe</td>
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<td>$^{59}$Fe</td>
<td>100±22</td>
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Values are means ± SD ($n = 3–6$) for $^{59}$Fe remaining in ferritin after 48 h (hepatoma cells; Caco2 cells) or 24 h (K562 cells). DFO, deferoxamine; FAC, ferric ammonium citrate. Cultured cells were induced to accumulate ferritin and labeled with $^{59}$Fe by being exposed to $^{59}$Fe-labeled FAC overnight (10 μg Fe/ml). They were then washed and recultured for 24 or 48 h (as indicated) in medium containing DFO, minus (−Fe) or plus (+Fe) nonradioactive iron (FAC; 10 μg Fe/ml), before ferritin $^{59}$Fe radioactivity was assayed using rocket immunoelectrophoresis. Data from several experiments were combined. Thus units (percent) are relatives to cells not treated with Fe (−100%).

(18). Others showed that serum deprivation of rat hepatocytes promoted the transfer of cytosolic ferritin to lysosomes, and that this resulted in an enlargement of the labile iron pool (30). More recently and while we were carrying out our own work, Kwok and Richardson (19), followed up on their finding that the antitumor drug, doxorubicin, increased the amount of iron retained in ferritin. They reported for melanoma cells that treatment with leupeptin, chymostatin, or chloroquine had the same effect (20). Conversely, there have been reports that the proteasome (rather than lysosomes) degrades ferritin, although this may require prior damage by oxidation (35, 36, 38).

The studies reported here confirm that in three different cell types, cytosolic ferritin is largely degraded in lysosomes. Moreover, they indicate that release of iron from ferritin is dependent on the degradation of the ferritin protein shell by lysosomal proteases.

### RESULTS

#### Turnover of ferritin and its iron in hepatic, intestinal, and erythroid cells.

To establish conditions for following release of iron from ferritin, rat hepatoma, Caco2, and K562 erythroleukemic cells (the latter induced to produce hemoglobin) were separately loaded with iron to induce ferritin, and then exposed to DFO to induce ferritin iron release. In each case, iron to medium without added iron, changed daily. As in the previous studies (45), 6- or 12-μl portions of 15-fold concentrated (Centricon 30; Millipore, Bedford, MA) heat supernatant were applied to rocket immunoelectrophoresis, usually in the presence of rat liver ferritin standards to allow quantitation. The image and density of the rockets were recorded using phosphorimaging of the $^{59}$Fe. Changes in the density of the rockets over time indicated the rate of release of Fe from ferritin. Rocket areas were used to follow changes in ferritin protein concentration over time, with various treatments. The results were corrected for differences in cell number within individual flasks, by assaying whole cell lysates for protein by the Bradford dye-binding method, using the protocol and reagent from Bio-Rad (Richmond, CA), and bovine serum albumin as the standard.

#### Localisation of ferritin in cytoplasm and lysosomes.

This was determined by fractionation of post nuclear supernatants on iodixanol gradients, using a modification of the approach of Graham and colleagues (3, 10, 12). Before harvest, cells were exposed to lysosomal tracker 1 or Lyso Tracker Red (150 nM) for 1 h. Trypsinized cultures, washed and suspended in PBS-Tricine (0.85% NaCl and 10 mM Tricine-NaOH, pH 7.4), were disrupted by decompression in a nitrogen cavitation bomb (Parr, Moline, IL) and centrifuged for 5 min at 1,300 g. The pellet was rinsed, and supernatants were combined for application to iodixanol (30%), in 0.85% NaCl with 30 mM Tricine-NaOH, pH 7.4 in 13 × 48 mm tubes. Gradients were generated and organelles separated by centrifugation in the NVT 90 rotor of a Beckman-Coulter ultracentrifuge, for 3 h at 70,000 rpm, 4°C. Fractions (usually 15) were collected from the bottom of the gradient and analyzed for radioactivity (to detect ferritin and other concentrated Fe pools); for fluorescence or β-hexosaminidase activity (39) (to detect lysosomes); and for ferritin (by rocket immunoelectrophoresis).

#### Statistics.

Results are expressed as means ± SD for the number of determinations shown in parentheses. Statistical analysis of the data was made by one-way ANOVA. $P$ values <0.05 were considered significant.

### MATERIALS AND METHODS

#### Materials.

Leupeptin, chloroquine, and iodixanol were obtained from Fisher Scientific (Pittsburgh, PA). Chymostatin and lactacystin were obtained from Sigma (St. Louis, MO). Lysosomal tracker 1 was the kind gift of Drs. David E. Lewis and Scott C. Hartsel (Department of Chemistry, University of Wisconsin-Au Claire). Lyso Tracker Red DND99 was obtained from Molecular Probes (Eugene, OR).

#### Cell culturing and treatments.

Rat hepatoma cells (H4-II-E-C3), and human Caco2 and K562 cells were obtained from the American Type Culture Collection (Manassas, VA). Hepatoma cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% horse and 5% fetal bovine serum, as previously described (45) in T25 flasks. Caco2 cells were cultured in DMEM, with 5% horse and 15% fetal bovine serum (49). K562 cells were grown in Iscove’s modified Eagle’s medium, with 10% fetal bovine serum, and 0.3 mM tributyrin, at pH 7.6 (Na2CO3), to induce hemoglobin synthesis (7, 48). Generally, cells were used for experiments when 80% confluence was reached.

#### Measurement of ferritin iron release and loss of ferritin protein.

This was as previously described, using $^{59}$Fe, rocket immunoelectrophoresis, and autoradiography, by phosphorimaging (45). Briefly and unless otherwise mentioned, cells and cellular ferritin were loaded with $^{59}$Fe overnight, by incubation with $^{59}$Fe-labeled FAC (180 μM). In one set of studies, cells were grown in the usual serum-containing culture medium, to which 18 μM $^{59}$Fe-FAC was added to the medium with 20% serum. Washed cells were then incubated in fresh medium containing 100 μM DFO mesylate (Sigma), with or without 180 μM FAC or other additives, for various periods of time before analysis of ferritin $^{59}$Fe and ferritin protein concentration. In the case of cells pregrown on 18 μM iron, iron deprivation was induced by switching to medium without added iron, changed daily. As in the previous studies (45), 6- or 12-μl portions of 15-fold concentrated (Centricon 30; Millipore, Bedford, MA) heat supernatant were applied to rocket immuno-electrophoresis, usually in the presence of rat liver ferritin standards to allow quantitation. The image and density of the rockets were recorded using phosphorimaging of the $^{59}$Fe. Changes in the density of the rockets over time indicated the rate of release of Fe from ferritin. Rocket areas were used to follow changes in ferritin protein concentration over time, with various treatments. The results were corrected for differences in cell number within individual flasks, by assaying whole cell lysates for protein by the Bradford dye-binding method, using the protocol and reagent from Bio-Rad (Richmond, CA), and bovine serum albumin as the standard.

#### Measurements of iron uptake.

Cells were treated or not treated overnight with 180 μM FAC in DMEM containing 5% horse and 15% fetal bovine serum. After being rinsed with fresh, non-iron-added medium, uptake of iron was measured by the addition of $^{59}$FeCl3 (0.15 μCi) to each flask, and measuring the radioactivity in washed cell lysates after 3 h of incubation.

#### Localisation of ferritin in cytoplasm and lysosomes.

This was determined by fractionation of post nuclear supernatants on iodixanol gradients, using a modification of the approach of Graham and colleagues (3, 10, 12). Before harvest, cells were exposed to lysosomal tracker 1 or Lyso Tracker Red (150 nM) for 1 h. Trypsinized cultures, washed and suspended in PBS-Tricine (0.85% NaCl and 10 mM Tricine-NaOH, pH 7.4), were disrupted by decompression in a nitrogen cavitation bomb (Parr, Moline, IL) and centrifuged for 5 min at 1,300 g. The pellet was rinsed, and supernatants were combined for application to iodixanol (30%), in 0.85% NaCl with 30 mM Tricine-NaOH, pH 7.4 in 13 × 48 mm tubes. Gradients were generated and organelles separated by centrifugation in the NVT 90 rotor of a Beckman-Coulter ultracentrifuge, for 3 h at 70,000 rpm, 4°C. Fractions (usually 15) were collected from the bottom of the gradient and analyzed for radioactivity (to detect ferritin and other concentrated Fe pools); for fluorescence or β-hexosaminidase activity (39) (to detect lysosomes); and for ferritin (by rocket immuno-electrophoresis).

#### Statistics.

Results are expressed as means ± SD for the number of determinations shown in parentheses. Statistical analysis of the data was made by one-way ANOVA. $P$ values <0.05 were considered significant.
loading was with FAC (180 μM) overnight. Figure 1A shows examples of autoradiographs of rockets obtained for the cultures at different times after the start of DFO exposure. The areas of the rockets are proportional to the ferritin protein concentration. The density of the autoradiographic rockets indicates the $^{59}$Fe present in the ferritin. For all three cell types, it is clear that both the quantity and $^{59}$Fe radioactivity of the ferritin decreased markedly over the 24–64 h examined, when cells were exposed to the chelator.

The data for changes in ferritin protein and radioactivity from multiple studies are summarized in Fig. 1B. Data show changes in ferritin concentration (left) and ferritin $^{59}$Fe (right) over time in the hepatoma, Caco2, and erythroleukemic (K562) cells (top to bottom), corrected for cell number. It is noteworthy that overnight exposure to the same concentration of iron as FAC induced different levels of ferritin in the three cell types. Hepatoma cells accumulated almost four times more ferritin than did Caco2 cells (~180 vs. 50 ng/mg cell protein), and the erythroleukemic cells were in between (~75 ng/mg). This suggests that the different cell types vary in their abilities to take up non-transferrin-bound (ionic) iron (see more below).

In all three cell types, cell concentrations of ferritin decreased in an apparent first order manner in response to the iron deprivation induced by DFO (Fig. 1B, left). In hepatoma cells, ferritin protein had a half-life of ~24 h; that in Caco2 cells was ~17 h; and that in K562 cells ~13 h. Thus, at least in these conditions, ferritin turned over more rapidly in erythroleukemia cells and colon carcinoma cells than in hepatoma cells.

The content of $^{59}$Fe remaining in ferritin followed a very similar pattern and had similar cell-specific half-lives (Fig. 1B, right). As previously reported for the same hepatoma cells with $^{35}$S-met- and $^{59}$Fe-labeling, the release of ferritin iron paralleled that of ferritin protein degradation. This was also the case in the other cell types examined. Half-lives for loss of $^{59}$Fe from ferritin and for loss of ferritin protein were the same, within experimental error (Fig. 1B).

Effects of iron treatment on release of iron from ferritin and on ferritin protein concentrations. In previous studies with hepatoma cells (45), we had shown that continued exposure to excess nonradioactive Fe (180 μM FAC) completely inhibited loss of $^{59}$Fe from ferritin and degradation of ferritin protein. In this study, we also found a marked inhibition of iron release...
from ferritin (Table 1). Following ferritin induction with \(^{59}\text{Fe}\)-labeled FAC, exposure of the washed hepatoma cells to DFO without and with nonradioactive iron (as FAC) for 48 h greatly reduced the loss of \(^{59}\text{Fe}\) from the ferritin: with FAC, there was more than twice as much \(^{59}\text{Fe}\) still in ferritin after 48 h as in its absence. The results for erythroblastocytic cells over 24 h were very similar. However, iron was much less effective in the case of the Caco2 cells, only 20–30% more \(^{59}\text{Fe}\) being retained in ferritin after 48 h.

To determine whether the ability of these different cell types to take up iron from FAC was influencing these results, we also measured initial rates of iron uptake (Table 2). As might be expected from their interior location and the fact that transferrin is the main delivery mechanism, the erythroblastocytic cells were not nearly as adept at taking up this form of iron as were the two other cell types. However, the Caco2 cells took up iron from FAC even better than the hepatic cells. In combination with the results in Table 1, this indicates that Caco2 cells may handle iron differently so that it has less influence on iron in its ferritin compared with what occurs in hepatic and erythroid cells.

**Effects of inhibitors of lysosomal proteases and the proteasome on ferritin iron release.** To determine whether lysosomal proteases were involved in the degradation of ferritin and release of its iron, we examined the effects of inhibitors of lysosomal proteases, as well as chloroquine, which increases lysosomal pH (26), thus inhibiting the activity of most lysosomal enzymes. The effects of leupeptin are shown in Fig. 2. Its presence considerably slowed ferritin iron release in all three cell types. As in the previous studies, ferritin iron release was induced by exposure of iron-loaded cells to DFO, and leupeptin was added to one-half of the cultures in a given experiment. Leupeptin increased the half-life of \(^{59}\text{Fe}\) in ferritin from 23 to 36 h in the hepatoma cells, from 14 to 27 h in the Caco2 cells, and from 14 to \(\approx\)26 h in the K562 cells (Fig. 2, left). This indicated first that, when release of iron from ferritin was induced by iron deprivation, lysosomal proteases (inhibited by leupeptin) became involved in degrading the ferritin; second,
that inhibition of ferritin degradation inhibited release of its iron. The results also implied that degradation of this iron storage protein is required for its iron to be made available for biological functions.

The effectiveness of this protease inhibitor in preventing ferritin degradation was verified by measuring ferritin concentrations in the cells during the treatments (Fig. 2, right). In all cases, leupeptin prevented or dramatically slowed the decline in ferritin content. Leupeptin was particularly effective in the hepatic cells.

A second inhibitor of lysosomal proteases (chymostatin; Ref. 29) had the same effects. Figure 3, left, shows the amounts of $^{59}$Fe remaining in ferritin after 48 or 24 h of treatment with DFO in the presence and absence of chymostatin. In the hepatoma cells (top), about three times as much Fe remained in ferritin after 48 h when chymostatin or leupeptin (or both) were there to prevent ferritin degradation. This was also the case in the erythroleukemic cells over 24 h (bottom). The inhibitors also prevented iron release in the Caco2 cells (middle), but the degree of inhibition was less. At the doses applied and in a given cell type, the two inhibitors caused a similar reduction in release of ferritin Fe, and their combined effects were not additive, suggesting the same proteases were targeted. The chymostatin (and leupeptin) treatments inhibited degradation of ferritin protein (Fig. 3, right), again directly implicating ferritin degradation in the release of its iron. It is noteworthy, however, that the proportional losses of ferritin iron and ferritin protein were not entirely identical: threefold more iron but 6-fold more protein remained in the case of the hepatic cells after 48 h, whereas in the erythroid cells there was 4 times more iron and 2 times the ferritin protein; and in Caco2 cells there was 60% more iron and 250% more ferritin protein. This implies that more ferritin iron was released than ferritin protein was degraded (although actual rates of ferritin protein degradation were not measured).

Additional evidence that lysosomal proteases were involved in ferritin degradation and iron release was obtained with...
chloroquine, which would be expected to inhibit all lysosomal enzyme activities. Again, in the hepatoma cells over 48 h, chloroquine caused threefold more iron to be retained by ferritin, and 60–80% more in the case of the Caco2 cells (Fig. 4, left). Chloroquine caused the erythroleukemic cells to release half as much iron from ferritin over 24 h. Similar, though not entirely parallel, effects on loss of ferritin protein were observed (Fig. 4, right). Again, the degree of loss of ferritin iron was greater than loss of ferritin protein. Together, these studies showing marked inhibition of ferritin iron release by inhibitors of lysosomal proteases provide strong evidence that they are required for release of iron from ferritin when cells are deprived of iron, and storage iron in ferritin must be made available.

To determine whether the cytoplasmic proteasome might also be involved, we applied the well-known inhibitor, lactacystin (17). As shown in Fig. 5, this inhibitor had no statistically significant effect on the release of iron from ferritin or on ferritin degradation in the hepatoma and Caco2 cells, implying no involvement of proteasomal degradation. In the erythroleukemic cells, on the other hand, the higher concentration of lactacystin tested did have a small but statistically significant effect on ferritin protein degradation and iron release (over 24 h), implying it might also have at least a small role in ferritin turnover in these cells.

Treatment with DFO lowers the proportion of ferritin in the cytoplasm relative to that in lysosomes. Because all of the data indicated that lysosomes were important for release of iron from ferritin and for ferritin degradation, we did some initial studies to assess where ferritin would be found in our cells after overnight exposure to iron, and whether subsequent exposure to DFO (to induce iron deprivation) would result in a shift to the lysosomes from the cytoplasm. Figure 6A shows the distribution of ferritin (rocket area) and ferritin-radioactivity (rocket density) of fractions obtained by sedimenting hepatoma cell post nuclear supernatants on iodixanol gradients. Data on the left are for two cultures of cells not exposed to DFO; those on the right for two cultures exposed to DFO for 2 h. The total radioactivity associated with ferritin in the cytoplasm (fractions 1–4 at the bottom of the gradient) and that associated with lysosomes (fractions 6–8 or higher), with and without 2-h DFO.
treatment is given in Fig. 6C. The location of lysosomes in the gradient was determined by fluorescent and enzyme markers (Fig. 6B). (The distribution of 59Fe radioactivity in the same gradients is shown as well.) The data show that in all cases, a considerable portion of cell ferritin was in the lysosomal fractions. Treatment with DFO markedly diminished the radioactivity and proportion of ferritin remaining in the cytoplasm (Fig. 6, A and C), consistent with its moving into the lysosomes for degradation. However, there was no concomitant lysosomal accumulation of ferritin, implying that the lysosomes readily degraded it upon entry.

Effects of leupeptin under “iron-normal” conditions. In the studies reported so far, cells were grown excess iron to increase ferritin and make it and its 59Fe more detectable. However, it seemed possible that a different mechanism of iron release from ferritin might be operative under iron-normal conditions. To begin to assess this possibility, we grew all three cell types in serum-containing medium to which we added 180 μM Fe(III) as FAC. This is one-tenth the amount we used to induce ferritin accumulation and labeling. Dupeicate flasks with washed cells were then either incubated for a further 2 h (left, −DFO) in culture medium, or treated with DFO (100 μM) (right, +DFO) added to the same medium, before they were lysed (by decompression) and analysed on iodixanol gradients for distribution of ferritin protein and ferritin 59Fe (by autoradiography of rockets). A: autoradiograph of 59Fe-ferritin in gradient fractions (numbered from bottom to top) containing ferritin associated with the cytoplasm (fractions 1–3 or 1–4) or lysosomes (higher fractions), the latter determined by measuring lysosomally located fluorescence. (Purified ferritin applied to the gradients (data not shown) sedimented to the bottom (fractions 1–5)). B: distribution of lysosomal markers (Lyso Tracker Red and β-hexosaminidase activity) in the gradient fractions in relation to 59Fe radioactivity (most of which is in ferritin). Data are mean values for duplicate runs in which all three variables were measured in each fraction. C: total ferritin-59Fe associated with cytoplasm and lysosomes (C and L, respectively), calculated from the total density of the ferritin rockets in each compartment.

At these “normal” levels of iron in the medium, however, we were unable to get measurable results with the other two cell types. Even when samples were additionally concentrated and extra volumes were loaded, insufficient ferritin was detected for accurate quantitation. Thus further work will be required to address this question in these cell types.

DISCUSSION

We have shown that the exposure of cells to 180 μM Fe as FAC results in the accumulation of ferritin, but that this varies with the cell type, hepatic cells being 2–4 times more responsive than the intestinal epithelial and erythroid cell models we employed. This resulted from a relatively rapid uptake of this form of iron, paired with a slower rate of ferritin turnover in hepatoma cells in the face of iron deprivation induced by the chelator DFO. Hepatoma cells took up iron almost as rapidly as Caco2 cells, but ferritin concentrations declined with a half-life of ~24 h compared with 14–17 h with other cell types. In contrast, the erythroleukemic cells took up iron only one-third or one-quarter as well, and had a faster rate of ferritin protein turnover, resulting in accumulation of ~40% as much ferritin. The Caco2 cells were the best at taking up FAC iron, but accumulated less than one-third as much ferritin as the hepatic cells and lost ferritin protein at a greater rate. These findings are consistent with fact that the intestinal epithelium is “built” to take up ionic iron across the brush border, after its release from digested foods; and that hepatic parenchymal cells are better than most other internally located cells in the body with regard to taking up such iron (1, 37). In contrast, erythroid cells rely mainly on plasma transferrin for their iron, which is delivered via receptor-mediated endocytosis involving transferrin receptors 1 and 2 (16, 44). A large capacity for ionic iron uptake plus a relatively slower capacity for ferritin degradation
and that this occurred primarily or exclusively in lysosomes. Analysis of intracellular ferritin distribution on gradients indicated that with iron treatment, ferritin accumulated in the cytoplasm, but that a significant portion was present in the lysosomal compartment. Even short-term treatment with DFO resulted in a rapid disappearance of ferritin from the cytoplasm, and its continued presence in lysosomes but no lysosomal accumulation. Because there were no lysosomal protease inhibitors present, this suggests that lysosomes are able to keep up with the degradation of ferritin that enters.

Ferritin degradation was markedly inhibited by simultaneously treating with inhibitors of lysosomal proteases (leupeptin, chymostatin) or lowering lysosomal acidity with chloroquine. Evidence that lysosomes are involved in degradation of ferritin had been reported by several other laboratories, using a variety of individual cells and techniques. On the basis of electron microscopy, it has long been assumed that when excess iron accumulates in cells, depositing in ferritin, there is migration of ferritin clusters into lysosomes, and that partial degradation there of the outer ferritin shell results in the formation of hemosiderin (8, 28). In addition exogenous ferritin delivered to the cytoplasm of HeLa cells by microinjection (15) or liposomes (13) finds its way into lysosomes over time.

Radisky and Kaplan (33) showed that cationic ferritin endocytosed by fibroblasts was degraded in lysosomes, and that this resulted in release of Fe that induced cytoplasmic ferritin synthesis. The latter group also showed that ferritin was degraded in isolated lysosomes and that the process was accelerated by ATP. In addition, they reported that ascorbate (which can reduce and chelate iron) decreased the rate of ferritin degradation. This goes against the concept that reduction and chelation of the iron in the ferritin core (after penetration of such agents) is responsible for ferritin iron release in vivo and supports the opposing view implied by our studies that degradation of the protein shell of ferritin is required. Although it is unclear how ferritin enters the lysosomes, studies by Bridges et al. (4) and others (13, 14) add credence to the concept that ferritin aggregates (clusters) before it can enter lysosomes. Researchers have not determined what this aggregation means chemically.

Ferritin may not only be in lysosomes, however, to release its iron. Recent reports (11, 31) indicate that ferritin also promotes lysosomal stability in the face of oxidative stress. This would suggest that ferritin is not only there to be degraded but also to protect this organelle. Thus induction of ferritin accumulation by iron treatment or uptake of exogenous apo-ferritin increased the amounts of lysosomal ferritin in macrophage (11) and bronchial epithelial cells (31), and this enhanced survival of the cells exposed to oxidative stress. These findings also imply that oxidative stress releases Fe from endogenous proteins, which accelerates formation of damaging radicals unless captured by ferritin.

It is noteworthy that ferritin itself is somewhat resistant to release of its iron by radicals such as superoxide (4), as demonstrated in vitro. Moreover, oxidation of the protein part of ferritin results in its accelerated degradation, as reported by Mehlhase et al. (27) for RAW264.7 macrophages exposed to peroxide. Here, unlike what we and others have reported, proteasomal degradation seems to be involved: treatment with MG132 (a proteasomal inhibitor, which however, can also inhibit lysosomal proteases; see Ref. 35) markedly inhibited
the degradation of ferritin induced by peroxide (27). Other in vitro and in vivo observations confirmed involvement of the 20 S proteasome in degradation of oxidized proteins and ferritin (36, 38), and that ubiquitination and ATP are not required. However, Rudeck et al. (36) observed that an increased proteasomal susceptibility of oxidized ferritin did not correlate with release of its iron.

Our major finding that release of iron from ferritin consistently (and in very different cell types) depended upon degradation of ferritin is in agreement with our previous studies with the same hepatic cells, where we showed with 35S-met labeling that manipulation of ferritin degradation (by treatment with DFO or iron) directly influenced the release of its iron (45). In the studies reported here, iron treatment again greatly reduced or eliminated turnover of ferritin protein in the hepatic cells and had the same effect on retention of its iron. Treatment with iron as FAC also retarded ferritin degradation in the other cell types and reduced parallel ferritin iron release. However, it was much less effective in erythroid cells, which can be explained by their much lower ability to take up this form of iron. The Caco2 cells are interesting and potentially different in that they readily took up iron, but it was not nearly as effective in preventing ferritin iron release. This implies a difference in compartmentalization.

Perhaps our most important observation is that, when iron is needed, the metal is released from ferritin by lysosomal proteases. In several very different cells, release of 59Fe from prelabeled endogenous ferritin was markedly inhibited by standard substances that specifically interfere with lysosomal function and lysosomal protease activity. Except in erythroid cells (and at higher concentrations), the proteasomal inhibitor, lactacystin, had no such effect, implying no involvement of the alternative, proteasomal degradation system. In most of our studies, the ferritin that responded to iron deprivation was preinduced with 180 μM iron as FAC, and deprivation was induced with DFO. However, at least in the hepatoma cells, exactly the same response was obtained when ferritin was induced with normal levels of iron delivered at least partly by transferrin, and deprivation was induced by exposing the cells to fresh medium without added iron. Preexposure and labeling with 18 μM 59Fe added to 20% serum-containing medium resulted in much less cellular ferritin than with 180 μM FAC. Removal of added iron from the culture medium resulted in loss of almost all the ferritin 59Fe over 90 h, and most of the loss of 59Fe in ferritin was prevented by treatment with leupeptin, which also resulted in ferritin protein accumulation. This implies that at least for the hepatoma cells, the form in which iron was delivered and the process used to reduce iron availability does not matter; either way, lysosomal degradation of ferritin protein accompanies release of its iron.

Our findings are consistent with that of the following studies implicating lysosomal proteases in ferritin iron release: Konijn and Vaisman et al. (18, 46) for erythroid cells producing hemoglobin; Ollinger and Roberg (30) for primary hepatocytes responding to serum and amino acid deprivation by autophagy of ferritin, and an increase in cytoplasmic DFO-chelatable iron pools; Pourzand et al. (32) showing in fibroblasts that release of DFO-available iron from ferritin by UV exposure was inhibited by leupeptin and chymostatin; and Kwok and Richardson (20), who showed in melanoma cells that leupeptin, chymostatin, or chloroquine had the same effect as doxorubicin, greatly increasing the amount of 59Fe retained in ferritin. Like we, they also found that high concentrations of lactacystin (10 and 25 μM) promoted retention of iron by ferritin, as did MG132, another proteasomal (but also lysosomal) inhibitor (35). Along with our findings, this suggests that although in all cases ferritin protein degradation is tied to release of its iron, in certain cells the cytoplasmic proteasome may also participate in degradative iron mobilization.

GRANTS

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17. Jeffers M, Taylor GA, Weidner KM, Omura S, and Vande Woude GF. Degradation of the Met tyrosine kinase receptor by ubiquitin-protea- somal inhibitor (35). Along with our findings, this suggests that although in all cases ferritin protein degradation is tied to release of its iron, in certain cells the cytoplasmic proteasome may also participate in degradative iron mobilization.